Expression of various sarcomeric tropomyosin isoforms in equine striated muscles

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Abstract

In order to better understand the training and athletic activity of horses, we must have complete understanding of the isoform diversity of various myofibrillar protein genes like tropomyosin. Tropomyosin (TPM), a coiled-coil dimeric protein, is a component of thin filament in striated muscles. In mammals, four TPM genes (TPM1, TPM2, TPM3, and TPM4) generate a multitude of TPM isoforms via alternate splicing and/or using different promoters. Unfortunately, our knowledge of TPM isoform diversity in the horse is very limited. Hence, we undertook a comprehensive exploratory study of various TPM isoforms from horse heart and skeletal muscle. We have cloned and sequenced two sarcomeric isoforms of the *TPM1* gene called TPM1α and TPM1κ, one sarcomeric isoform of the *TPM2* and one of the *TPM3* gene, TPM2α and TPM3α respectively. By qRT-PCR using both relative expression and copy number, we have shown that TPM1α expression compared to TPM1κ is very high in heart. On the other hand, the expression of TPM1α is higher in skeletal muscle compared to heart. Further, the expression of TPM2α and TPM3α are higher in skeletal muscle compared to heart. Using western blot analyses with CH1 monoclonal antibody we have shown the high expression levels of sarcomeric TPM proteins in cardiac and skeletal muscle. Due to the paucity of isoform specific antibodies we cannot specifically detect the expression of TPM1κ in horse striated muscle. To the best of our knowledge this is the very first report on the characterization of sarcmeric TPMs in horse striated muscle.

Keywords: Absolute copy number, Horse, qRT-PCR, Relative expression, TPM.

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Introduction

Tropomyosins, a family of actin-biding proteins, are present in all eukaryotes from yeast to humans. They play a critical role(s) in regulating the function of actin filaments in both muscle and nonmuscle cells (Lees-Miller and Helfman, 1991; Pittenger *et al*., 1994; Pieples and Wieczorek, 2000; Perry, 2001; Denz *et al*., 2004; Gunning *et al*., 2008).

Sarcomeric tropomyosin is a component of the thin filament whereas muscle myosin isoforms comprise the thick filament in myofibrils. The dynamic interaction between the thick and thin filaments results in muscle contraction, which is triggered by nerve impulses that in turn stimulate the release of Ca^{2+} in the cytosol. Tropomyosin attached troponin(s) then binds to Ca^{+2} and undergoes a massive conformational change that aids the myosin associated ATPase activity to hydrolyze ATP and release energy. The energy thus released initiates muscle contraction. Muscle contraction is a function that various animals, including humans, exploit to move, stretch, and even keep themselves warm. Hence, various myofibrillar proteins including tropomyosin play critical role(s) in muscle contraction that is essential for walking, running, and exercising.

The horse is a unique and incredible athlete with remarkable capabilities. In fact, athletic performance is a vital criteria used for the selection of superior horses. It is well established that athletic performance of animals including humans can be improved by proper and rigorous physical exercise/training, which again depends on the expression of various genes related to energy metabolism as well as muscle contraction (McGivney *et al*., 2009; Eivers *et al*., 2010, 2012). However, very little is known about exercise-related gene expression patterns in equine muscles for example heart, and skeletal muscles.

Some myofibrillar protein genes like, TNNC2 (Tn-C type 2) ACTN1 (alpha actin, skeletal muscle) and TPM1 (tropmyosin 1) were found to be up regulated in equine skeletal muscle after exercise (Eivers *et al*., 2012). TPM1 and TPM2 along with the transcripts of some other myofibrillar protein genes are among the most highly abundant transcripts in equine skeletal

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muscle (McGivney *et al*., 2010). Yet, to the best our knowledge, none of the sarcomeric TPM isoforms from equine heart and skeletal muscles have been cloned or sequenced. Nor have extensive studies been done to evaluate the full repertoire of TPM sarcomeric isoforms expressed in equine cardiac and skeletal muscles.

In vertebrates, tropomyosin is encoded by four tropomyosin genes *TPM1, TPM2, TPM3*, and *TPM4* (Lees-Miller and Helfman, 1991; Pittenger *et al*., 1994; Pieples and Wieczorek, 2000; Perry, 2001; Denz *et al*., 2004; Gunning *et al*., 2008) except for zebrafish where six tropomyosin genes are present (Booth *et al*., 1998; McGivney *et al*., 2010). In mammalian systems the TPM sarcomeric isoforms deemed to be important in heart and skeletal muscle includeTPM1α , TPM1κ , TPM2α , and TPM3α (Lees-Miller and Helfman, 1991; Perry, 2001; Gunning *et al*., 2008; Dube *et al*., 2016).

