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Arginylation regulates myofibrils to maintain heart function and prevent dilated cardiomyopathy

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Abstract

Protein arginylation mediated by arginyltransferase (ATE1) is essential for heart formation during embryogenesis, however its cell-autonomous role in cardiomyocytes and the differentiated heart muscle has never been investigated. To address this question, we generated cardiac muscle-specific *Ate1* knockout mice, in which *Ate1* deletion was driven by α -myosin heavy chain promoter (α MHC-*Ate1* mouse). These mice were initially viable, but developed severe cardiac contractility defects, dilated cardiomyopathy, and thrombosis over time, resulting in high rates of lethality after 6 months of age. These symptoms were accompanied by severe ultrastructural defects in cardiac myofibrils, seen in the newborns and far preceding the onset of cardiomyopathy, suggesting that these defects were primary and likely underlay the development of the future heart defects. Several major sarcomeric proteins were arginylated in vivo. Moreover, *Ate1* deletion in the hearts resulted in a significant reduction of active and passive myofibril forces, suggesting that arginylation is critical for both myofibril structural integrity and contractility. Thus, arginylation is essential for maintaining the heart function by regulation of the major myofibril proteins and myofibril forces, and its absence in the heart muscle leads to progressive heart failure through cardiomyocyte-specific defects.

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Keywords

arginylation; dilated cardiomyopathy; myofibrils

1. Introduction

Protein arginylation is a poorly understood posttranslational modification mediated by arginyltransferase ATE1 [1], which transfers arginine (Arg) from arginyl-tRNA onto proteins [2–6]. While Ate1 preferentially transfers Arg to amino acid residues with acidic side chains (Wang et al., 2011), a large number of proteins arginylated in vivo on different sites have been identified [7, 8]. The chemistry of the arginylation reaction, as well as its downstream effects on the majority of protein targets, are not well understood.

In yeast, *Ate1* gene is not essential for cell viability, but *Ate1* knockout in mice results in embryonic lethality between embryonic days E12.5 and E17.5 and severe defects in cardiovascular development and angiogenesis [9]. Among these defects, especially prominent are severe abnormalities in cardiac morphogenesis, including thin myocardium, underdeveloped septa, and non-separation of the aorta and pulmonary artery (persistent truncus arteriosus, PTA) [9]. Analysis of the heart muscle and cardiomyocytes isolated from *Ate1* knockout embryos at E12.5 – E14.5 reveals defects in cardiac contractility, heart integrity, and myofibril development [10], including myofibril disorganization, sarcomere collapse, and disintegration of the intercalated disks that become more prominent at later developmental stages (E14.5 and on). However it is not clear whether any of these defects are cell-autonomous or whether they arise through impairments in tissue signaling and/or are secondary to the onset of the embryonic lethality, leading to the heart muscle disintegration as the animals die.

To address these questions and test the specific role of arginylation in cardiomyocytes during heart development and postnatal function, we generated cardiac muscle-specific *Ate1* knockout mice, in which *Ate1* deletion via Cre-mediated recombination is driven by cardiomyocyte-specific alpha myosin heavy chain (α MHC) promoter [11]. These mice, termed α MHC-*Ate1*, survived to adulthood, but developed age-related dilated cardiomyopathy and thrombosis, accompanied by early myofibril defects and resulting in high rates of lethality after 6 month of age. Our results demonstrate a cell-autonomous role of arginylation in cardiomyocytes, where it is essential for the maintenance of the normal heart function and prevention of cardiomyopathy and heart failure. Our study also provides a mouse model of age-related heart failure with symptoms reminiscent of human heart disease, which has a potentially broad outreach in developing novel heart disease therapeutics.

2. Materials and Methods

2.1. Transgenic mice

To obtain α MHC-*Ate1* mice, *Ate1*-floxed mice [12, 13] were crossed with the mouse strain expressing Cre recombinase under α MHC promoter [11]. For the reporter analysis shown in Fig. S1, α MHC-Ate1 mice were crossed to R26R ROSA reporter strain and fixed embryos were stained with X-gal as described in [12].

2.2. Anatomical and histological analysis

For anatomical examination, mice found dead or euthanized were dissected to assess the morphology of the chest cavity (for fluid accumulation) and the hearts. To determine

heart:body weight ratios, mice were weighted before dissection and excised hearts were weighted separately after excision from the chest cavity.

