EXPRESSION OF THE SKELETAL CALSEQUESTRIN ISOFORM IN NORMAL AND REGENERATED SKELETAL MUSCLES AND IN THE HEARTS OF RATS WITH ALTERED THYROID STATUS

Tomáš Soukup[1], Vadym Sulimenko[2], Vladimíra Marková[2], Kateřina Kopecká[1], Gisela Zachařová[1], Jiří Paleček[1].

[1]Institute of Physiology and [2]Institute of Molecular Genetics, Academy of Sciences, Prague, v. v. i., Czech Republic.

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Calsequestrin in skeletal and cardiac muscles

Corresponding author:

RNDr. Tomáš Soukup, PhD.
Institute of Physiology,v.v.i.
Academy of Sciences of the Czech Republic
Vídeňská 1083
CZ-142 20 Prague 4, Czech Republic
Tel: +420-24106 2544
Fax: +420-24106 2488
E-mail: tsoukup@biomed.cas.cz

SUMMARY

We have investigated expression of skeletal calsequestrin (CSQ1) and fiber type composition in normal and regenerated fast and slow skeletal muscles and in the left heart ventricles of euthyroid (EU), hypothyroid (HY) and hyperthyroid (TH) adult inbred Lewis strain rats. The CSQ1 level was determined by SDS-PAGE followed by Western blot analysis. CSQ1 gene expression was assessed using reverse transcription and subsequent real time polymerase chain reaction. Muscle regeneration was achieved by intramuscular grafting of either soleus or extensor digitorum longus (EDL) from 3- to 4-week-old rats to either EDL or soleus muscle of 2-month-old rats. The fiber type composition was assessed by a stereological method applied to stained muscle cross sections. We found that the protein and *mRNA* levels for CSQ1 were highest in the EDL muscle, the relative CSQ1 protein levels in the soleus muscle were two times lower and the transcript levels more than 5 times lower compared to the EDL. In the left heart ventricle, protein isoform and CSQ1 transcript were also present, although at protein level, CSQ1 was hardly detectable. TH status increased and HY status decreased the expression of CSQ1 in the EDL, but its relative levels in the soleus and in the heart did not change. The regenerated soleus transplanted into EDL, as well as EDL transplanted into soleus exhibited protein and *mRNA* levels of CSQ1 corresponding to the host muscle and not to the graft source. TH status increased the percentages of the fastest 2X/D and 2B fibers at the expense of slow type 1 and fast 2A fibers in the EDL and that of fast 2A fibers in the soleus at the expense of slow type 1 fibers. HY status led to converse fiber type changes. We suggest that the observed changes in CSQ1 levels in TH and HY compared to EU rats can be related to fiber type changes caused by alteration of the thyroid status rather than to the direct effect of thyroid hormones on CSQ1 gene expression.

Key words: skeletal calsequestrin – calcium and muscle contraction – thyroid hormones – western blot and qRT-PCR analysis – muscle transplantation - fast and slow muscle fibers

INTRODUCTION

Calsequestrin (CSQ, 55kD) is the most abundant calcium binding protein (CaBP) of skeletal and cardiac muscle. It is an acidic protein that binds Ca^{2+} with low to moderate affinity, but with high capacity. It is located in the lumen of the sarcoplasmic reticulum (SR) in close proximity to the junctional SR domains containing ryanodine receptors (RyRs, for review see Franzini-Armstrong 2009). It maintains free Ca^{2+} concentrations relatively low, which is important for easier and more efficient inward transport of the released calcium by SERCA pumps. CSQ is a component of the macromolecular complex involved in excitation contraction coupling (ECC), the process linking surface membrane depolarization to Ca^{2+} release from the SR (for review see Sandow 1965, Dulhunty 2006, Beard *et al.* 2004, 2009). CSQ is produced as a skeletal and a cardiac isoform, which are products of the two different genes. Its genes, as well as protein structure are relatively simple, but the latter is well suited to bind Ca^{2+} as well as other proteins, namely RyR, junctin and triadin. This strongly enhances CSQ ability to regulate calcium movements (for review see Zhang *et al.* 1997, Berchtold *et al.* 2000, Beard *et al.* 2004, 2009). The skeletal isoform (sCSQ, CSQ1) is found in fast-twitch and slow-twitch muscles, while the cardiac isoform (cCSQ, CSQ2) is considered as the only transcript in cardiac and a minor transcript in adult slow-twitch muscle (for review see Berchtold *et al.* 2000, Beard *et al.* 2004, 2009, Wei *et al.* 2009, Novák and Soukup 2011). Functional changes of the CSQ complex and its mutations can result in pathology, including impairment of ECC, skeletal muscle myopathies or cardiac arrhythmias (for review see Berchtold *et al.* 2000, Marks *et al.* 2002, Tomelleri *et al.* 2006, Knollmann 2009, MacKrill 2010, Novák and Soukup 2011).

Many hormones exert a strong systematic influence on skeletal muscles during development as well as in adulthood. For thyroid hormones, it has been shown in many species including rats that both hypothyroid and hyperthyroid states modify the myosin heavy chain (MyHC) isoform content in skeletal (and cardiac) muscles, which can result in transformation of slow to fast muscle fibers or

vice versa (for review see d`Albis *et al.* 1993, Larsson *et al.* 1994, Schiaffino and Reggiany 1996, Soukup and Jirmanová 2000, Vadászová *et al.* 2004, Vadászová-Soukup and Soukup 2007, Arnostova *et al.* 2011, Schiaffino 2010, Novák and Soukup 2011). Changes of fiber type composition in adult differentiated muscles are, however, only in a range of several percent, while changes in regenerated muscle are much more pronounced (Snoj-Cvetko *et al.* 1996a, b, Erzen *et al.* 1996). For the physiological change of muscle performance one should expect changes of both contractile and ECC machinery including changes of CaBPs. In order to better understand the chronic effects of thyroid hormone alterations on skeletal and heart muscles, we have investigated expression of CSQ1 and fiber type composition in fast extensor digitorum longus (EDL) and slow soleus (SOL) skeletal muscles and CSQ1 levels also in the heart left ventricles (LV) of euthyroid (EU), hypothyroid (HY) and hyperthyroid (TH) adult rats at *mRNA* and/or protein levels. The preliminary results were published previously (Novák *et al.* 2008, 2010a, Novák and Soukup 2011).

MATERIAL AND METHODS

Animals Experiments were performed on adult inbred Lewis strain rats of both sexes obtained from the authorized laboratory rat-breeding unit of the Institute of Physiology, Academy of Science of the Czech Republic, v. v. i., Prague (Accreditation No. 1020/491/A/00). They were housed at 23±1 °C and at 12-hour light-dark cycle periods (6:00 a.m. to 6:00 p.m.) with *ad libitum* access to water and a complete laboratory diet. The maintenance and handling of experimental animals were in accordance with the EU Council Directive (86/609EEC) and the investigation was approved by the Expert Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, v. v. i., Prague. The animals were anesthetized with intraperitoneal injections of 1ml (100 mg) of Narketan (Ketaminum ut hydrochloridum) per 1000 g of body weight, followed by 0.5 ml (10 mg) of the myorelaxant Xylapan (Xylazinum ut hydrochloridum) per 1000 g of body weight

(Vetoquinol S.A. France and Vetoquinol Biowet Poland, respectively) and sacrificed by an overdose of the anesthetic.

Heterochronous isotransplantation The SOL or EDL muscles of 3- to 4-week-old inbred Lewis rats were intramuscularly grafted either into the host EDL or SOL muscles of 2-month-old animals of the same strain (Jirmanová and Soukup 1995, Soukup and Jirmanová 2000) under anesthesia described above and the muscles were analyzed approximately 8 months later.

Alteration of the thyroid status The hyperthyroid status (TH) was induced in 4-week-old animals or in 2-month-old rats in case of transplantation and maintained during the experiment by intraperitoneal injections of 3,3',5-triiodo-L-thyronine (sodium salt, T_3, 0.15 μg/kg body weight) three times a week. The hypothyroid (HY) status was induced in 4-week-old animals or in 2-month-old rats in case of transplantation and maintained during the experiment with a 0.05 % solution of methimazole (2-mercapto-1-methylimidazole) in drinking water. The euthyroid (EU) rats were age-matched littermates of the TH and HY animals. The effect of the procedure was checked by measuring total T_3 (tT_3) and total T_4 (tT_4) levels by a radioimmunoassay using commercial RIA kits (Immunotech - Beckman Coulter Co., Prague, Czech Republic) for human and rat sera (Pavelka 2002, Fig. 5A), by measurement of GPDH activity (Rauchová *et al.* 2004, 2011, 2013) and of heart and thyroid gland weights (Soukup *et al*. 2001, Fig. 5B,C); all these parameters are known to be affected by thyroid hormone level alterations and their measurements clearly demonstrated efficiency of our procedure.

