Age-dependent Changes in a Chaperone Complex in the Mouse Heart

Short title: Aging induced downregulation of Unc45b, Hsp70 and Myosin in mouse heart

Purnima Devaki Bhat[1], Aswan Nalli[2], Luke Shoemaker[3], Jennifer Q. Kwong[3], Prasanna Krishnamurthy[2] and Guy M. Benian[3 *]

[1]Program in Neuroscience, College of Arts and Sciences, University of Alabama at Birmingham, Birmingham, AL 35233

[2]Department of Biomedical Sciences, Heersink School of Medicine and School of Engineering, University of Alabama at Birmingham, Birmingham, AL 35233

[3]Department of Pathology, Emory University, Atlanta, GA 30322

*Correspondence at pathgb.edu

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Abstract

The exquisitely organized sarcomere, the unit of contraction of striated muscle, is a very stable structure with a slow turnover rate of its components. The myosin chaperone UNC-45 and its co-chaperones, Hsp90 and Hsp70, are required for the initial folding of the myosin head domain and the assembly of myosin into thick filaments. There is increasing evidence that the UNC-45 chaperone system has an important role during aging to preserve sarcomere organization and myosin levels, and its decline may be a key factor in sarcopenia, the reduced skeletal muscle mass and function found in the elderly without an underlying condition. Cardiac muscle is another type of striated muscle but the role of the UNC-45 system has not yet been examined in the aging heart. Here we show that in the mouse heart, during aging, there is a decline in the levels of myosin, Unc-45b and Hsp70, but not Hsp90, and no obvious changes in sarcomere organization. In contrast, it is known that in skeletal muscle, there is a decline in both Unc-45b and Hsp90, and we show here that there is no decline in Hsp70. The decreased level of Hsp70 specifically in the heart could have broader implications than seem apparent as it could affect the folding and assembly of many other proteins in the aging heart and contribute to cardiomyopathies. Since Hsp70 mediates protein stabilization and prevents protein aggregation, its age-dependent reduction could potentially affect diseases such as amyloidosis.

Introduction

Muscle contraction results from the pulling inwards of thin filaments anchored to Z-lines by myosin head domains located on the surface of thick filaments. Muscle myosin is a hexamer consisting of two myosin heavy chains, and four myosin light chains, and has two head domains, two necks, and a rod that is comprised of the two myosin heavy chains in an α-helical coiled coil structure. The myosin rods assemble into the thick filament shaft with the myosin heads displayed on their surface in a specific pattern. The myosin heads are nanometer scale motors that efficiently convert the energy of ATP hydrolysis, through a series of conformational changes, to the swinging of the neck of myosin while a single head is attached to a thin filament. UNC-45 is a chaperone required to fold the myosin head after translation and for assembly of myosin into thick filaments during development (Barral et al., 1998, 2002). UNC-45 may also be involved in refolding the myosin head after stress in adulthood (Etard et al., 2008; Kachur and Pilgrim, 2008). A role for UNC-45 in mature muscle is even more plausible given that there is a relatively slow turnover of myosin in assembled thick filaments (Solomon and Goldber, 1996). Recently, we have reported that UNC-45 has an important role during adult aging to preserve myosin levels and myofibril organization (Matheny et al., 2024). Moreover, we found that additional expression of UNC-45 in old adults leads to maintenance of myosin levels and prevents the disorganization of myofibrils, suggesting that increasing UNC-45 might be a way to prevent or treat sarcopenia, the age dependent decline in sarcomere organization and function in skeletal muscle that occurs without an underlying condition.

UNC-45 was first discovered by genetic analysis in C. elegans, by finding a temperature sensitive mutant that when grown at the restrictive temperature results in an adult worm with highly disorganized myofibrils and reduced muscle function (Epstein and Thomson, 1974). It was later found that unc-45 null alleles are embryonic lethal (Venolia and Waterston, 1990; Barral et al., 1998). Whereas C. elegans and Drosophila have single UNC-45 genes, humans and mice have two genes for UNC-45, one encoding a Unc-45b, expressed in striated (skeletal and cardiac) muscle cells, and the other, Unc-45a, expressed in all cell types (Price et al., 2002).

All UNC-45 homologs consist of a C-terminal UCS domain which binds to the myosin head, an N-terminal TPR domain that binds to HSP-90 (Barral et al, 2002) and HSP-70 (Gazda et al., 2013), and a central region that acts as an inhibitor of the myosin power stroke (Bujalowski et al., 2018). Evidence that UNC-45 acts as a chaperone for the myosin head includes showing that: (1) UNC-45 prevents the thermal aggregation of myosin heads (Barral et al., 2002); (2) through a clever single molecule experiment that UNC-45 can refold an unfolded myosin head (Kaiser et al., 2012); and (3) UNC-45 can protect the myosin head's Mg-ATPase activity from being reduced by heat denaturation (Gaziova et al., 2020). The crystal structure of nematode UNC-45 shows that it forms linear multimers, two to five subunits each, in which the repeating unit spans 17 nm, which is similar to the absolutely conserved repeating unit of 14.3 nm of the staggered display of pairs of myosin heads along the surface of thick filaments (Gazda et al., 2013). Therefore, such UNC-45 multimers could help direct or stabilize this arrangement of myosin heads during thick filament assembly. In addition, this architecture suggests the possibility that in the assembled thick filament, refolding of myosin heads that were damaged by oxidative or thermal stresses as muscle is used and ages, might be facilitated. Support for such a role in mature muscle is that in at least C. elegans, antibodies to UNC-45 co-localize with