For histological analysis, fetuses at E13.5 and isolated adult mouse organs were fixed in 4% paraformaldehyde in PBS, paraffin embedded, and sectioned. For observation and analysis of general organ morphology sections were stained with hematoxylin and eosin. Electron microscopy in the hearts of newborn and adult mice was performed as described elsewhere[10].

Comparisons of age-matched littermate control and α MHC-Ate1 mice were performed in electron microscopic and histological sections shown in the figures.

2.3. Echocardiography and Electrocardiograms

Echocardiography and electrocardiogram were performed at the Small Animal Imaging Facility of the University of Pennsylvania. The detail is in the website (<http://www.uphs.upenn.edu/radiology/research/labs/saif/services/ultrasound.html>).

2.4. Mass Spectrometry

Myofibrils were isolated as described in[14] with minor modification. Briefly, the hearts were homogenized in relaxing solution (10 mM MOPS pH 7.0, 64.4 mM K^+ propionate, 5.23 mM Mg^{2+} propionate, 9.45 mM Na_2SO_4 , 10 mM EGTA, 7 mM ATP, 10 mM creatine phosphate, pCa2+ 9.0), cells were skinned with 0.5% Triton X-100 in relaxing solution, and washed 3 times in relaxing solution. The final myofibril pellets were solubilized and analyzed by mass spectrometry as described in[7, 8].

2.5. Measurements of active and passive myofibril forces

Small sections of ventricles extracted from control and mutant hearts were tied to wood sticks, gently cleaned in rigor solution (50mM Tris pH 7.0, 100 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, and 10 mM EGTA), and stored in rigor/glycerol solution (1:1 v/v) at $-20^\circ C$ for up to 2 weeks. On the day of experiments, small pieces of the heart were further dissected and homogenized in rigor solution (homogenizer VWR AHS250, Canada) following standard procedures[15–17], which resulted in a solution containing small bundles of myofibrils. The myofibril bundles were transferred to an experimental, temperature-controlled chamber, which was kept at a constant temperature of $15^\circ C$. The sample was rinsed several times with rigor solution, and then the relaxing solution (see above) was added to the media. Small bundles of 2–6 myofibrils were chosen for mechanical testing based on striation pattern and number of sarcomeres (between 10 and 20). Using micromanipulators (Narishige NT-88-V3, Japan), the myofibrils were glued between an atomic force cantilever (AFC, model ATEC-CONTPT, Nanosensors, USA; mean stiffness = 0.2 Newton per meter (N/m)) and a stiff glass microneedle (stiffness $>2000nN \cdot \mu m^{-1}$) produced with a pipette puller (KOPF 720, David Kopf Inst, Tujunga, USA) and lifted off the glass slide by $\sim 1\mu m$. The stiff microneedle was connected to a motor arm, allowing for computer-controlled length changes in the myofibrils during the experiments. Before use, every new AFC was calibrated against a needle of known stiffness. The attached myofibril was centered in the microscope optical field under low magnification (10X–20X), and further imaging was performed using a CCD camera (Go-3, QImaging, USA; pixel size: $3.2\mu m \times 3.2\mu m$; final resolution $0.035\mu m$) under high magnification provided by a phase contrast lens (Nikon plan-fluor, 60X, NA 0.70), and increased 1.5X by an internal microscope function. The contrast between the dark bands of myosin (A-bands) and the light bands of actin (I-bands) provided a dark-light intensity pattern representing the striation pattern produced by the sarcomeres, which allowed measurements of the average sarcomere length during the experiments [16, 17].

For the force measurements, myofibrils were activated/deactivated by quickly changing the solutions surrounding the myofibrils (relaxing, pCa^{2+} 9.0 and activating, pCa^{2+} 4.5) using a multi-channel perfusion system (VC-6M, Harvard Apparatus, USA) attached to a double-barreled pipette, as previously described[16]. The double-barreled pipette was made with a vertical pipette puller and was subsequently polished to reach an inner diameter of $\sim 600\mu m$, each of the two channels with an inner diameter of $\sim 300\mu m$. The pipette was placed close to the myofibrils ($\sim 100\mu m$), and the solutions were continuously dragged from the experimental chamber through a back channel by using a peristaltic pump (Instech P720, Harvard Apparatus, USA); the flow rate achieved with this system was $\sim 5\mu l \cdot sec^{-1}$. When surrounded by the activating solution (pCa^{2+} 4.5), the myofibrils contracted and produced force, which caused deflection of the AFC; the deflection was detected and recorded using a recently developed laser system that allows high time-resolution measurements of mechanical properties of myofibrils [18]. Since the stiffness of the cantilever was known, the force could be calculated. Two tests were performed to investigate the active force and passive properties of the myofibrils, respectively: (i) myofibrils were activated isometrically at an initial SL of $2.2\mu m$, and (ii) myofibrils were stretched passively between sarcomeres of $1.8\mu m$ and $2.6\mu m$ with a magnitude of $0.2\mu m$ and a speed of $10\mu m/sec$.