Specimen manipulation Specimens were excised from 12 EU, 7 HY and 8 TH rats. Dissected EDL and SOL muscles and heart LV were frozen in liquid nitrogen and divided into three portions. One was quickly placed into a RNA later stabilization reagent (Qiagen, Valencia, USA) and kept at 4°C until *RNA* isolation in order to demonstrate CSQ transcripts. The second was kept at -80 ^{o}C for SDS-PAGE and Western blot analysis of CSQ1. The last portion was immediately cut on a Leica 3000 cryocut for the detection of muscle fiber types. Hearts and thyroid glands were quickly

weighed. Serum was prepared from blood samples by centrifugation for 15 min (1260 x g at 4 ºC) and frozen before it was used for measuring thyroid hormone levels.

SDS-PAGE and immunoblotting of CSQ Muscles were mechanically disrupted in a teflon/glass homogenizer in cold HEPES buffer (50 mM HEPES adjusted to pH 7.6 with NaOH, 75 mM NaCl, 1 mM $MgCl_2$ and 1 mM EGTA) supplemented with protease (Roche; Complete EDTA-free protease mixture) and phosphatases (1 mM Na_3VO_4 and 1 mM NaF) inhibitors and then centrifuged at 20,000 g for 15 min. The collected supernatant representing the cytosolic fraction was boiled with a SDS-sample buffer for 5 min. One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretic transfer of separated protein onto nitrocellulose membranes and details of the immunostaining procedure are described elsewhere (Dráber *et al.* 1988). For immunoblotting, rabbit antibodies to skeletal CSQ1 isoform (Sigma C0743) and GAPDH (Sigma C9545) were diluted 1:30000 and 1:300000, respectively. Peroxidase-conjugated secondary antibodies were diluted 1:10000. Bound antibodies were detected by WestPico Chemiluminescent reagents (Pierce). Exposed autoradiography films X-Omat AR (Eastman Kodak), images captured with LAS-3000 (Fuji) and photos of gels stained with Coomassie Brilliant Blue (CBB) were quantified by densitometry using AIDA software. The results were expressed in arbitrary units adjusted to CBB staining and GAPDH expression. Data are presented as mean ± SD of four gels.

RNA isolation and RT-PCR Total cellular *RNA* was extracted from each muscle sample using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The purity and integrity of the *RNA* preparations were checked spectroscopically and by agarose gel electrophoresis before the analytical procedures were carried out. One microgram of total *RNA* was converted to cDNA using the ImProm-II RT kit (Promega, Madison, USA) with random hexamer primers according to the manufacturer's instructions. 20 ml of the cDNA reaction were diluted 1:3 in DEPC-treated water to eliminate inhibition of Taq polymerase. 1 ml of diluted cDNA was amplified in 10 ml of PCR reaction mixture containing QuantiTect SYBR Green PCR Master Mix (QIAGEN, Valencia, CA, USA) and

0.5 mM of each gene-specific primer for the studied gene (CSQ1) or the housekeeping gene (b-2-microglobulin) according to the manufacturer's instructions. The sequences of primers designed in our laboratories using the LaserGene PrimerSelect program (DNAStar) were as follows:

CSQ1(GenBank no. XM_001063867)

forward: 5' -TTCCTCCTCCTGACCCTTTT– 3'

reverse: 5' –CCTGGAGCAACTGCTGACA– 3'

β-2-M (GenBank no. NM_012512)

forward: 5' –CGCTCGGTGACCGTGATCTTTCTG– 3'

reverse: 5' –CTGAGGTGGGTGGAACTGAGACACG– 3'

Real-time PCR reactions were performed on Mastercycler EP realplex (Eppendorf, Wesseling-Berzdorf, Germany) with the following thermo profile: initial denaturation at 95°C for 15 min, 50 cycles with 15 s denaturation at 94°C, 20 s annealing at 60°C (primer-specific temperature) and 15 s elongation at 72°C. Fluorescence was determined during each elongation step. At the end of each run, melting curve analysis was performed to ascertain the presence of a single amplicon. Standard curves were generated for each PCR run from single cDNAs by 3-fold serial dilution. Quantification was performed using the Realplex software version 1.5 (Eppendorf, Wesseling-Berzdorf, Germany) and applying second derivative maximum analysis. The expression of analyzed genes was normalized to expression of b-2-microglobulin. Non-template and non-RT control reactions were performed in each PCR run. To ensure that PCR products from qRT-PCR had the expected size, an agarose gel electrophoresis was performed. The data (means ± SD of four measurements) are presented as a percentage of the *mRNA* level in the EU EDL muscle.

Myofibrillar ATPase, immunohistochemistry and quantitative morphological analysis Muscle fiber types were determined on fresh frozen muscle cross sections 10 μm thick according to their activity of mATPase (E.C.3.6.1.3) after alkaline (pH 10.3) and acid (pH 4.5 and 4.3) pre-incubations (Guth and Samaha 1970) or the muscle sections were incubated with different mouse

monoclonal antibodies (mAbs) specific for rat MyHC isoforms BA-D5 (MyHC-1), SC-71 (MyHC-2a), BF-35 (all MyHC except -2x/d) and BF-F3 (MyHC-2b) (cf. Schiaffino *et al.* 1986, Developmental Studies Hybridoma Bank). Additionally, mAbs anti Slow (MyHC-1) and anti-Fast (MyHC-2), both provided by Biotrend or Medac/Novocastra, were used to further distinguish slow and fast MyHC isoforms. Primary antibody binding was revealed using donkey secondary antibody conjugated with HRP (Jackson Immunoresearch Laboratories, USA). (For further details see Soukup *et al.* 2002, 2009, Smerdu and Soukup 2008, Novák *et al.* 2010b, Říčný and Soukup 2011).

The numerical (N) proportions (%) of muscle fiber types were assessed by 2-D stereological methods using the principles of an unbiased counting frame and point counting (Zacharova and Kubínová 1995). The stereological measurements were performed using the C.A.S.T. Grid System (Olympus, Albertslund, Denmark). In order to attain realistic estimates of the parameters measured, the concrete arrangement of the stereological system (size of the counting frame, scanning interval) were chosen according to the muscle section size and fiber composition, on the basis of efficacy analysis described in our previous papers (Zacharova and Kubinova 1995, Zacharova *et al.* 1997, 1999, 2005).

Statistical analysis The data were expressed as mean ± SD and the differences between groups were evaluated using the SigmaStat program, Systat software, Germany.

RESULTS

Changes of CSQ1 expression

Using Western blot analysis (Fig. 1), we found that the mean protein levels of CSQ1 in the inbred Lewis strain rats were highest in the fast EDL in all thyroid states. Less than half of this amount was present in the SOL muscle and hardly detectable levels were found in the LV (Fig. 2). Also on the *mRNA* level, the CSQ1 transcript was highest in the EDL, more than 5 times less was found in the SOL and very low level was detected in the LV (Fig. 3). TH status induced at protein level a

relative increase (by about 25%) and HY status a decrease (by about 25%) in CSQ1 expression in the EDL muscle, but its ratio to the EDL in the SOL and LV did not change (Fig. 2). At *mRNA* level, CSQ1 relative transcript level in HY status in both EDL and SOL muscles was about 50% lower than in EU status, but no significant change was observed in the LV (Fig. 3). TH status did not induce significant differences in CSQ1 *mRNA* level compared to EU status.

The regenerated SOL muscle transplanted into EDL, as well as the EDL transplanted into SOL exhibited CSQ1 protein levels corresponding to the host muscle and not to the original muscle graft source in all thyroid states (Fig. 4). On the other hand, no difference in CSQ1 expression was observed in grafts transplanted to the same host muscles (Fig. 4). CSQ1 transcript level of the SOL transplanted into the EDL was higher than in the control SOL and lower than in the host EDL (Fig. 3, S/E EU).

The same proportions of CSQ1 expression at protein level under all thyroid states were found when the values were corrected to GAPDH or to CBB.