Myosin on the major portions of the thick filaments in already assembled sarcomeres of adult muscle (Ao and Pilgrim, 2000; Gazda et al., 2013). In zebrafish skeletal muscle, Unc-45b normally localizes to Z-disks, but moves to thick filaments during stress (cold and heat shock, chemical and physical)(Etard et al., 2008). Surprisingly, Unc-45b may also function as a chaperone for the cardiogenic transcription factor GATA4 (Chen et al., 2012). Unc-45b forms a physical complex with GATA4, although chaperone activity has not been demonstrated. Interestingly, recessive loss of function mutations in the Unc-45b gene in mice result in reduced levels of GATA4 (in addition to reduced levels of myosin) and arrest of cardiac morphogenesis and embryonic lethality.

Like humans, C. elegans develops sarcopenia, a reduction in the number and organization of sarcomeres and skeletal muscle function with age (Herndon et al., 2002; Matheny et al., 2024). We have reported that using a temperature sensitive unc-45 mutant to reduce the level and function of UNC-45 at the beginning of adulthood, sarcopenia develops earlier. We also observed a sequential decline of HSP-90 (adult day 3), UNC-45 (day 4) and then myosin (day 8), perhaps reflecting a cause and effect relationship (Matheny et al., 2024). The decline of these proteins is likely to be the result of protein degradation as the decline in their mRNAs occurs much earlier, especially for myosin (mRNA at day 1, protein at day 8). We have also shown that in mouse skeletal muscle Hsp90 and Unc-45b are reduced when comparing 3 month old to 24 month old mice (Matheny et al., 2024). No such studies have been undertaken to examine the Unc45 chaperone system in the aging heart, and a process similar to sarcopenia could play an important role in cardiac dysfunction with age.

Here we report our examination of the UNC-45 chaperone complex in the aging mouse heart. We find that when comparing 3 month old to 22 month old hearts, there is a decline in

myosin (myosin heavy chains), Unc-45b and Hsp70, but not Hsp90, and no obvious changes in sarcomere organization. The decline in Hsp70 in the heart was particularly dramatic, and in contrast to skeletal muscle, in which we observed no such decline. This decrease in Hsp70 has further ramifications since its downregulation could result in incomplete folding, accumulation and aggregation of other proteins in cardiomyocytes.

Cardiac aging at the cellular level consists of multiple changes including cardiomyocyte hypertrophy, increased cardiomyocyte apoptosis, increased numbers of fibroblasts, and increased collagen deposition (Vijayakumar et al. 2024). However, the molecular mechanisms for these alterations is poorly understood. Cardiomyocyte hypertrophy is thought to be a compensatory mechanism for the loss of cardiomyocyte numbers and consists of an increase in cell size, increase in protein synthesis, changes in sarcomere organization (Frey et al. 2004), and increased numbers of myofibrils in surviving cardiomyocytes. The results described here provide further molecular information about what happens during aging in the heart.

Results and Discussion

We harvested hearts from C57 Black mice at 3 months and 22 months of age, which translates roughly to 20 years and 65 years in humans, and prepared total protein lysates. These lysates were used to perform quantitative western blots to compare the levels of several proteins known to be part of an UNC-45 chaperone complex. As shown in Figure 1, we observed an approximately 30% decrease in the level of the UNC-45b client, myosin heavy chains, in 22 months vs. 3 month old hearts (Fig. 1A). In contrast, we observed no change in the level of the possible Unc-45b client protein GATA4 (Fig. 1B). We found approximately a 25% decline in the level of Unc-45b in both male and female mice (Fig. 2A). Interestingly, no significant decline in Hsp90 levels was found between the two ages (Fig. 2B), however, we found a dramatic (~60%) decline in the level of Hsp70 (Fig. 2C). This was in contrast to 3 months versus 22 month old quadriceps skeletal muscle where no such decline in Hsp70 was observed (Fig. 3). Hsp70 chaperone is generally induced beyond the basal level by stress such as heat (Tavaria et al., 1996; Morano, 2007). Under those conditions, it binds its protein substrates to prevent their denaturation and aggregation, thus, playing a major role in the health of cells. It has a weak ATPase activity as well.