2.6. Western Blotting

Whole hearts (for newborns) and fragments of left ventricular muscle (for adults) were minced on ice and resuspended in SDS sample buffer, and analyzed by SDS-PAGE and Western blotting using anti-ATE1 [12, 19], α -actinin 2 (kindly provided by Deepak Nihalani, School of Medicine, University of Pennsylvania, 1:200), anti-troponin T (Labvision, MS-295, 1:200), anti-tropomyosin (Santa Cruz Biotechnology, sc-74480, 1:100), or anti-myosin light chain 3 (Epitomics, 2939-1, 1:100).

2.7. Immunohistochemistry

For immunohistochemistry, paraffin-embedded heart sections were deparaffinized with xylene, re-hydrated with sequential methanol:water series (95:5, 75:25, 50:50 and 25:75), washed with water, boiled in 0.01M sodium citrate for 20 minutes for antigen retrieval, blocked with PBS supplemented with 0.1% Triton X-100 and BSA for 1 hour at room temperature, and treated with primary antibodies diluted in PBS supplemented with 0.1% Triton X-100 overnight at $4^{\circ}C$. Primary antibodies were used as listed above (section 2.6); in addition, we also performed staining with anti-titin (Developmental Studies Hybridoma Bank, 9D10, 1:100). After treatment with primary antibodies, samples were washed with 0.1% Triton X-100 in PBS, treated with fluorescent dye-conjugated secondary antibodies, washed with PBS, and mounted in Aqua Poly/Mount (Polysciences, Inc., 18606). Samples were observed under a fluorescence microscope.

2.8. X-gal staining in fetuses

For reporter analysis shown in Fig. S1, α MHC-Ate1 mice were crossed to R26R ROSA reporter strain and fixed embryos were stained with X-gal as follows[12]. Fetuses were fixed with 4% paraformaldehyde at $4^{\circ}C$ for 1 hour, rinsed for 30 minutes with rinse buffer (0.2 M sodium phosphate pH 7.3, 2 mM magnesium chloride, 0.02% IGEPAL and 0.01% sodium deoxycholate) 3 times, and incubated overnight at $37^{\circ}C$ in the staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal in rinse buffer). Samples were post-fixed with 4% paraformaldehyde and stored in 70% ethanol.

2.9. Electron microscopy

Electron microscopy in the hearts of newborn and adult mice was performed as follows[10]. Hearts excised from newborn and adult mice were washed in PBS and fixed in 2.5%

glutaraldehyde and 2% paraformaldehyde in buffer C (0.1 M sodium cacodylate, pH 7.4) overnight at 4°C, followed by two 10-minute washes in buffer C and post-fixation in 2% osmium tetroxide in buffer C. For staining, fixed embryos or hearts were washed twice for 10 minutes each in buffer C, once for 10 minutes in distilled water, incubated 1 hour at room temperature in a 2% aqueous solution of uranyl acetate and then washed twice for 10 minutes each in distilled water. For embedding, stained embryos or hearts were dehydrated by incubation for 10 minutes each in 50%, 70%, 80%, 90% and 100% ethanol, followed by two 5-minute incubations in propylene oxide (PO), overnight incubation in 1:1 PO:Epon (Poly/Bed 812, Polysciences), and then 1 day in 100% Epon. Epon-embedded embryos or hearts were kept for 2 days at 60°C for Epon polymerization, sectioned, stained with 1% uranyl acetate in 50% methanol and with a 2% (w/v) solution of bismuth subnitrite at 1:50 dilution, and then overlaid onto Formvar-coated grids for electron microscopy.