Changes of fiber type composition

The same group of animals as in the case of CSQ1 expression was analyzed for determination of fiber type composition using either monoclonal antibodies specifically recognizing MyHC isoforms (Fig. 6) or mATPase reaction (Figs 7 and 8). Immunohistochemical evaluation showed that the EDL of EU rats contained 92.5 % of fast 2B, 2X/D and 2A fibers complemented by 7.5 % of slow type 1 fibers (Table 1, EDL EU). The number of 2B plus 2X/D fibers increased in the EDL from 71.0% in EU to 81.2% in TH rats, while the number of 2A and type 1 fibers decreased by about 5% each (Table 1, EDL TH). In the HY rats, in contrast, the number of type 1 and 2A fibers of the EDL increased by about 3 and 10% respectively, while that of 2X/D and 2B decreased by 5 and 8%, respectively, compared to EU animals (Table 1, EDL HY).

The analyzed SOL muscles were in EU rats almost entirely composed of type 1 fibers (99.2 %) supplemented by occasional 2A or 2C fibers (the latter reacting with both anti slow and anti fast MyHC antibodies) and no pure 2X/D and 2B fibers were determined by immunostaining (Table 1, SOL EU). In the SOL of TH rats, however, , **t**he percentage of 2A and 2C fibers increased from about 1% to about 32% with an equivalent decrease of slow type 1 fibers, but no immunochemically pure 2X/D or 2B fibers appeared (Table 1, SOL TH). Hypothyroid SOL (Table 1, SOL HY) attained almost 100% of slow type 1 fibers, which was a small shift compared to EU animals, but a significant difference compared to TH rats.

The regenerated muscles grafted into the opposite muscle type (SOL to EDL or EDL to SOL) showed a significant switch of their fiber type composition towards the host muscle. On the other hand, grafts transplanted into the same muscle type (EDL to EDL, SOL to SOL) did not change their fiber type proportions significantly.

Discussion

Our present experiments demonstrated a high expression of CSQ1 in the fast EDL muscles composed in the majority of fast type 2X/D and 2B fibers. Its expression in the slow SOL muscles containing mainly slow type 1 and a few fast 2A fibers was less than one half compared to the EDL muscle. HY status decreased and TH status increased expression of CSQ1 in the EDL, but neither altered thyroid status had any effect on CSQ1 expression in the SOL. The increased thyroid hormone level thus tends to increase both the amount of CSQ1 and the percentage of the fastest 2B and 2X/D fibers in the EDL muscle. Our results thus indicate that 2X/D and 2B fibers contain more CSQ1 than type 1 and 2A fibers and that the thyroid hormone induced changes in CSQ1 expression in the EDL may be related to changes in their proportion.

Comparison of CSQ 1 levels and fiber type changes

Skeletal muscle fiber types 1, 2A, 2X/D and 2B fibers (for review see e.g. Hämäläinen and Pette 1993, Schiaffino and Reggiani 1996, Berchtold *et al.* 2000, Pette and Staron 2001, Pette 2002, Schiaffino 2010) were recognized in Lewis rats by immunostaining with specific monoclonal antibodies against individual MyHC isoforms, mATPase reaction or *mRNA* expression (Zacharova *et al.* 1999, 2005, Soukup and Jirmanová 2000, Soukup *et al.* 2002, 2009, Vadászová *et al.* 2006a,b, Smerdu and Soukup 2008, Zurmanova *et al.* 2008, Novák *et al.* 2010b, Soukup and Zurmanova 2012). The EDL muscles of adult Lewis strain rats contain a low number of slow type 1 fibers and a prevailing percentage of fast fibers. On the other hand, the SOL of the Lewis strain rats is composed of a great majority of slow type 1 fibers complemented by a variable but very low number of fast 2A or mixed 2C fibers. The values in Table 1 based on immunohistochemical evaluation closely resemble the recently published quantitative data based on mATPase reaction that, however, differentiates only 3 muscle fiber types (Novák *et al.* 2010b). Our comparison of fiber type and CSQ level changes in the EDL muscle indicates that the observed increase of the CSQ1 level in TH status can be caused by an increase in the fastest 2B and 2X/D fibers at the expense of 2A and type 1 fibers, while the decreased level of CSQ1 in HY rats can result from a decline in the proportion of these fibers and in an increase in 2A and type 1 fibers. No changes of CSQ1 levels in the SOL can be explained by the lack of fast 2X/D and 2B fibers, assuming that type 1 and 2A fibers express corresponding amount of CSQ1. Murphy *et al.* (2009) found more than a 3x higher content of CSQ1 in EDL fast type 2 fibers compared to SOL slow type 1 fibers and they also presumed the existence of differences in CSQ1 levels between subgroups of fast type 2 fibers. These results and our immunohistochemical analysis differentiating the 2B and 2X/D fibers are consistent with the idea that the 2B and 2X/D fibers contain higher levels of CSQ1 than do 2A and type 1 fibers.

The skeletal isoform CSQ1 is the only isoform found in fast-twitch muscles and is the major isoform in slow-twitch muscles (for review see Beard *et al.* 2004, 2009, Novák and Soukup 2011) and, according to our results, relative CSQ1 levels in the SOL were less than half of the EDL level.

The CSQ1 level should be naturally higher in fast-twitch compared to slow-twitch muscles, as the amount of released calcium that must be taken up at a given moment is much higher in fast than in slow muscles. Similarly to our measurements, the content of CSQ1 in homogenates of rat fast EDL and slow SOL was determined as 1.4±0.1 and 0.43±0.3 mg/g muscle wet weight, respectively (Murphy *et al.* 2009) and the CSQ1 to cardiac CSQ2 ratio in the rabbit soleus muscle was reported to be ~3:1 (Damiani *et al.* 1990) or higher (Fliegel *et al.* 1989).

Murphy *et al.* (2009) also described the amount of CSQ1 in the SOL II (2A) fibers as intermediate and about twice as high as in type 1 fibers. However, they used 5-8 months old Long Evans hooded rats containing according to them about 20% of 2A fibers. Still, they estimated the content of type 1 fibers as ~30% of EDL fibers. As the SOL of adult Lewis rats contains almost 99% of slow type 1 fibers in EU (see also Novák *et al.* 2010b) and practically 100% in HY rats (see also Novák and Soukup 2011), we can conclude that type 1 fibers should, at least in Lewis rats, also contain "intermediate" CSQ1 levels. We cannot ascribe this intermediate content to type 2A fibers that are so few in Lewis rats. Furthermore, type 1 fibers must have a similar CSQ1 content as 2A fibers seeing that the increase in the content of 2A fibers by 30% in TH rat SOL is not reflected by a corresponding change in the CSQ1 content. However, there is still a possibility that the anti-CSQ1 antibody cross-reacts to some extent with CSQ2, although the product information clearly states that C 0743 anti CSQ1 rabbit polyclonal antibody specifically recognizes rat and mouse CSQ1 (~60kDa) by immunoblotting. Unfortunately, we were not able to benefit from the determination of CSQ2 by C 2491 antibody to uncover whether the altered thyroid status has any effect on its levels. Our results thus suggest that the observed changes in CSQ1 expression in rat muscles resulting from chronic alteration of thyroid hormone levels are more likely part of complex fiber type changes induced by thyroid hormones and revealed by the switch of MyHC isoforms and muscle fiber types.

The character of muscle contraction (twitch) is to a large extent dictated by sarcoplasmic reticulum (SR) properties and apparently the CSQ content is related to the appearance of the SR.

Schiaffino *et al.* (1970) found that most fibers in the EDL muscle have a richly developed SR, which is consistent with rapid calcium release and uptake during the contraction-relaxation cycle, contrasting with the major type 1 slow fibers of the soleus muscle with a relatively poorly developed SR. Normalization of CSQ to GAPDH, which is a cytosolic housekeeping protein, may not reflect change in SR volume, which is likely to be affected by thyroid hormone alterations in accordance with fiber type changes. However, because we attained in both muscles under all thyroid states a corresponding proportion of CSQ1 expression adjusted to CBB as to GAPDH and because all fast EDL fibers possess rich SR (Schiaffino *et al.* 1970), we believe that the observed differences in CSQ1 content are fiber type specific.