Hence, in this study, we have cloned and sequenced the above sarcomeric tropomyosin cDNAs from equine striated muscles. Recently, we have described sarcomeric TPM4α expression in human hearts and, to a much more limited degree, human skeletal muscle (Dube *et al*., 2016).

However, in several mammals, for example rodents, the *TPM4* gene is truncated. As a result, the *TPM4* gene does not code a sarcomeric isoform in rodents. Also, the predicted isoforms of the horse *TPM4* gene (GenBank mRNA accession # XM_014734753.1) did not include the sarcomeric isoform because of a stop codon in exon 2. Further, no definitive role on muscle contraction in mammals has yet been assigned for TPM4α , the sarcomeric isoform of the *TPM4* gene. Hence, in this study, we have not included TPM4α.

Materials and Methods

Conventional and nested RT-PCR for amplification of TPM1α, TPM1κ, TPM2α and TPM3α

Total RNAs from whole horse (*Equus ferus caballus*) heart and skeletal muscle were procured   from Zyagen (San Diego, CA) and BioChain (Newark, CA). 1st strand cDNA was made using oligo (dT)$_{12-15}$ using SuperScript II (Life Technologies, Grand Island, NY) following manufacturer's specifications as described earlier (Thurston *et al*., 2009; Nan *et al*., 2015). First, PCR amplification for TPM1α and TPM1κ were carried out with the P1 and P2 primer-pair (Table 1 and Fig. 1).

For nested PCR, the first amplified DNAs from heart and skeletal muscle were diluted 200 fold with distilled water and 2 µl from each sample was amplified with the P5 and P2 primer-pair   for TPM1κ amplification and P3 and P2 primer-pair for TPM1α amplification. P8 was used as probe for both isoforms. The primer combinations P5 and P6; P1 and P7 were used in the direct sequencing of TPM1κ. Nucleotide sequences of

various primer-pairs used for amplification of different isoforms are provided in Table 1.

Following amplification the DNA product was run on a 1.5% agarose gel. DNA bands of appropriate size(s) were extracted using the MinElute Gel Extraction kit provided by QIAGEN. One portion of isolated DNA was used for determination of nucleotide sequences and a portion of the isolated DNA was ligated into a T/A cloning vector (Invitrogen) using the manufacturer's specifications as previously described (Thurston *et al*., 2009; Nan *et al*., 2015).

This resulting ligation mixture was then used for the transformation of competent One Shot *E. coli* cells (provided by Invitrogen) as per the manufacturer's protocol. For identification of the particular isoform we performed colony hybridization with [^{32}P]-labeled Exon 2b specific probe for TPM1α or exon 2a specific probe for TPM1κ, as listed in Table 1. The hybridization positive colonies were picked up; grown overnight and plasmid DNA were isolated using the QIAprep Spin Miniprep kit (QIAGEN). The isolated plasmid DNA as well as the gel extracted DNA (as stated above) were sequenced at the Cornell University Core DNA sequencing facility. For amplification of TPM2α and TPM3α we employed TPM2α and TPM3α specific primer-pairs designed using the predicted sequences available in GenBank. The amplified DNAs were cloned into T/A cloning vector (Invitrogen) and subsequently sequenced as stated above.

Real-time quantitative RT-PCR.

qRT-PCR analyses of cDNA templates were performed using the LightCycler 480 Real-Time PCR System as described before (Dube *et al*., 2014; Nan *et al*., 2015). Reactions were carried out in a 384-well plate using the LightCycler 480 SYBR Green I Master kit (Roche). Briefly, each well contained a total volume of 10 µl reaction solution, of which 2 µl was cDNA template and 8 µl was SYBR green mix (5 µl 1 x SYBR green Master mix, 2.8 µl of PCR-grade water, and 0.2 µl of 10 µM primer pair). Primers for real-time PCR for various TPM isoforms are listed in Table 1. In case of TPM1α and TPM1κ, cDNA was made with an oligonucleotide specific for exon 9a/b that allowed us to make an isoform specific cDNA corresponding to the mRNA containing exon 9a/b as in various sarcomeric isoforms. Amplification in the absence of a cDNA template was also evaluated to insure a lack of signal due to primer dimerization and extension or carryover. Data were analyzed using both relative and absolute quantification methods. Relative quantification of qRT-PCR data was performed using the delta CT (sample CT minus 18S rRNA CT) and delta delta CT (sample delta CT minus comparator delta CT) methods (Pfaffl, 2001; Livak and Schmittget, 2001).