2.10. Statistical Analysis

Two-group analysis (wild type vs α MHC-*Ate1*) was performed by t-test (two-tailed). For myofibril force measurements, the active force produced by the two groups of myofibrils was compared using unpaired t-test. The passive forces produced by the two groups during passive stretches in different sarcomere lengths were compared using a two-way ANOVA for repeated measures. A level of significance of $p < 0.05$ was accepted for all analyses.

3. Results

3.1. Generation of α MHC-*Ate1* mice

To develop a mouse model with cardiomyocyte specific *Ate1* knockout, we used our previously developed '*Ate1*-floxed' mouse line [12, 13] and crossed it with α MHC-Cre mice [11], in which Cre recombinase is expressed under cardiomyocyte-specific α -myosin heavy chain promoter that activates upon differentiation, resulting in *Ate1* deletion in the heart muscle. These mice, termed α MHC-*Ate1* mice, were used in the present study. To confirm the efficiency and specificity of the Cre transgene expression, we crossed α MHC-Cre mice to the R26R Rosa reporter mouse strain, in which LacZ expression occurs following Cre-mediated excision of a repressor element and thus is confined specifically to the Cre-expressing tissues. X-gal staining of α MHC-Cre embryos at E10.5 showed specific Cre expression in the embryonic heart (Supplemental Fig. 1 online).

α MHC-*Ate1* mice exhibit postnatal lethality and develop severe dilated cardiomyopathy and contractility defects over time

Unlike the complete *Ate1* knockout mice that die soon after mid-gestation (E12.5 to E14.5) with severe defects in heart formation, α MHC-*Ate1* mice were born without apparent abnormalities and survived for several months without visible impairments in their appearance, behavior, or fertility. Examination of their heart morphology at the embryonic day E13.5 showed no visible differences from controls (Supplemental Fig. 2 online, left and middle panels), unlike the complete *Ate1* knockout mice that have severe cardiac morphogenesis defects at that age (Supplemental Fig. 2 online, right panel, and [9]). However, α MHC-*Ate1* mice started dying after 3 months of age at significantly higher rates than control (Fig. 1a). In control mice, regardless of the presence of the Cre transgene the death rarely occurred before 300 days of age; the earliest death of α MHC-Cre *Ate1*^{+/+} mouse was observed at 323 days, suggesting that the early death of α MHC-*Ate1* mice was not due to Cre expression in the heart. Analysis of the older live and deceased α MHC-*Ate1* mice showed severely enlarged hearts (Fig. 1c, left panels), with thinner heart walls and larger chambers characteristic for dilated cardiomyopathy (Fig. 1c, middle and right panels). Those mice that were found dead also had large thrombi in the atria and ventricles (Fig. 1c, middle panels) and accumulation of effusion in the thoracic and abdominal cavity,

suggesting the symptoms of congestive heart failure. Measurements of heart to body weight ratios showed that the hearts of 12-months old mice were severely enlarged compared to control (Fig. 1b). A similar trend was observed in the younger mice, which showed no increase in mortality rates but had somewhat enlarged hearts compared to the control, with differences reaching statistically significant levels by 7–9 months (Supplemental Fig. 3 online).

To test whether cardiac abnormalities and age-related lethality in α MHC-*Ate1* are associated with defects in heart contractility, we performed echocardiography using control and α MHC-*Ate1* mice at 3 months of age (when no visible health problems are observed, but the hearts appear somewhat enlarged in α MHC-*Ate1* mice) and 12-months old mice (when a large portion of α MHC-*Ate1* mice have died and hearts exhibit severe morphological defects). At 3 months old, no visible contractility defects could be observed in α MHC-*Ate1* mice compared to control (Fig. 1d, left panels). However, at 12 months old, cardiac contractility in α MHC-*Ate1* mice was severely weakened, as evidenced by a decrease in fraction shortening (percent difference between the size of the relaxed (diastolic) and contracted (systolic) ventricular chamber) (Fig. 1d, middle panels). At least in one observed case (Fig. 1d, middle bottom), the heart was ‘twitching’ rather than truly contracting, so that despite the observed periodic movement of the heart walls, the chamber size did not change at all over time. Furthermore, analysis of the electrocardiograms obtained from the 12-month old mice during echocardiographic imaging (Fig. 1d, right panels) showed visible differences from the control, with longer PQR distance indicating a delay between the atrial and ventricular contraction (double-headed arrows), flat or missing S waves (arrowheads), and/or flattened T waves (arrows), suggesting abnormalities in de-/repolarization and/or related responses.