The regenerated muscles grafted into the opposite muscle type innervated by the collaterals of host axons (Jirmanová and Soukup 1995, 2001, Soukup and Novotová 2000) showed a significant switch of their fiber type composition towards the host muscle, on the other hand, grafts transplanted into the same muscle did not change their fiber type proportions significantly. As expected, changes observed in the regenerated muscles after isotransplantation into the opposite host muscle (SOL to EDL and EDL to SOL) were much more pronounced compared to changes in non-grafted muscles. Both CSQ1 levels and fiber type composition were altered by tens of percent, almost acquiring levels typical for the host muscles themselves. These changes were apparently only slightly affected by the grafting procedure itself, as grafting into the same muscle conserved more or less the CSQ1 level and fiber type composition typical for a given muscle. Similarly, altered CSQ levels were found in transforming skeletal muscles (Maguire *et al.* 1997) and altered calcium-sequestering proteins after chronic stimulation were found in rabbit muscles (Leberer *et al.* 1986). Comparison of CSQ1 expression and fiber type changes in regenerated muscle is thus consistent with the premise that both the level of CSQ1 and fiber type composition change in concurrence and that the expression of CSQ1 apparently proceeds in a fiber type-specific manner. CSQ gene transcription would thus be regulated by T_3 coordinately with other proteins rebuilt

during muscle fiber type transformation. The final answer can be obtained e.g. by single fiber analysis of MyHC isoforms and the CSQ1 content.

Evaluation of thyroid status and effects of thyroid hormones on CaBPs

Experimental studies analyzing the effects of thyroid hormone levels on CaBPs expression have mainly focused on cardiac muscle apparently due to the profound impact of thyroid hormones on heart function (for review see Dillmann 1990, Gupta 2007, Tribulová *et al.* 2010). Much less is known about the regulation of Ca^{2+} transport systems due to altered concentrations of thyroid hormones in skeletal muscle (e. g. Simonides and van Hardeveld 1985, Arai *et al.* 1991, Connelly *et al.* 1994, Hudecová *et al.* 2004, for review see e.g. Berchtold *et al.* 2000). These studies of *mRNA* and protein changes in the TH and HY states suggest that CaBPs of the SR responsible for calcium release and uptake (DHPR, RyR, SERCA, NCX) are all coordinately regulated in response to thyroid hormone level in skeletal and heart muscles. The aforementioned CaBPs were increased in TH and decreased in HY status after acute 4- or 8-day (Arai *et al.* 1991) or chronic exposition (Hudecová *et al.* 2004). Studies related to CSQ changes in animals with altered thyroid status are exceptional. Apart from a brief mention in CSQ review (Novák and Soukup 2011), it has only been reported that acute changes of thyroid status for 4 and 8 days in the rabbit soleus may suggest that hyperthyroidism increases expression of CSQ1 compared to CSQ2, while hypothyroidism usually has the opposite effect (Arai *et al.* 1991). Taken together, these results suggest that SR calcium storage capacity (CSQ) is less affected by thyroid hormone alterations than are calcium release (RyRs) and uptake (SERCA, NCX).

Conclusions

We found corresponding changes of CSQ1 levels and fiber type composition both in normal and regenerated muscles and therefore we suggest that CSQ1 changes may be related to complex fiber

type transformation caused by thyroid hormone alterations rather than by the direct effect of thyroid hormones on CSQ1 gene transcription.

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Text to tables

Table 1

Mean fiber type composition (expressed as percentage ± SD) from of the extensor digitorum longus (EDL) and soleus (SOL) muscles based on immunohistochemical staining of adult hypothyroid (HY, treated with methimazole), euthyroid (EU) and hyperthyroid (TH, treated with triiodothyronine) adult Lewis rats. 1 - slow type 1 fibers, 2A - fast 2A and 2C, 2X/D - fast 2X/D and 2B - fast 2B fibers. n = number of analyzed muscles. *significant difference ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) compared to EU status.

Text to figures

Fig. 1 An illustrative Western blot of skeletal calsequestrin (CSQ1) in the fast extensor digitorum longus (EDL) and slow soleus (SOL) skeletal muscles of euthyroid (EU), hyperthyroid (TH, treated with triiodothyronine) and hypothyroid (HY, treated with methimazole) adult Lewis rats. After separation of muscle proteins by SDS-PAGE, CSQ1 was revealed on Western blots by specific rabbit polyclonal antibody C 0743 (Sigma). Corresponding amounts of GAPDH are shown in the bottom panel as loading control (from Novák and Soukup, Physiol Res 60: 439-452, 2011, with the kind permission of the Journal).

Fig. 2 The mean relative content of CSQ1 determined on Western blots in the fast extensor digitorum longus (EDL), slow soleus (SOL) muscles and heart left ventricles (LV) of euthyroid (EU), hyperthyroid (TH, treated with triiodothyronine) and hypothyroid (HY, treated with methimazole) adult Lewis rats. The results are expressed in adjusted arbitrary units. The CSQ1 content in the EDL of EU rats was considered as 100. *significant difference ($p \leq 0.05$) compared to the EU EDL (obvious difference of the LV is not marked), $^{\#}$significant difference ($p \leq 0.05$) between TH and HY states.

Fig. 3 Real-time RT-PCR of CSQ1 *mRNA* levels in the fast extensor digitorum longus (EDL), slow soleus (SOL) and left ventricle (LV) of euthyroid (EU) and hypothyroid (HY, treated with methimazole) adult Lewis rats. S/E indicates unilateral intramuscular isotransplantation of the SOL (S) muscle into the EDL (E) muscle. Note that the CSQ1 *mRNA* levels are highest in the EDL, low in the SOL and very low in the LV. The expression of analyzed genes was normalized to the expression of beta-2-microglobulin. The data (means ± SD of four measurements) are presented as a percentage of the *mRNA* level in the EU EDL muscle.

Fig. 4 A,B

The mean relative content of CSQ1 determined on Western blots of regenerated EDL or SOL muscles transplanted into EDL (**A**, E/E, S/E) and SOL (**B**, S/S, E/S) muscles in comparison with the contralateral EDL or SOL muscles in euthyroid (EU), hyperthyroid (TH, treated with triiodothyronine) and hypothyroid (HY, treated with methimazole) adult Lewis rats. The CSQ1 content in the EDL of EU rats was considered as 100. S/E indicates unilateral intramuscular isotransplantation of the SOL (S) muscle into the EDL (E) muscle, S/S isotransplantation of the SOL into the SOL, E/S isotransplantation of the EDL into the SOL and E/E of the EDL into the EDL muscle. *significant difference ($p \leq 0.05$) between the graft and the EDL in the same thyroid status, $^{\#}$significant difference ($p \leq 0.05$) between the graft and the SOL in the same thyroid status (only transplantation with different donor and host muscles are marked).

Fig. 5 Absolute serum levels of total triiodothyronine (**A**) and relative weights of thyroid gland (thyroid gland/body weight, **B**) or heart (heart/body weight, **C**) in hypothyroid (HY, treated with methimazole), euthyroid (EU) and hyperthyroid (TH, treated with T_3) adult Lewis rats used for the CSQ1 analyses. *significant difference ($p \leq 0.05$) compared to EU, $^{\#}$significant difference ($p \leq 0.05$) between TH and HY rats.

Fig. 6 A-D

Examples of cross sections demonstrating immune reactivity of the extensor digitorum longus muscle of adult Lewis euthyroid rats. **A**: BA-D5 staining type 1 fibers, **B**: SC-71 staining 2A fibers, **C**: BF-35 staining all fibers except 2X/D and **D**: BF-F3 staining 2B. Bar indicates 100 μm.