Because skeletal muscle sarcopenia includes sarcomeric disorganization, we examined sarcomere organization in young vs. old hearts. Immunostaining with antibodies to myosin heavy chain for thick filament/A-band organization, and antibodies to α-actinin for Z-disks organization, revealed no differences in these structures in old vs. young (Fig. 4). Immunostaining for the intercalated disk marker, N-cadherin, also did not reveal any obvious differences (Fig. 4).

In Matheny et al. (2024) we primarily studied sarcopenia in C. elegans, but also showed that comparison of 3 month and 24 month old mice, revealed that in mouse quadriceps muscle, we observed an approximately 75% decline in both Unc-45b and Hsp90. As shown in Figure 2C, we found no difference in the level of Hsp70 in quadriceps muscle in 3 vs. 22 months old mice. Therefore, at least for the mouse, for both skeletal muscle and the heart, there is an age dependent decrease in Myosin and Unc-45b, and a only in skeletal muscle, a decline in Hsp90, and only in cardiac muscle a decline in Hsp70. This illustrates a significant difference between skeletal and cardiac muscle through which Hsp protein suffers during aging between these muscle types: Hsp90 in skeletal muscle, Hsp70 in cardiac muscle.

Normally, Hsp70 binds to partially synthesizing peptides and prevents them from aggregation. Once the client protein is fully synthesized, Hsp70 can transfer the client protein to other chaperones. It is also that peptides can be transferred from Hsp70 to Hsp90 by the Hsp70/Hsp90 Organizing Protein (Bracher and Verghese, 2015). As mentioned above, the same binding site in the TPR region of UNC-45 binds to both Hsp90 and Hsp70, although Gazda et al. (2013) reports that *in vitro* Hsp70 binds with a ~9 fold less affinity than does Hsp90 (KD of 120 μM vs. KD of 14 μM); when equal amount of Hsp70 and Hsp90 are mixed with UNC-45, Hsp90 is preferentially bound. It is not clear if this hold true also *in vivo*. We do not know the function of binding of either Hsp90 or Hsp70 to UNC-45, but there are at least two possibilities. One is that the UNC-45 multimer provides a multisite docking platform for Hsp90 and Hsp70 to act as chaperones on Myosin. Nevertheless, there is demonstrated chaperone activity for UNC-45 itself, although perhaps the folding or re-folding of Myosin heads requires both the UNC-45 chaperone and either an Hsp90 or Hsp70 chaperone. Another possibility is that the binding of Hsp90 or Hsp70 stabilizes UNC-45. Another issue is that we do not know the relative levels of Hsp90 and

Hsp70 in skeletal vs. heart muscle. Perhaps the reason that we find age dependent decline in one or the other is that the one that shows the decline starts out as having a limited amount. An additional consideration is that Hsp70 has many more functions in addition to binding to UNC-45, and possibly acting as a chaperone on myosin, and those functions may decline with age in cardiac muscle. In this tissue Hsp70 is likely to fold many proteins, and a decline in Hsp70 could affect multiple processes in cardiac muscle, not just the level and or organization of myofibrils. Finally, our results need to be interpreted with some caution, as the extracts we have made are from whole hearts and the heart is cellularly heterogeneous.

In addition, in the heart, although cardiomyocytes are the prominent cell type, there are also fibroblasts, endothelial cells, smooth muscle cells and even inflammatory cells. In fact, in the young heart 65-80% of the total heart volume is from cardiomyocytes, but during normal aging up to 30% of these are lost from apoptosis (Sheydina et al. 2011), and there is no regeneration. We think the downregulation of both Unc45 and Hsp70 with age in unlikely due to this loss of cardiomyocytes since the levels of GATA4 or Hsp90 were not downregulated (see Fig. 1B and Fig. 2B). Similarly, there was no downregulation of another protein called Ola1 or Obg-like ATPase 1 (Fig. 5), an ATPase protein involved in cardiomyopathies (Dubey et al., 2024). It is possible that no significant apoptosis occurs at 22 months of age, but later on.

While there may be a loss of cardiomyocytes with aging at some point, perhaps beyond 22 months of age, as a seemingly adaptive mechanism to maintain heart function, the remaining cardiomyocytes undergo hypertrophy (Vijayakumar et al., 2024) due to an increase in myofibrils per cell. The myofibrillogenesis which includes new myosin translation and new thick filaments certainly require Unc-45 and Hsp70, thus our observation of significant declines in these proteins suggests that the newly synthesized Myosin may not be optimally folded or optimally functional

and thus contribute to a decline in contractile activity and heart pumping. Nonetheless, the finding that both Unc45b and Hsp70 are downregulated with age in the heart offers certain insight into cardiac dysfunction. Their reduced levels may play a role in the worsening of cardiomyopathy phenotype during aging, regardless of the causative mutations. Given that Hsp70 has a role in protein stabilization and preventing protein aggregation, its age-dependent reduction could potentially affect diseases such as amyloidosis.

