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Supporting Material

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SUPPLEMENTARY MATERIAL

METHODS

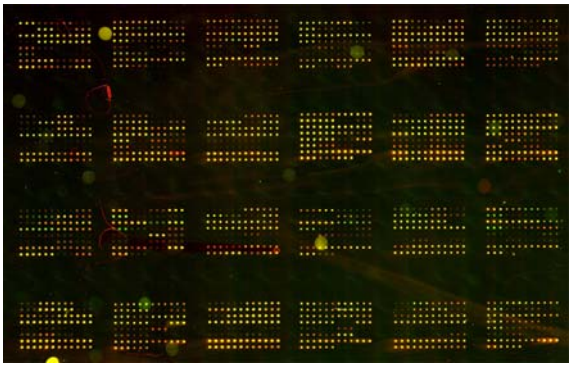
Western blotting. For Western blotting of MARPs, samples were run on 15% Acrylamide Gels, and transferred to PVDF membrane using a semi-dry transfer unit (Bio-Rad, Hercules, CA). The blots were stained with PonceauS to visualize total transferred protein. The blots were then probed with rabbit polyclonal antibodies for CARP, Ankrd2, and DARP (Miller et al., 2003). To normalize for loading differences, MARP labelling was normalized to actin, determined from the PonceauS-stained membrane. Secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra were used for detection. One-color IR western blots were scanned (Odyssey Infrared Imaging System, Li-Cor Biosciences, NE. USA) and the images analyzed with One-D scan EX. For Western blotting with the anti-titin antibody 9D10, samples were run on 1% agarose gels and transferred as described above. The blots were probed with 9D10 antibody (which is specific for the PEVK region of titin) and visualized as described above. 9D10 labelling was normalized to total titin obtained from the PonceauS stained blot.

Passive tension characteristics. The procedures for skinned muscle contractility were as described previously (Granzier and Irving, 1995;Fukuda et al., 2005b), with minor modifications. Soleus and TC muscles from adult and neonatal (day 5) mice were skinned overnight at ~4°C in relaxing solution (in mM; 20 BES, 10 EGTA, 6.56 $MgCl_2$, 5.88 NaATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate, pH 7.0 at 20°C) containing 1% (w/v) Triton X-100. Preparations were washed thoroughly with relaxing solution and stored in 50% glycerol/relaxing solution at -20°C. Small muscle bundles (diameter ~0.06 mm) were dissected from the skinned muscles. Muscle bundles were attached to a strain gauge and a high-speed motor using aluminum foil clips. Experiments were performed at 20°C. Sarcomere length (SL) was measured online by laser-diffraction using a He-Ne laser beam. The XY (width) and XZ (depth, using a prism) bundle diameters were measured with a 40 x objective. The muscle bundle cross-sectional area (CSA) was calculated from the average of three width and depth measurements made along the length of the muscle bundle, and passive tension was determined by dividing the passive force by CSA. Muscle bundles were set at slack length in relaxing solution and passive force was recorded while SL was increased to ~3.2 μm

(velocity, 0.1 muscle length/s), after which length was held constant for 30 seconds to observe stress relaxation, followed by a release to slack sarcomere length. The stress relaxation response in each recording was fit with a second-order exponential decay function. Subsequently, thick and thin filaments were extracted by immersing the preparation in relaxing solution containing 0.6 M KCl (35 min at 20°C) followed by relaxing solution containing 1.0 M KI (35 min at 20°C). Following the extraction procedure, the muscle bundles were stretched again at the same velocity and the passive force remaining after KCl/KI treatment was assumed to be collagen based and titin-based passive force was determined as total passive force minus collagen-based passive force (Granzier and Irving, 1995;Fukuda et al., 2005a;Fukuda et al., 2005b).

Transcript studies. For each muscle type we used muscles from 3 neonatal (1 day old) and 3 adult (105 days old) mice. Muscle tissues were collected in RNAlater from Ambion Inc. (Austin, TX, USA). The tissue was homogenized using a Pro200 homogenizer from *PRO* Scientific Inc. RNA was isolated using the Qiagen RNeasy Fibrous Tissue Mini Kit. RNA was amplified using the SenseAmp kit from Genisphere and Superscript III reverse transcriptase enzyme from Invitrogen. Reverse transcription and dye coupling (Alexa Fluor 555 and Alexa Fluor 647 were used) was done using Invitrogen's superscript plus indirect cDNA labeling module. Half of each sample was incorporated with Alexa Fluor 555 and the other with Alexa Fluor 647. We used a home-made titin exon microarray that consisted of 50mer oligonucleotides representing each of 358 mouse titin exons (selected for GC content 45-55 %; Tm ~ 70^{o}C and minimal tendency for hairpin structures and dimerization). Exons were spotted in triplicate on Corning Ultra GAPS glass slides. Slides were then baked at 90^{o}C for 90 minutes and stored in a non-electric desiccator. Hybridization (Ambion: Slide-Hyb buffer #1) was for 16 hours at 42^{o}C after which slides were scanned at 595 nm and 685 nm with an Array WoRx scanner. Spot finding was done with SoftWoRx Tracker. Spot files were analyzed using CARMA, which provides a quantitative and statistical characterization of each measured exon. Normalization between the two channels of each array was achieved by performing a locally weighted regression (lowess) transformation that adjusts for intensity and location dependent effects (for details, see Greer et al. (Greer et al., 2006). Exon location was randomized (the layout of the microarray will be made available upon request).

FIGURE



Example of a microarray chip hybridized with cDNA from mouse TC.

Reference List

1. Fukuda,N., Y.Wu, G.Farman, T.C.Irving, and H.Granzier. 2005a. Titin-based modulation of active tension and interfilament lattice spacing in skinned rat cardiac muscle. *Pflugers Arch.* 449:449-457.

2. Fukuda,N., Y.Wu, P.Nair, and H.L.Granzier. 2005b. Phosphorylation of titin modulates passive stiffness of cardiac muscle in a titin isoform-dependent manner. *J Gen. Physiol* 125:257-271.

3. Granzier,H.L. and T.C.Irving. 1995. Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys. J* 68:1027-1044.

4. Greer,K.A., M.R.McReynolds, H.L.Brooks, and J.B.Hoying. 2006. CARMA: A platform for analyzing microarray datasets that incorporate replicate measures. *BMC. Bioinformatics.* 7:149.

5. Miller,M.K., M.L.Bang, C.C.Witt, D.Labeit, C.Trombitas, K.Watanabe, H.Granzier, A.S.McElhinny, C.C.Gregorio, and S.Labeit. 2003. The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J Mol Biol.* 333:951-964.