Fig. 7A-C

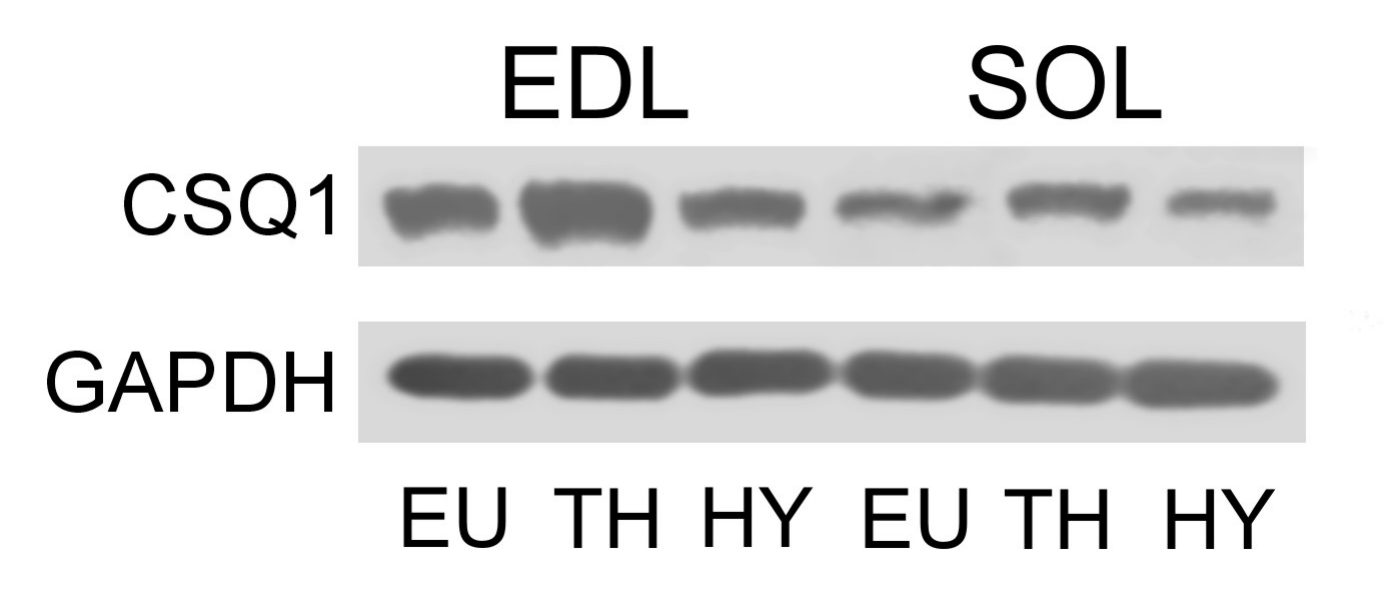
Examples of cross sections demonstrating mATPase reaction after pH 4.5 pre-incubation of the extensor digitorum longus muscles of adult hypothyroid (**A**), euthyroid (**B**) and hyperthyroid (**C**) inbred Lewis strain rats. Note a higher number (9.2%) in HY (**A**) and a lower number (1.3%) in TH (**C**) of darkly stained type 1 fibers compared to EU (**B**, 7.4%). In TH (**C**) also note higher incidence of medium stained 2B fibers (75.4%) compared to HY rats (68.3%). Bar indicates 100 μm.

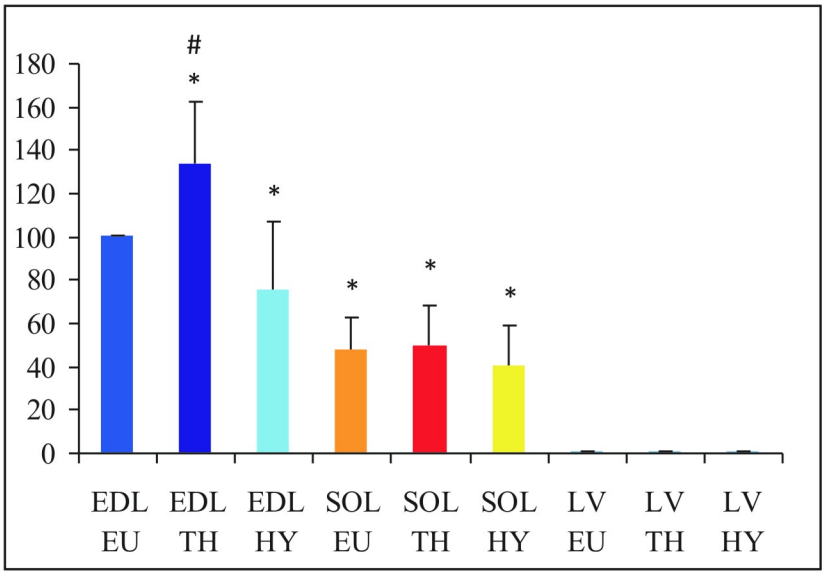
Fig. 8 A-C

Examples of cross sections demonstrating alkali-stable mATPase reaction after pH 10.3 pre-incubation of the soleus muscles of adult hypothyroid (**A**), euthyroid (**B**) and hyperthyroid (**C**) Lewis strain rats. The same image was achieved using the SC-71 monoclonal antibody. Slow type I fibers are marked as 1 and fast type 2A fibers as 2A. Bar indicates 50 μm.

Table 1

THYROID STATUS	**HY**	**EU**	**TH**
EDL	*n=13*	*n=20*	*n=10*
1	10.7±5.2*	7.5±7.0	2.9±3.2***
2A	30.9±10.4**	21.5±6.3	15.9±6.4
2X/D	19.6±4.5	24.5±8.2	31.9±4.0
2B	38.8±10.9***	46.5±7.6	49.3±8.7
THYROID STATUS	**HY**	**EU**	**TH**
SOL	*n=13*	*n=24*	*n=10*
1	99.6±1.2	99.2±1.2	68.2±10.2***
2A	0.4±1.2	0.8±1.2	31.8±10.2***
2X/D	0.0	0.0	0.0
2B	0.0	0.0	0.0