Materials and Methods

Genotypes: Young and old mice were of the genotype C57BL/6J were used. Young age is 2 or 3 months old and old is 22 months old. Mice were all healthy and hearts and skeletal muscle were harvested and samples were prepared as described below.

Western blot analysis

Heart tissues (1 cm^3) from C57 black mice aged three months and 20-22 months were collected. The tissues were homogenized in chilled 650 µL RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1% SDS, and PIC [final concentration: 1x, Sigma Aldrich P2714]) using a Bead Ruptor Elite (Speed: 5 m/s, Time: 30 s, Cycles: 15), with the tissue kept on ice between cycles. The lysates were centrifuged at 13,000 rpm for 5 minutes in an Eppendorf microfuge. The supernatant (~520 µL) was transferred to a clean 1.5 mL tube on ice and mixed with 200 µL of 4X Loading Dye and 80 µL of 10X Reducing Agent (Invitrogen NuPAGE NP007 and NP009, respectively). The samples were heated at 95°C for 5 minutes and stored at -20°C until use. Five µL of each sample were loaded per lane on a 4-12% Tris-acetate gel (Invitrogen XP04125BOX), and proteins were transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). After transfer, membranes were stained with 25 mL Ponceau S Stain (Sigma Aldrich P7170) for 5 minutes, washed extensively with water, and imaged for loading control. Membranes were blocked with 5 mL of 1x TBST (Tris Buffered Saline with 0.1% Tween-20) containing 5% powdered milk for one hour at room temperature. After blocking, membranes were washed and incubated in 5 mL of primary antibody solution (primary antibody diluted in 1x TBST + 1% powdered milk) overnight at 4°C on a BenchRocker (Benchmark Scientific). The primary antibodies used were against UNC45B

(rabbit polyclonal at 1:2,500, Proteintech 21640-1-AP), HSP70 (rabbit polyclonal at 1:7,000, Proteintech 10995-1-AP), HSP90 (1:5,000, Proteintech 13171-1-AP), and Myosin (1:1,000, Abcam ab50967).The primary antibody solutions were removed, and membranes were washed three times for 15 minutes each in 1x TBST. Following the washes, membranes were incubated with secondary antibody solutions diluted in 1x TBST + 1% powdered milk to a final volume of 5 mL. Secondary antibodies included HRP-conjugated Goat anti-Rabbit (1:15,000) and Goat anti-Mouse (1:100,000) (Jackson Laboratories), and were incubated for 2 hours at room temperature on a BenchBlotter 2D Rocker (Benchmark Scientific). Membranes were washed three times for 15 minutes each in 1x TBST to remove excess antibodies. Finally, membranes were treated with Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma) according to the manufacturer's instructions and imaged using an Amersham Imager 600 (GE Healthcare Life Sciences).

Immunofluorescence microscopy

Hearts were isolated, snap frozen in isobutane, and embedded into cryogenic molds using OCT medium. 10 mm thick sections were obtained by cryosectioning using a cryostat, and the sections collected onto the surface of Colorfrost Plus (ThermoFisher) slides, dried, and stored at -80° until further use. We then used the acetone fixation and immunostaining protocol as described in Blondelle et al. (2019). Primary antibodies used: anti-myosin heavy chain (mouse monoclonal at 1:200, University of Iowa Hybridoma Bank , cat. no. A4.1025), anti-a-actinin (mouse monoclonal at 1:800, Millipore cat. no. A7811), and anti-N-cadherin (mouse monoclonal at 1:100 dilution, Santa Cruz cat. no. sc-59987). The secondary antibody, used at 1:200 dilution, was anti-mouse Alexa 594, purchased from Invitrogen. Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M

microscope and an Apochromat x63/1.4 numerical aperture oil immersion objective. The color balances were adjusted using Adobe Photoshop (Adobe).