Thus, *Ate1* knockout in the heart muscle results in age-related progressive dilated cardiomyopathy, abnormal cardiac contractility, and thrombosis, leading to high rates of lethality at 12 months of age.

3.3. Lack of protein arginylation results in ultrastructural defects in the heart muscle

To address if the cardiac abnormalities in α MHC-*Ate1* mice are accompanied by defects in the cardiac muscle at the ultrastructural level, we analyzed sections of the heart tissue from control and α MHC-*Ate1* mice at different ages by electron microscopy (Fig. 2 and Supplemental Fig. 4 online). Analysis of the aging mice (~12 months old) revealed prominent defects that affected the overall organization of the heart muscle as well as the structure of the individual myofibrils. At the tissue level, the distance between individual cardiomyocytes (Fig. 2a–c) was larger in α MHC-*Ate1* mice than in control. In more extreme cases, cardiomyocytes appeared to be ‘floating’ in the extracellular matrix, rather than firmly connected to each other (Fig. 2c and Supplemental Fig. 4a online). Overall, these defects suggested severe disruption of the inter-myocyte connections and the overall integrity of the heart muscle.

At the intracellular level, several prominent defects in the myofibril structure could be observed (Fig. 2d–m). While in control hearts myofibrils were well aligned and consisted of ‘sharp’ Z-bands and uniformly organized sarcomeres with evenly spaced filaments, α MHC-*Ate1* mice exhibited a range of myofibril defects, including partial loss of parallel alignment, diffuse Z-bands (that appeared ‘fuzzy’ on the micrographs of the mutant mice compared to the control and indicated possible supercontraction (Fig. 2e, h, i, k, m)), and in severe cases – partially disintegrating sarcomeres, with unevenly spaced filaments ‘fraying’ out of the myofibrils (Fig. 2f, m). In some cases, normal-looking myofibrils were interrupted by ‘split’ sarcomeres (Supplemental Fig. 4b online) and overall sarcomere disintegration appeared even more severe (Supplemental Fig. 4c online). Strikingly, similar defects were also

observed in the hearts of the 3-months old mice at the stages where heart dilations begin to develop in the mutant mice, but no visible cardiac contractile defects are seen (Supplemental Fig. 4d,e online).

To test whether these ultrastructural defects could be the underlying reason of the gross abnormalities and lethality in α MHC-*Ate1* mice or whether they are secondary to the observed phenotypic changes, we analyzed the ultrastructure of the heart muscle in newborn pups (postnatal day P0), at the age when they exhibit no heart-related changes, are viable, and apparently healthy (Fig. 2g-i). Strikingly, these mice already exhibited some of the ultrastructural defects seen in the aging adults, including diffusing Z-bands and overall myofibril mis-alignment and disorganization. Areas with well developed myofibrils in these mice appeared to be more difficult to find compared to the control, suggesting a possible delay or defect in myofibril formation. Thus, our results suggest that lack of arginylation results in abnormalities in myofibril ultrastructure during embryogenesis and that these abnormalities likely underlie the age-related development of dilated cardiomyopathy in α MHC-*Ate1* mice.

3.4. Arginylation regulates sarcomeric proteins

To address the underlying molecular mechanisms that lead from impaired arginylation to the defective myofibrils and cardiac contractility, we isolated cardiac myofibrils from adult wild type mice by heart homogenization and Triton X-100 extraction (Supplemental Fig. 5 online), and analyzed the resulting preparation by mass spectrometry to detect arginylated proteins [7, 8]. This analysis, using our previously published algorithm for detecting arginylated proteins on the N-termini of the peptides [7, 8] revealed nine myofibril proteins arginylated on specific sites (Table 1). Some of these proteins were mono- or dimethylated on posttranslationally added arginines (indicated as * and **, respectively in Table 1) [20]. Strikingly, the majority of these proteins have known roles in establishing normal sarcomeric structure and maintaining sarcomere integrity [21–24].