EDL
SOL
CSQ1
GAPDH
EU TH HY EU TH HY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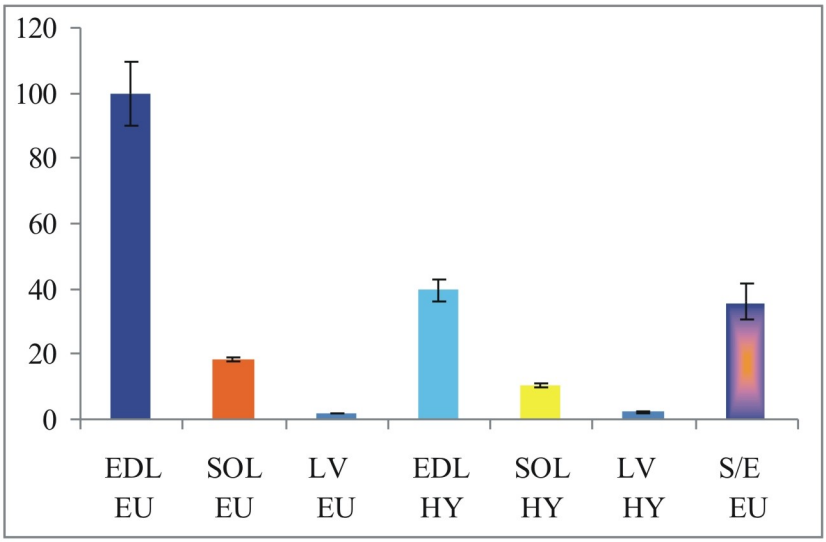
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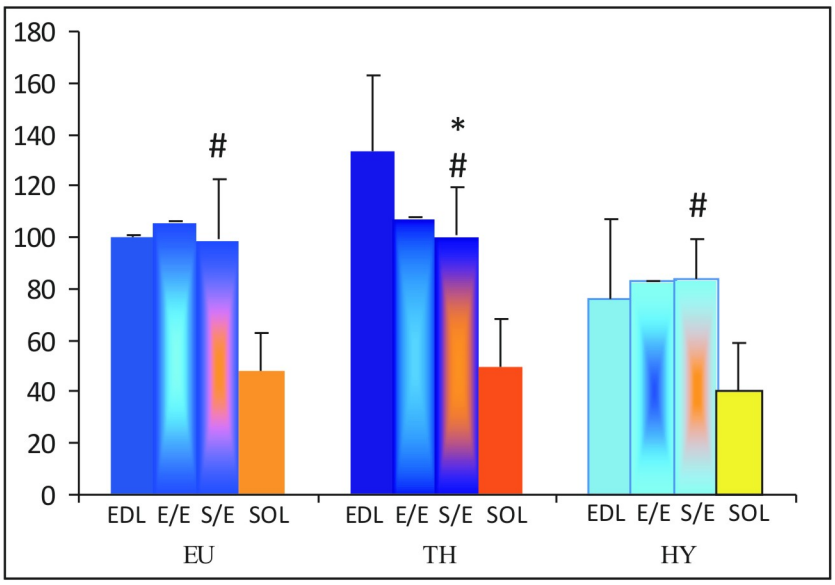
A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EU
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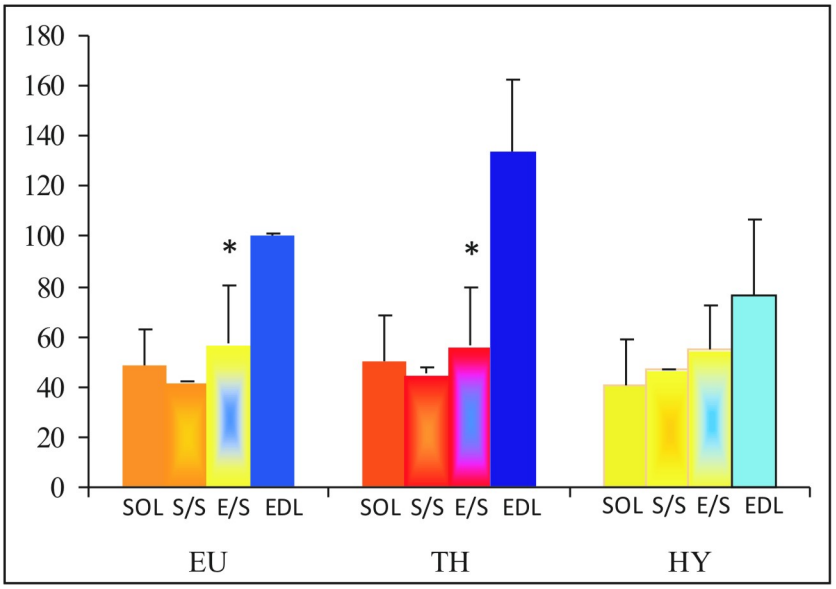
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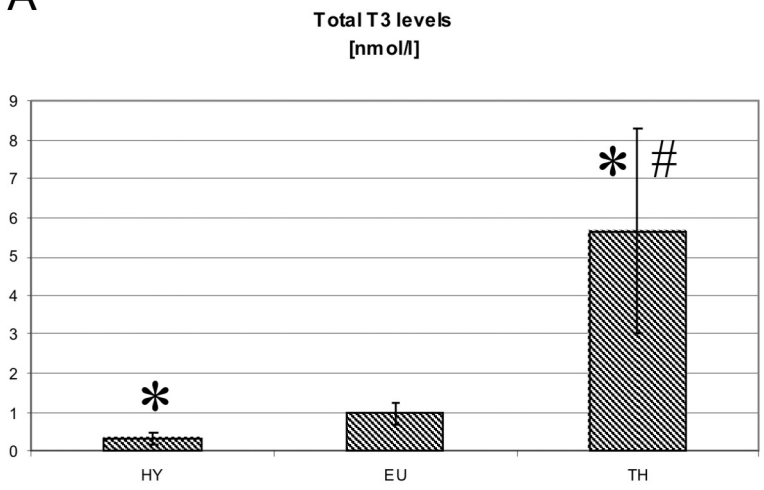