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Figure legends

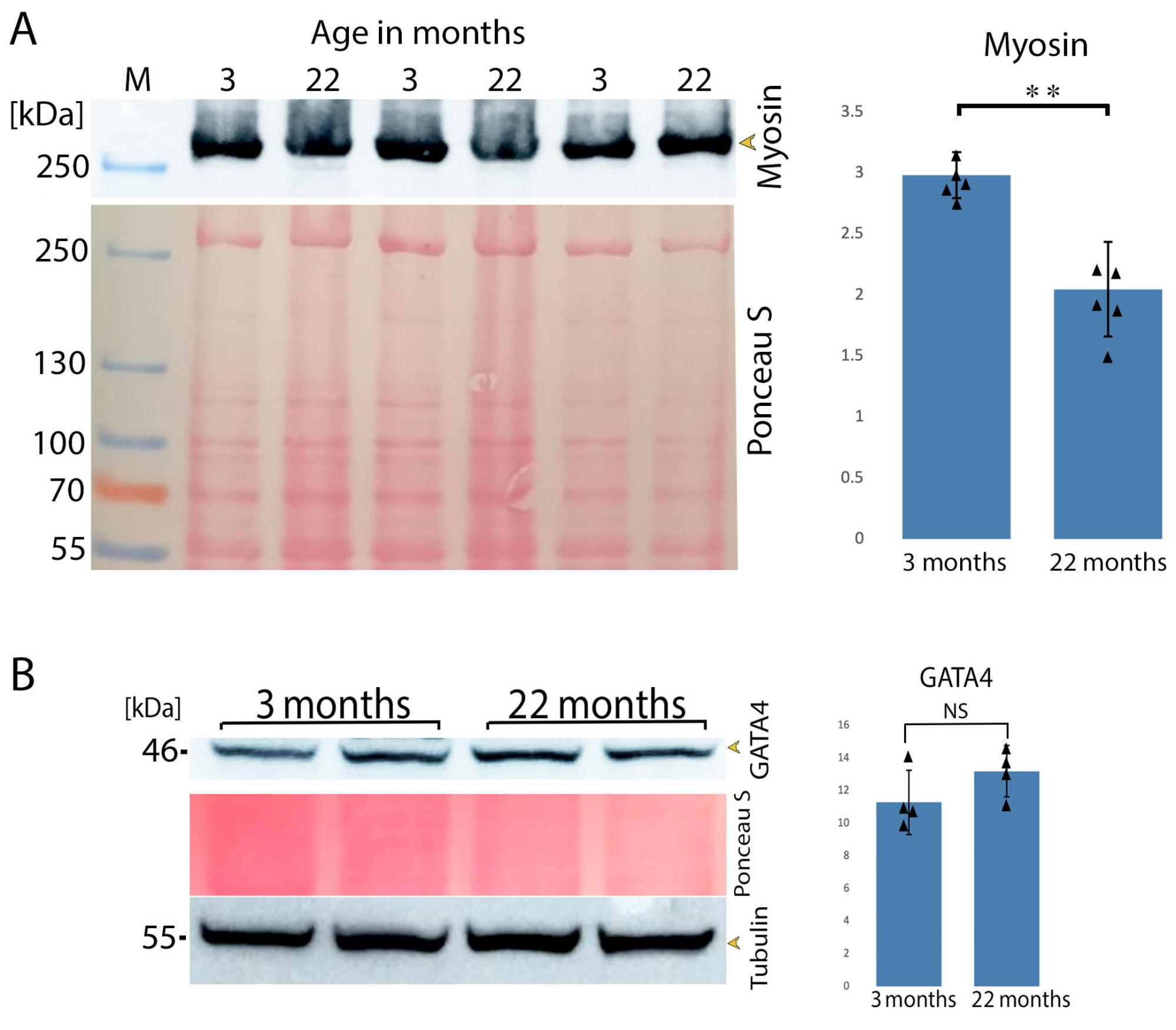
Figure 1: Myosin but not GATA4 levels are reduced in aged mice. Western analysis of hearts from 3 months and 22 months old mice using an antibody against Myosin (A) and GATA4 (B). Ponceau S was used for loading control for Myosin and both Ponceau S and Tubulin were used as control for GATA4. The results were subjected to statistical analysis using a student's t test.**: P<0.01; NS: not significant.

Figure 2: Unc45b and Hsp70 but not Hsp90 are reduced in aged mice. Western analysis of hearts from 3 months and 22 months old male and female mice using an antibody against Unc45b (A), Hsp90 (B) and Hsp70 (C). Ponceau S was used for loading control. The results were subjected to statistical analysis using a student's t test. **: P<0.01; ***: P<0.001; NS: not significant.

Figure 3: Hsp70 levels are not affected with age in skeletal muscle. Histogram showing the Western results of 3 months and 22 months old mice quadriceps muscle samples using an antibody against Hsp70. The difference was not statistically significant.

Figure 4: Sarcomere organization is not affected with age in cardiomyocytes. Sarcomeric organization was examined in young vs. old hearts by immunohistochemistry. Immunostaining was done with antibodies to myosin heavy chain for thick filament/A-band organization, and antibodies to α-actinin for Z-disks organization. Longitudinal views are shown. No differences were observed. Immunostaining for the intercalated disk marker, N-cadherin also did not reveal any obvious differences. Scale bar, 20 μm.