To assess the functional properties of these proteins that could be affected by arginylation, we compared the abundance of some of these proteins in the wild type and α MHC-*Ate1* heart homogenates by Western blotting and their distribution within the myofibrils on the heart sections from these mice by immunohistochemistry. These experiments showed that while the overall abundance of the analyzed proteins in both conditions was similar (Fig. 3a, b) and overall localized similarly in wild type and α MHC-*Ate1* myofibrils (see examples in Supplemental Fig. 6 online), the localization of tropomyosin was apparently misregulated in a larger portion of *Ate1* knockout myofibrils compared to the control (Fig. 3c, d). While in a normal situation these proteins localized as expected, to the thin filaments in between the Z-bands (identified by α -actinin co-staining in Fig. 3c), and formed either a single or a double band in contracted or relaxed myofibrils, respectively (Fig. 3c, top and middle rows), in some cases in α MHC-*Ate1* mice tropomyosin appeared to abandon this localization pattern and instead co-localized with the α -actinin at the Z-bands (Fig. 3c, bottom row).

Quantifications of immunostained sections showed that in 2 out of the 3 analyzed α MHC-*Ate1* mice approximately 10% of the myofibrils showed this defect, compared to ~2% cases or less observed in wild type (Fig. 3d).

3.5. Arginylation regulates passive and active myofibril forces

To test whether arginylation of the myofibril proteins affects myofibril mechanical properties and/or forces generated during the myofibril contraction, we performed measurements of passive stretch forces and active contractile forces of the myofibrils isolated from age-matched control and α MHC-*Ate1* mice using atomic force microscopy. Remarkably, while in the younger mice (5–6 months old) no significant differences were

observed (Supplemental Figure 7), in older mice (400+ days old) both active and passive forces produced by the myofibrils were significantly lower in α MHC-Ate1 mice compared to control (Fig. 4). The active forces in the mutant reached no more than 2/3 of the control values (Fig. 4a), which were typical for those measured in cardiac myofibrils from other mammals (e.g. [15, 25]). The passive forces in the mutants were progressively lower with stretching compared to the control myofibrils (Fig. 4b), suggesting that the mutant myofibrils are more compliant and more likely to weaken or disintegrate under normal cardiac contraction over time.

Thus, arginylation affects both the acto-myosin contractility during heart contraction (active force) and the structural integrity of the myofibril itself, maintaining its strength and elasticity during the normal heart function (passive force).

4. Discussion

Our results demonstrate a cell-autonomous role of arginylation in the development and function of the heart muscle, and identify arginylation as a novel mechanism that maintains cardiac health and prevents the development of dilated cardiomyopathy in mice (Fig. 5). It has been previously shown that arginylation is essential for cardiac morphogenesis and the development of heart muscle in embryogenesis [9, 10], however only the use of the current α MHC-Ate1 mouse model made it possible to identify the specific role of arginylation in cardiomyocytes and the heart muscle through the development and adult life. Our study has provided the first model of arginylation-related heart failure that can be used to address the corresponding mechanisms in human heart disease and to develop a new generation of arginylation-based cardiac therapeutics.

Unlike in the complete *Ate1* mouse knockout, heart-muscle-specific deletion of *Ate1* does not lead to visible morphogenic defects by impairing myocardium size, heart septation, or the formation of the outflow tract. Consistent with this observation, deletion of *Ate1* in migratory neural crest cells – the embryonic cell lineage that contributes to the formation of these structures – does not lead to cardiac morphogenic defects [12]. These facts suggest that *Ate1*-dependent developmental defects in cardiac morphogenesis, unlike the impairments in the heart muscle structure and functions, originate from longer-range tissue signaling rather than directly from the heart-forming cells. It is also possible that arginylation affects specification and/or differentiation of cardiomyocytes during the stages of heart formation that precede the activation of the α MHC promoter.

It has been previously shown that during development α -cardiac actin is arginylated to a relatively high extent, suggesting that arginylation of this protein may be required for myofibril formation [10]. Strikingly, our current data reveals no arginylated α -cardiac actin in the adult cardiac myofibrils, however we find a prominent set of other proteins that are arginylated, most of them directly relevant to the establishment and maintenance of the sarcomeric structure. Unlike in our previous studies [7], where we had to employ special enrichment methods to identify arginylated proteins, arginylation detection in the myofibrils required no enrichment procedures, suggesting that each of these proteins was arginylated to a relatively high extent. It should be noted that mutations in these same proteins have been previously shown to result in cardiomyopathies in human patients (reviewed in [26–29]), providing a possible functional link between arginylation and their role in heart disease. Addressing this link constitutes an important direction of further studies.