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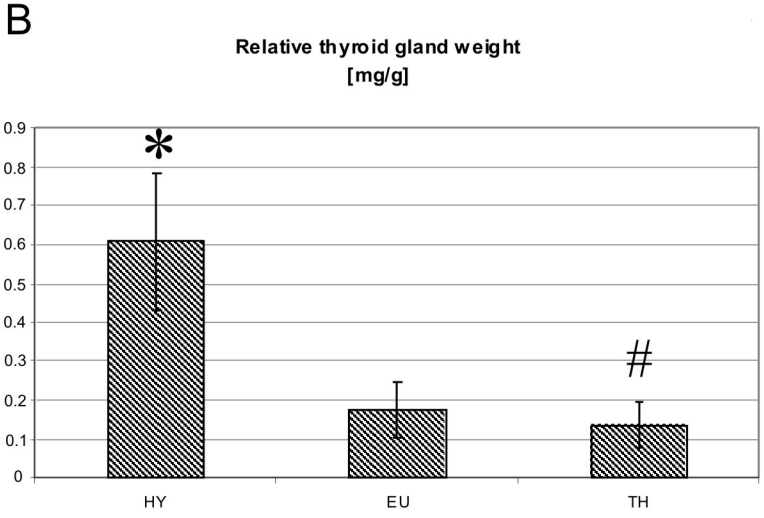
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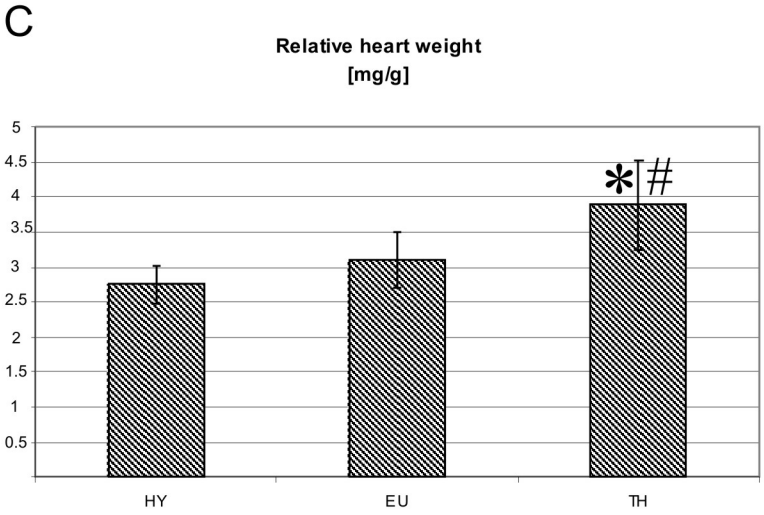
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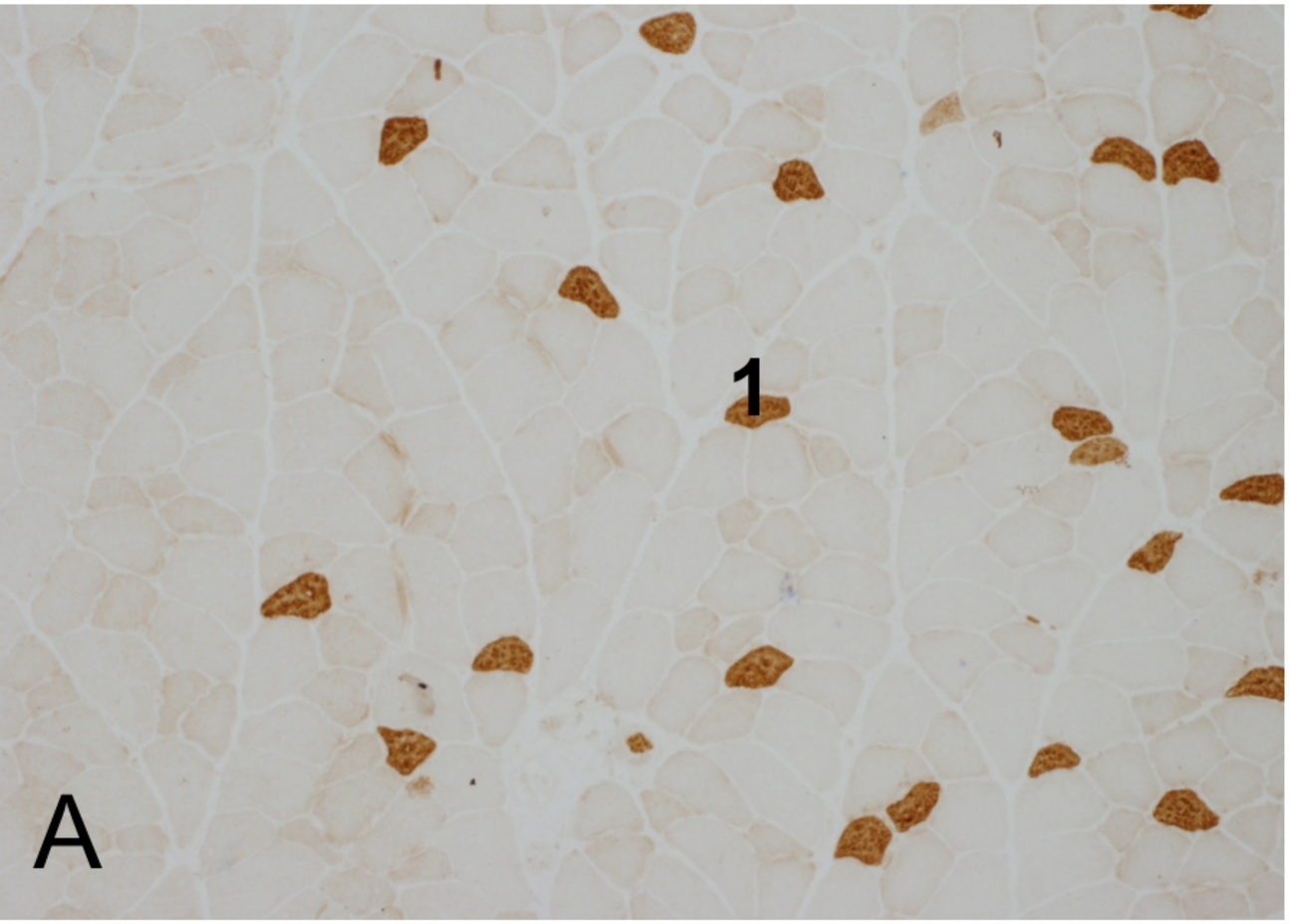
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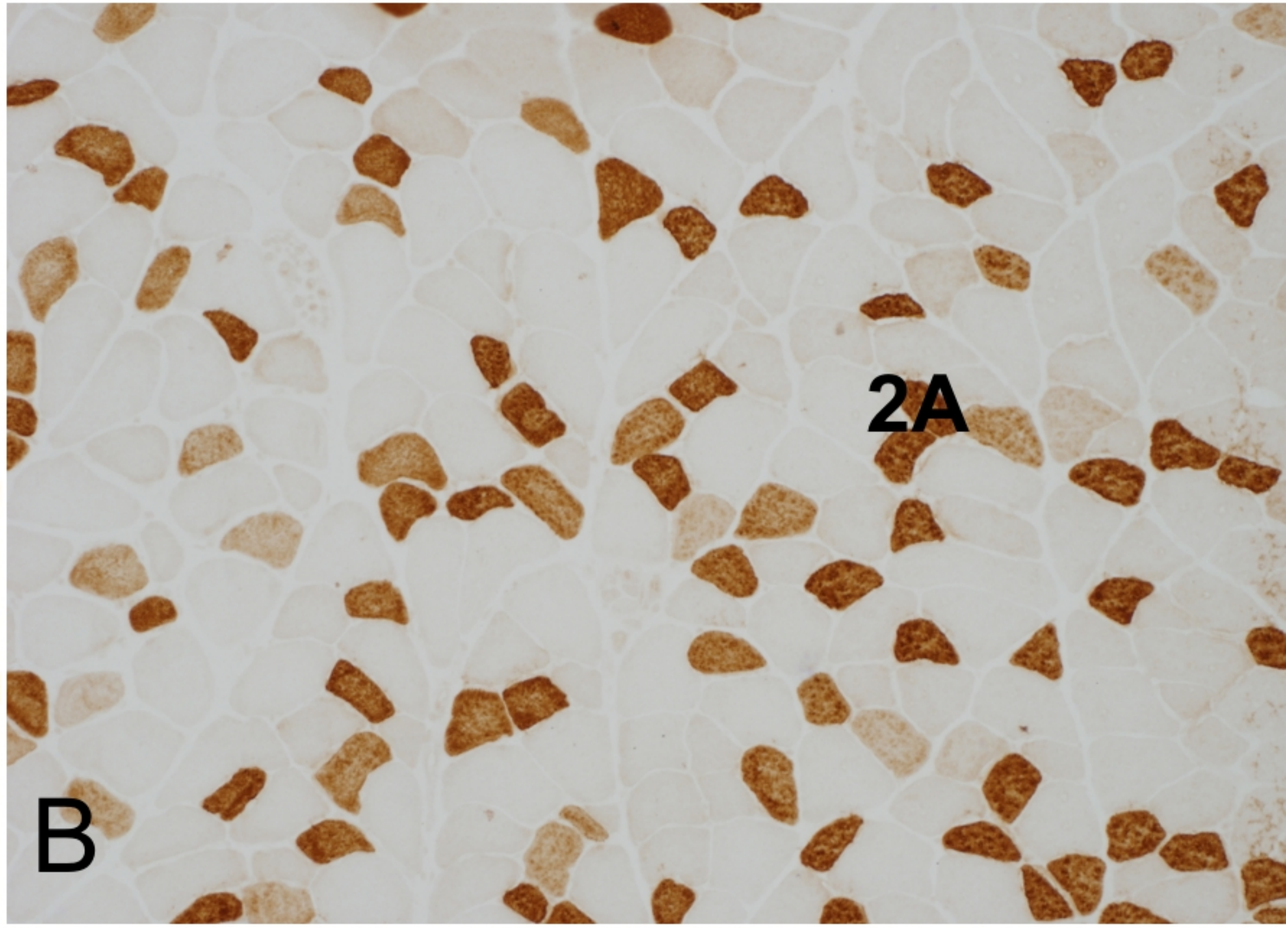