Figure 5: The levels of OLA1 is not affected with age in cardiac muscle. Western analysis of hearts from 3 months and 22 months old mice using an antibody against Ola1. Ponceau S was used for loading control. The results were subjected to statistical analysis using a student's t test. The difference was not statistically significant.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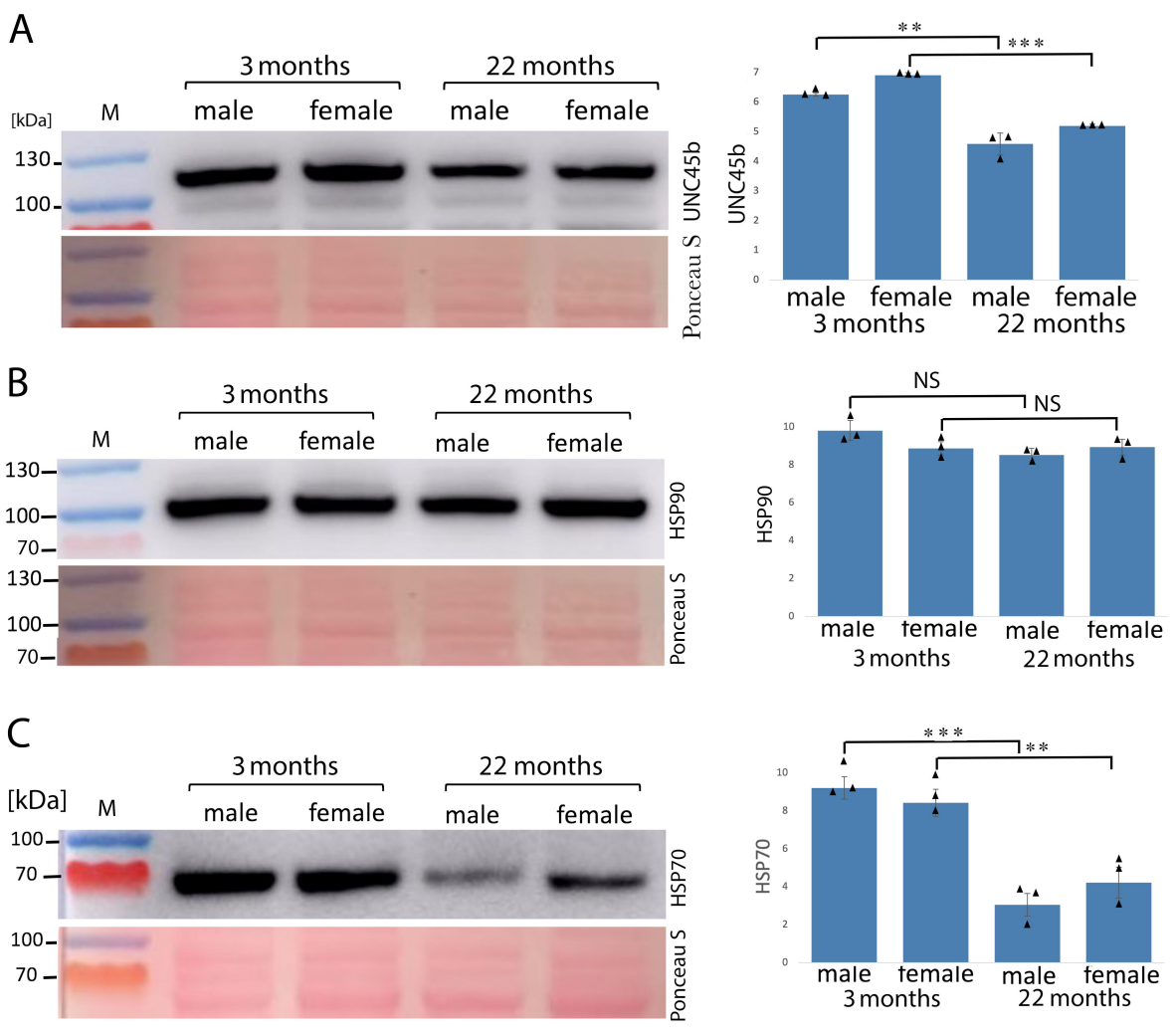
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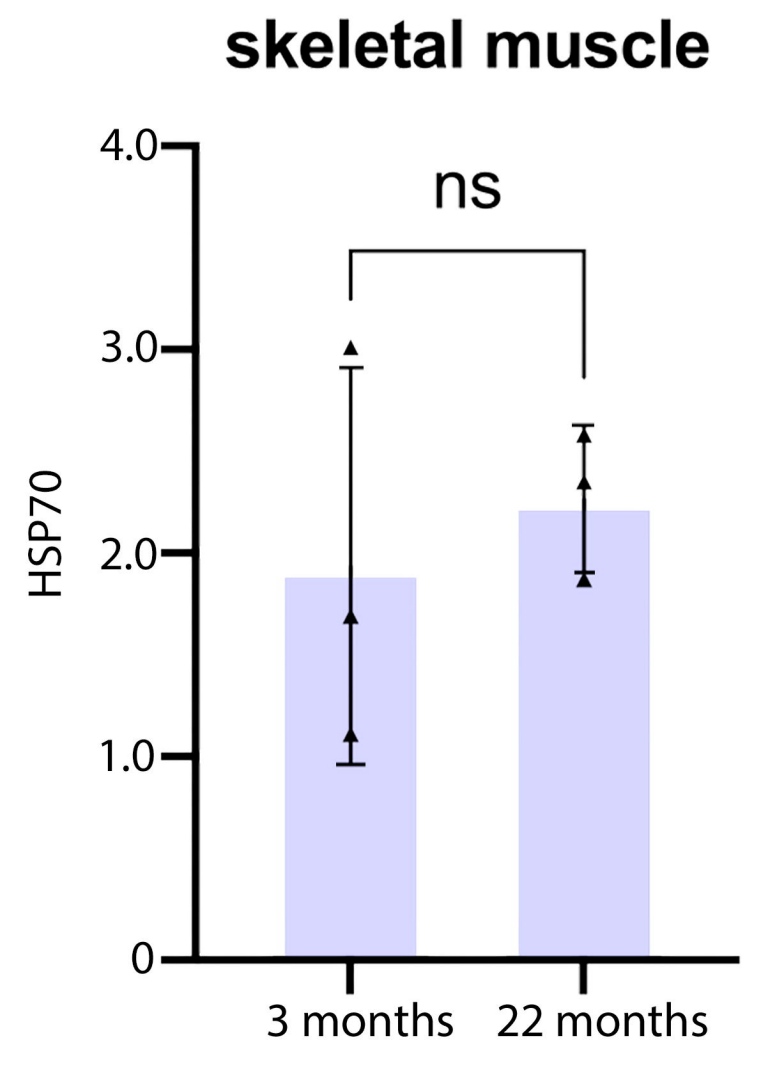
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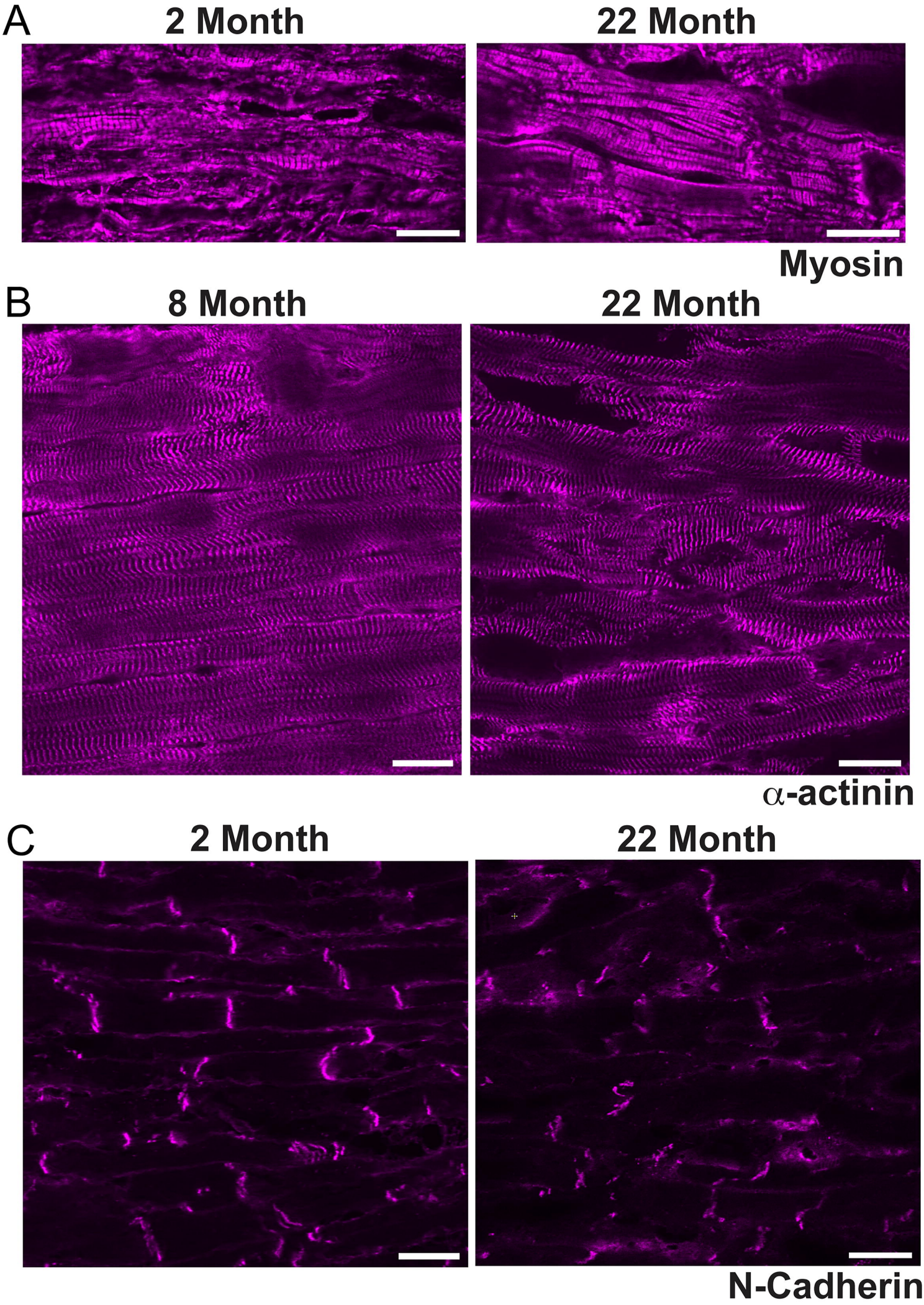