Our results show that posttranslational regulation is essential for maintaining both the active contractile forces and the passive-elastic forces of the heart. Both types of forces are essential for optimal contractile function. The reduction in the active contractile force suggests that the myosin motor function may itself be regulated by arginylation via direct

and/or indirect mechanisms. In support of this, myosin heavy and light chain have been found arginylated in our experiments (Table 1). The reduction of passive forces in the mutants suggests that the mutant myofibrils are more compliant and therefore are weaker under stress associated with normal cardiac contraction. Such altered properties likely contribute to the longer-term heart dilation and weakening of the heart muscle observed in α MHC-Ate1 mice. Remarkably, similar changes, with more compliant myofibrils, have been observed in other studies for conditions of dilated cardiomyopathy [30–33]. Since in our experiments myofibril preparations are removed from extracellular matrix components that could add to the stiffness measurements, the passive forces measured in these preparations are mostly attributed to the giant protein titin, which scaffolds multiple myofibril components and is a key protein in maintaining the structure of the myofibrils. Indeed, changes in titin's characteristics have been observed in varying heart diseases [31–33]. Moreover, it has been shown that the ratio between the titin isoforms N2BA:N2B is increased in dilated cardiomyopathy – the N2BA isoform is more compliant and is associated with lower stiffness levels in myofibrils. Like myosin, titin has been found among our arginylation targets (Table 1), suggesting that its structural role within the heart muscle may be directly regulated by arginylation.

Despite the prominence of structural and functional defects in the hearts of α MHC-Ate1 mice, only tropomyosin showed significantly abnormal localization compared to control. Lack of the visible defects for other proteins in our assays could be explained by the fact that arginylation may play highly specialized roles in fine-tuning of these proteins' levels and their interaction with their binding partners within the myofibrils. Indeed, if striking mislocalization of any of these key proteins was observed in the absence of arginylation, such an effect would likely lead to more severe phenotypes, causing perinatal or even embryonic lethality. The fact that α MHC-Ate1 mice develop the symptoms of cardiomyopathy only later in life suggests that subtle but persistent mechanisms in the absence of arginylation gradually perturb the myofibrils and cardiomyocyte function under constant duress during heart contraction. This phenotype makes α MHC-Ate1 mice highly reminiscent of those human patients that are apparently healthy but start developing severe heart problems at or after ~60 years of age (which corresponds to 8–12 months old in mice).

The higher incidence of localization of tropomyosin inside, rather than adjacent to the α -actinin-stained Z-bands, may indicate that in such myofibrils this protein loses its tight association with the actin filaments – an effect that could severely affect myofibril integrity even if observed only in a small percentage of cases. However, such altered localization could also be due to hypercontraction, during which the increased force of the sarcomere contraction causes the thin filament ends to perforate the Z-bands, eventually destroying the myofibril structure. In support of this mechanism, we see visible Z-band diffusion in α MHC-Ate1 myofibrils at as early as the newborn stage, suggesting that these Z-bands either have not formed normally or are forced to endure constant duress of super-contracting muscle. Addressing these possible mechanisms of myofibril regulation constitutes an exciting direction of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- New role of posttranslational arginylation in the heart muscle
- New regulatory mechanism of myofibril maintenance and contractility
- New insights into posttranslational regulation of the sarcomeric proteins
- New correlation between cardiac myofibril forces, heart defects, and arginylation

Figure 1. *Ate1* knockout in the heart results in dilated cardiomyopathy and postnatal lethality (a) Survival curve of wild type (WT) and α MHC-*Ate1* (CKO) mice. 61 WT and 131 CKO mice were used to derive the P0 100% values. (b) Heart/Body weight ratio of WT and CKO mice at the age of 10–12 months. Columns are significantly different from each other ($p=0.001$). 14 WT and 24 CKO were analyzed (error bars represent SEM). (c) Left panel: whole hearts fixed with formalin; Center panel: the hearts were cut in half to observe the inside; Right panel: heart sections stained with hematoxylin and eosin (H&E). 10 WT and 18 CKO mice were analyzed. (d) Left panels: echocardiograms of WT (top) and CKO (middle and bottom) mice at 3 (left) and 12 (middle) months of age. Right panel: electrocardiogram (ECG) of WT (top) and CKO (middle and bottom) mice at 12 month of the age (12 m). Double-headed arrows: PQR distance; arrowheads: S waves; arrows: T waves. 6 WT and 6 CKO mice at 3 months and 4 WT and 7 CKO at 12 months were analyzed.