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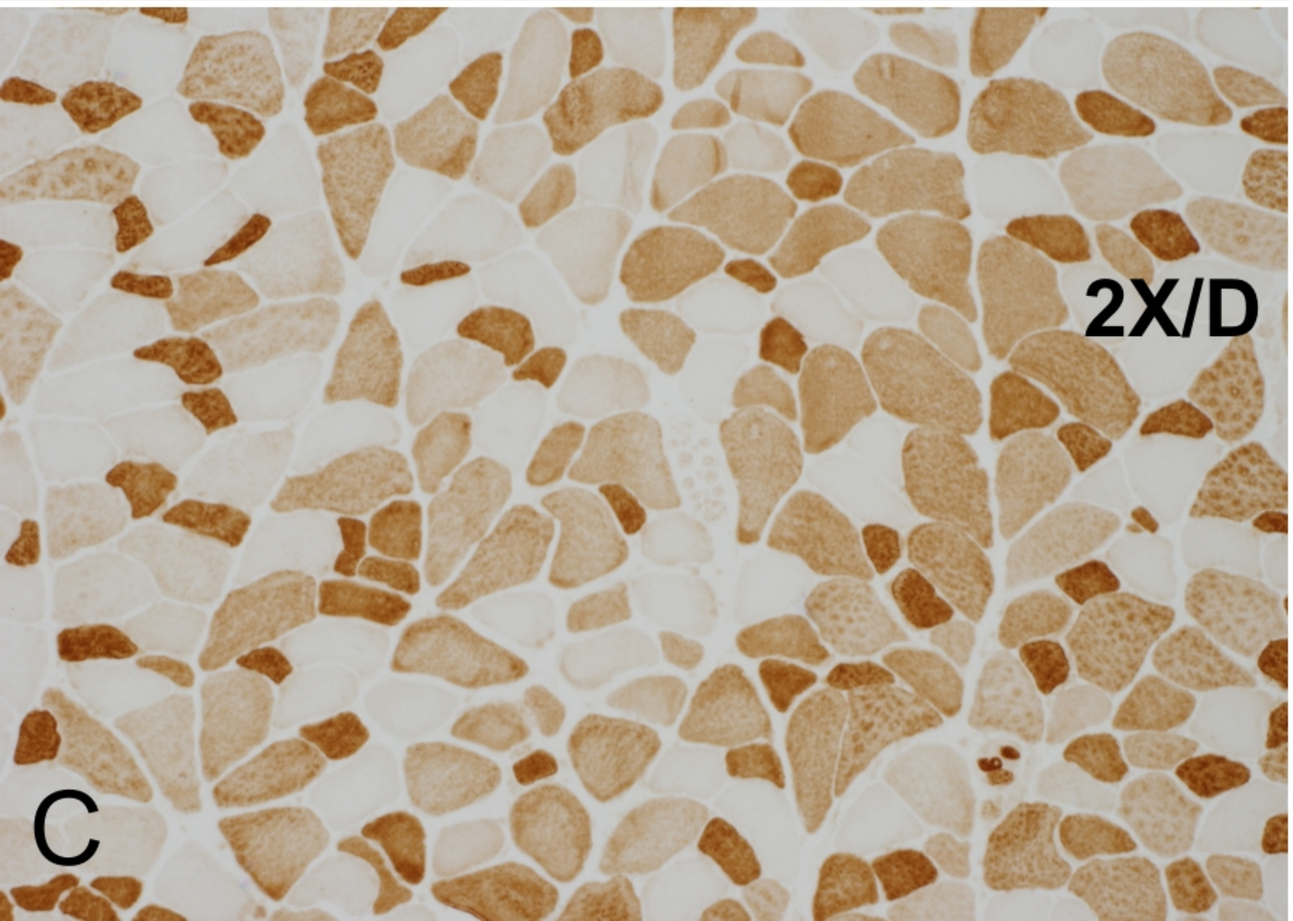
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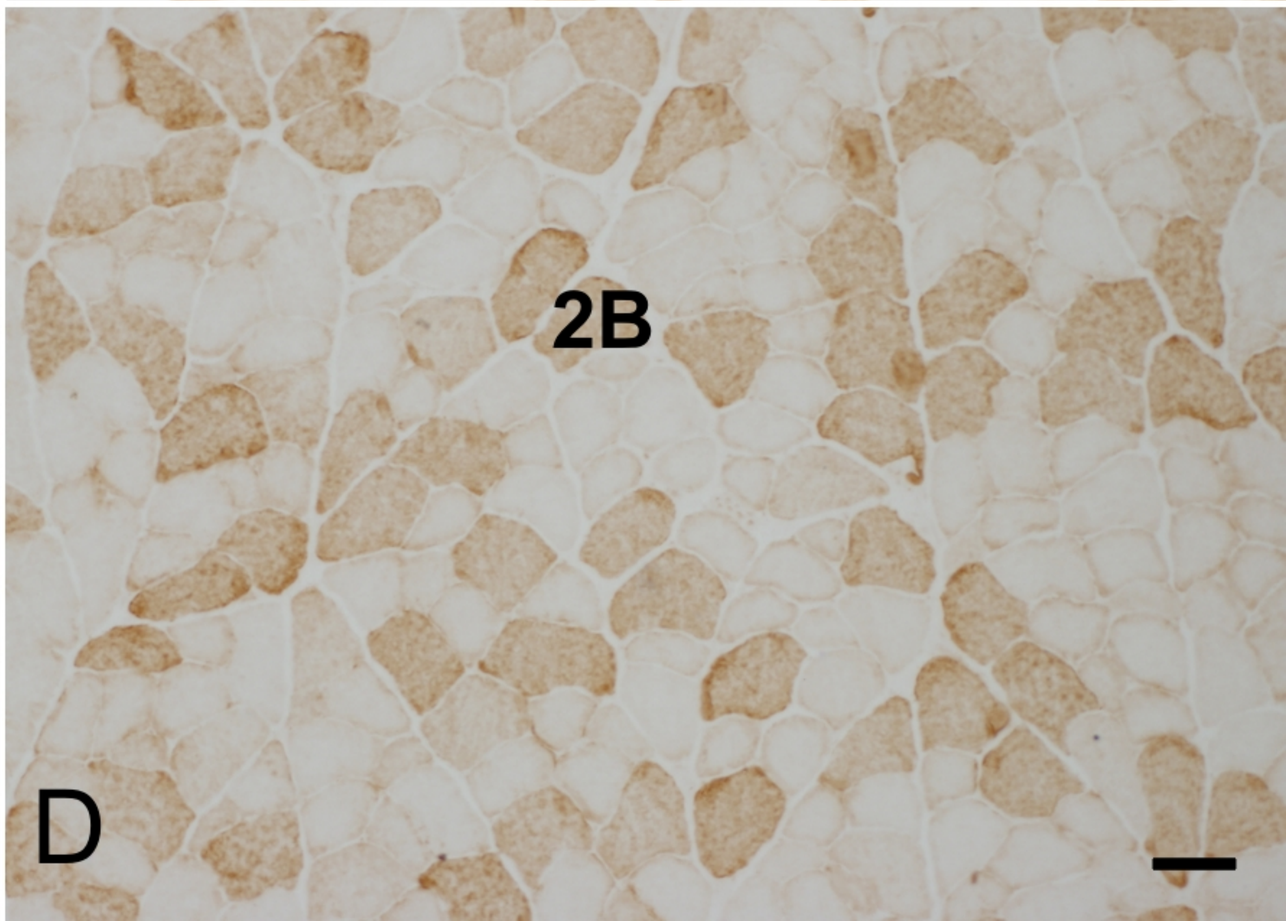
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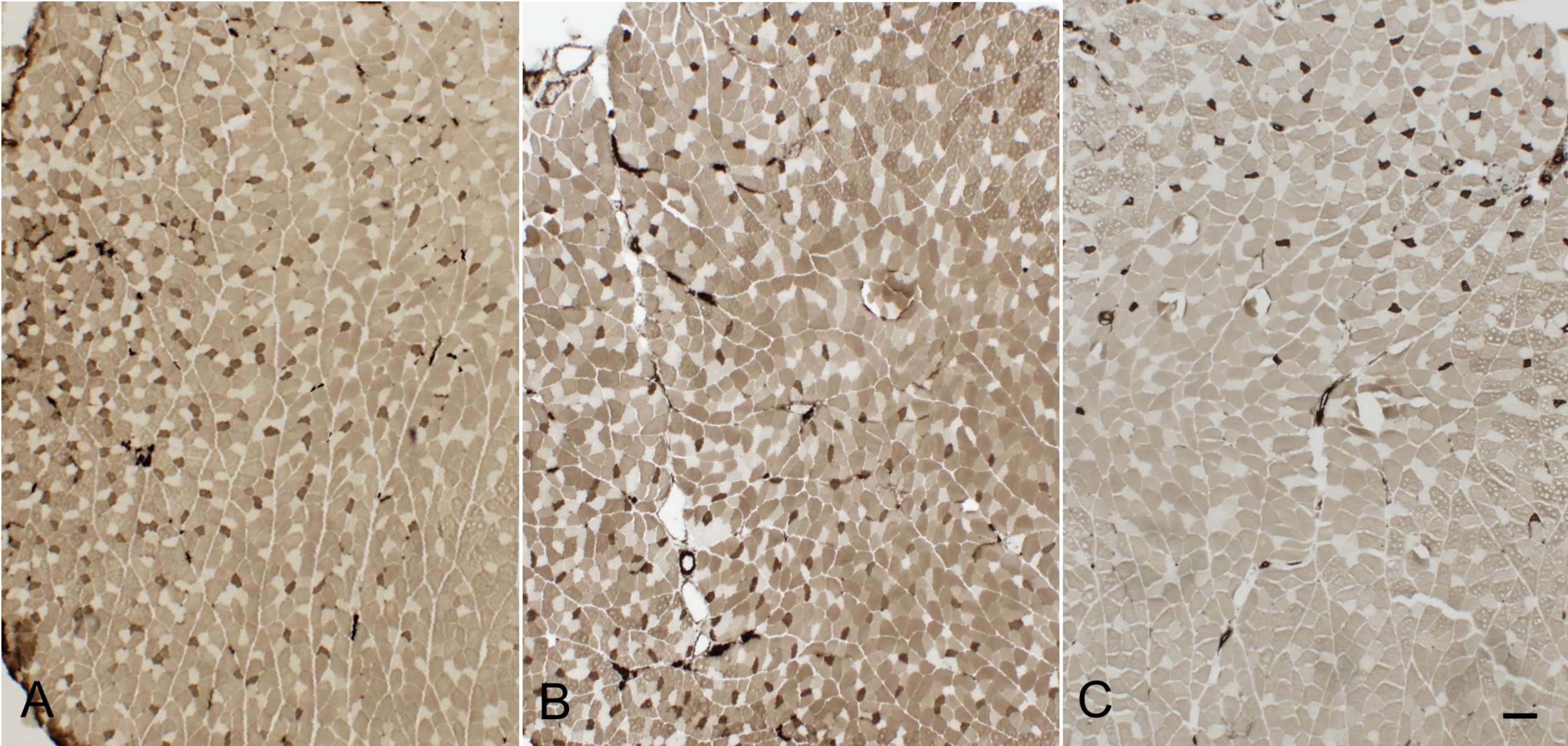
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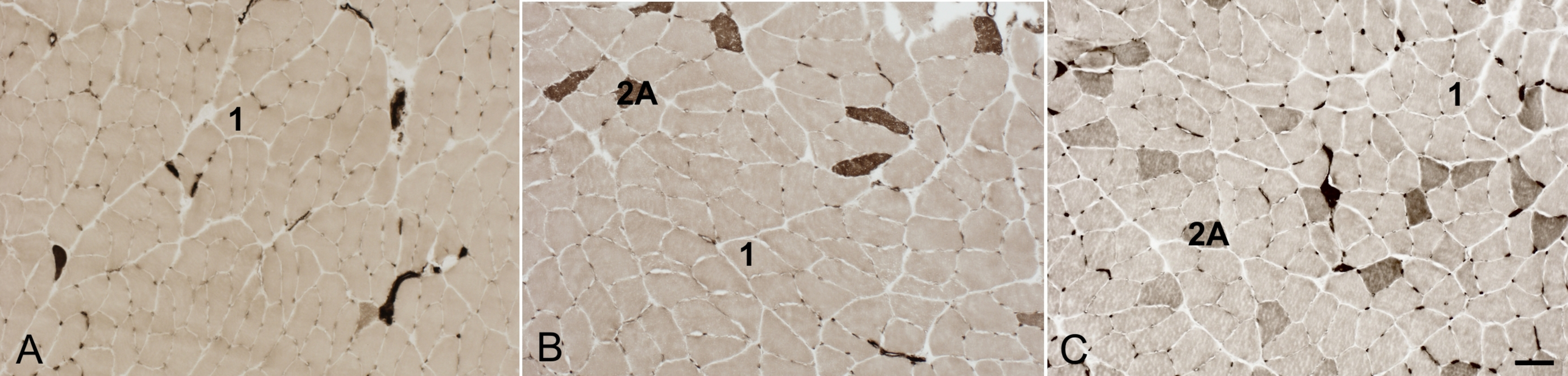
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