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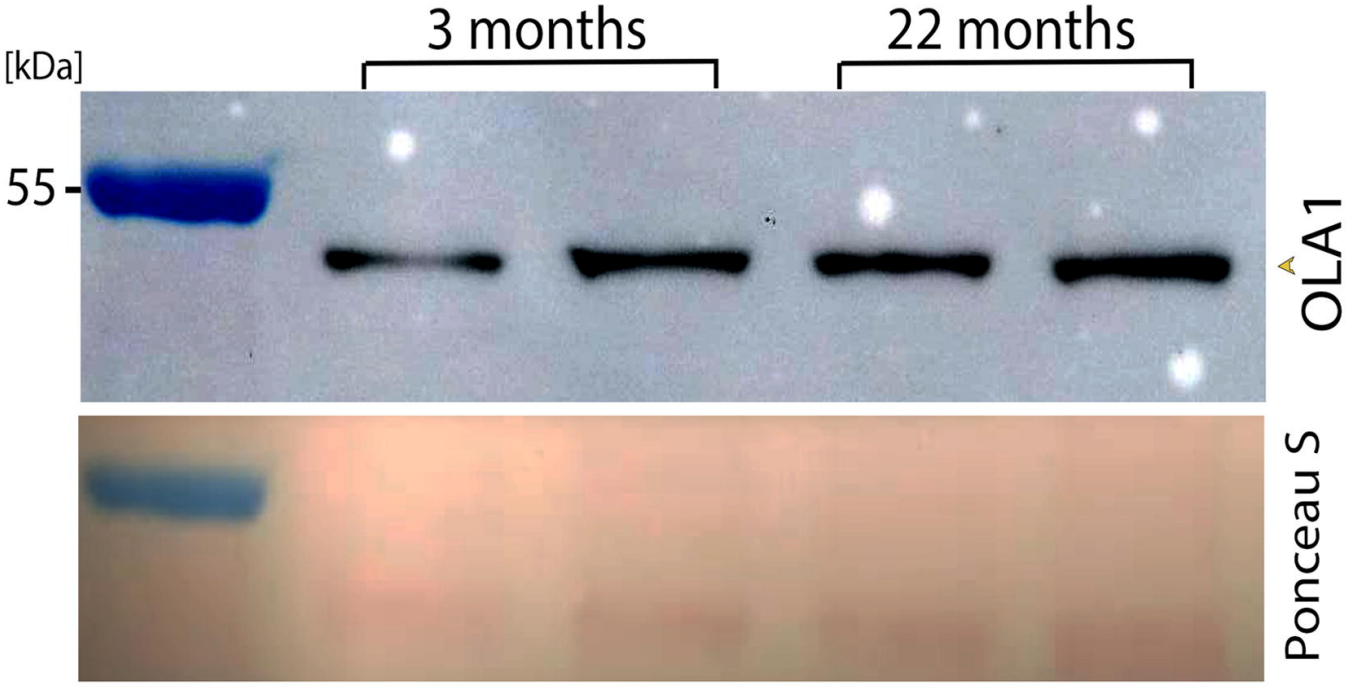
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OLA1
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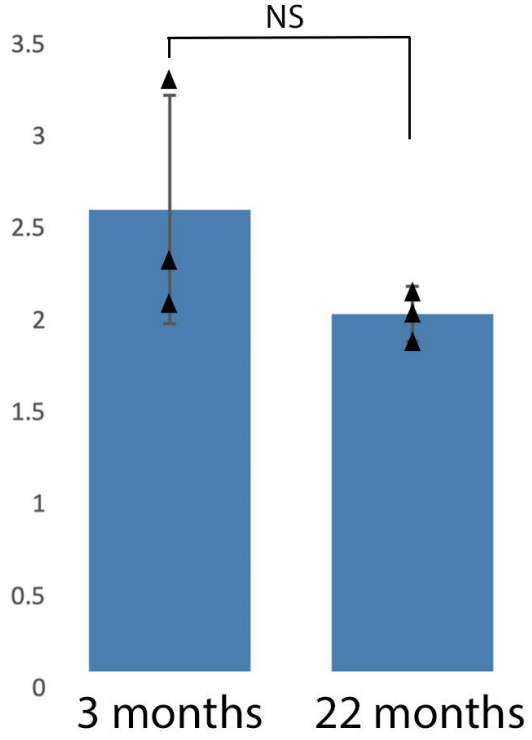
NS
3.5
3
2.5
2
1.5
1
0.5
0
3 months
22 months