Figure 2. Comparison of the ultrastructure of cardiac muscle between WT and CKO hearts by electron microscopy

Distribution of cardiomyocytes in adult (~12 months of age) and newborn (P0) wild type (WT) and α MHC-Ate1 (CKO) mouse hearts observed at different magnifications as indicated on the left-hand side for each group of images. 4 and 2 age-matched pairs of WT and CKO mice were analyzed in adult and newborn mice, respectively. Bars, 10 μ m in a–c, 500 nm in d–m.

Figure 3. *Ate1* knockout in the heart affects some of the myofibril proteins

(a) Western blotting of heart muscle homogenates probed with antibodies to the proteins arginylated in cardiac myofibrils in WT and CKO newborn (n) and adult mice (2 mice in each group). *Ate1*: arginyltransferase; Actn2: α -actinin 2; Tnnt2: cardiac troponin T2; Tpm: Tropomyosin; Myl3: myosin light polypeptide 3. (b) Left: Coomassie blue gels of total heart homogenates (Ex) and isolated myofibrils (MF) from WT and CKO adult mice, showing that the levels of actin and myosin heavy chain (MHC) are overall similar in these preparations. Right: immunoblots of the wild type heart homogenate and myofibril preparations with anti-Ate1 shows that Ate1 is highly prominent in the heart and directly associates with cardiac myofibrils. (c) Localization of tropomyosin and α -actinin 2 in

cardiac myofibrils. Left panel; localization of tropomyosin (Tpm); center panel: localization of α -actinin 2 (Actn2); right panel: merged pictures (Merged); top panel: Tpm appears as single bands in WT mice; middle panel: Tpm appears as double bands in WT mice; bottom panel: Tpm appears as single bands co-localized with Actn2 in CKO mice. (d) Ratio of myofibrils in which the Tpm and Actn2 distributed as single bands (as shown in (c), top panel), double bands (as shown in (c), middle panel) or co-localized (as shown in (c), bottom panel) in 2 WT and 3 CKO mice.

Fig. 4. *Ate1* knockout in the heart results in reduction of active and passive myofibril forces
 (a) Typical force measurements performed with two myofibril samples isolated from aged WT (black) and CKO (red) hearts. In both cases, myofibrils were activated at an initial SL of 2.2μm and after full activation the force was maintained stable until the myofibrils were relaxed. Note that the absolute force is significantly decreased in the mutant heart. (b) Consecutive stretches performed with non-activated myofibrils isolated from control (black) and mutant (red) hearts. Stretches were started at 1.8μm and were performed at magnitudes of 0.2μm at a speed of 10μm/1sec. Mean (± S.E.M.) values for (c) total active forces and (d) passive forces for myofibrils tested during these experiments. Multiple myofibrils from 4 age-matched control and αMHC-*Ate1* mice at 415 and 485 days old (2 pairs at each age) were analyzed.

Figure 5. Model of the regulation of cardiac formation and function by protein arginylation
Lack of arginylation causes dilated cardiomyopathy associated with abnormal and supercontracted sarcomeres.

Table 1

Arginylated proteins in cardiac myofibrils.

Name	Symbol	Accession	Arginylated Residue
alpha actinin 2	Actn2	NP_150371.4	L510
fibrillin 1	Fbn1	NP_032019.2	E640 ^{**} , Q671, L1266 [*] , E1794 [*]
laminin gamma 1	Lamc1	NP_034813.2	E803 [*]
myosin heavy polypeptide 6	Myh6	NP_034986.1	L747 [^] , K999 [^] , L1001 ^{^**} , V1027 [^] , L1486 [^] , Q1534, L1578 [*] , N1647
myosin light polypeptide 3	My13	NP_034989.1	A20, T81 ^{**} , M117
titin N2B	Ttn	NP_082280.2	L7960, V15013, C24818 [*]
tropomyosin alpha-1	Tpm1	NP_077745.2	L50, K77
cardiac troponin T2	Tnnt2	NP_001123650.1	A184

[^] also found arginylated in myosin heavy polypeptide 7 (Myh7; NP_542766.1; L745, K997, L999, V1025, L1484, and L1576)

^{*} found as monomethylated Arg as described in [20]

^{**} found as dimethylated Arg as described in [20]