Table 1. Primer-pairs and probes used for amplification and detection of TPM1α, TPM1κ,TPM2α, and TPM3α.

Isoform:	As indicated in	Type of amplification		
TPM1α & TPM1κ	Fig. 1	Con RT-PCR	qRT-PCR	Nucleotide sequence
MTPM1-1A(+)	P1	X	-	5'−ATGGACGCCATCAAGAAGAA−3'
MTPM1-2A(+)	P5	X		5'−CGGAGGACGAGCGGGACCGGG−3'
MTPM1-2A probe	P4		X	5'−GAGCTGCACAAGGCGGAGGACAG−3'
MTPM1-2B (+)	P3		X	5'−ATGAACTGGACAAATACTCTGAG−3'
MTPM1-2A(-)	P7			5'−TGTCCTCCGCCTTGTGCAG−3'
MTPM1-3-4(-)	P6		X	5'−TCAATGACTTTCATGCCTCT−3'
MTPM1-9A(-)	P2	X		5'−CGCTCTCAACGATATGACTT−3'
TPM2α				
MTPM2-1A(+)		X		5'−ATGGACGCCATCAAGAAGAAG−3'
MTPM2-9A(-)		X		5'−TCAGAGGGAGGTGATGTCATTGA−3'
TPM2 (+)			X	5'−CTCAAGGAGGCAGAGACCCG−3'
TPM2 (-)			X	5'−GGCCACACTGGTGGGGGCTC−3'
MTPM2 Exon 3 probe				5'−ATTCAGCTGGTTGAGGAGGAGCTGG−3'
TPM3α				
MTPM3-1A(+)		X		5'−CGCCTGGCCACTGCCCTGCAA−3'
MTPM3-9A(-)		X		5'−GAGTCTGGTCCAGCATCCTT−3'
TPM3 (+)			X	5'−CTTGGAGCGCACAGAGGAAC−3'
TPM3 (-)			X	5'−GATCCAGAACAGAGCAGAAAC−3'
MTPM3 Exon 2 probe				5'−GAGAAGAAGGCTGCTGAT−3'

A comparative value was calculated using the formula $X^{-\text{delta delta CT}}$, where "*E*" equals the efficiency of specific primer pairs. This is similar to the $2^{-\text{delta delta CT}}$ method but corrects for the assumption that the reaction is occurring with 100% efficiency. Efficiencies (*E*) were determined using dilution series of isoform-specific plasmid clones with respective isoform-specific primers pairs.

The LightCycler 480 software plotted the CT at each concentration against the logarithm of the dilutions of the clone, generating a linear regression curve that calculated efficiency based on the formula $E = 10^{[-1/\text{slope}]}$. Efficiency of 18S rRNA was determined by serial dilution of horse cDNAs generated with specific primers (Dube *et al*., 2014; Nan *et al*., 2015).

For determination of absolute copy number, optical density was taken of various horse isoform specific clones (for example, TPM1α, TPM1κ, TPM2α, and TPM3α separately using a spectrophotometer. The copy number per volume of clone in solution was determined using the equation number of copies = (ng of plasmid DNA x 6.02 X 10^{23})/(bp length of plasmid x 1 x 10^9 x 650), which was simplified by Andrew Staroscik at the URI Genomics and Sequencing Center. A dilution series of each clone was done for 1 x 10^1–1 x 10^4 copies of template, which was used to create a standard curve after amplification (Booth *et al*., 1998; Livak and Schmittgen, 2001; Nan *et al*., 2015). For better accuracy, each sample in the dilution series was run in triplicate.

Western blot
Horse heart and horse skeletal thigh muscle protein extracts were obtained from ZYAGEN. Protein concentration was verified using the BioRad protein reagent kit. For each sample in a total reaction mixture of 30 µl was made, 5 µl protein sample, 7.5 µl NuPage LDS sample buffer (4x), 15.5 µl distilled water and 2 µl NuPage sample reducing agent (10x). The samples were run on NuPage 4-12% Bis Tris gel and then transferred to nitrocellulose membrane according to the manufacturer's instruction as described earlier (Thomas *et al*., 2010).

The membrane was stained with Ponceau S solution in order to verify the transfer of the protein onto the membrane itself. The Ponceau stained membrane paper was then blocked in 5% milk protein solution for ~1 hour at room temperature to prevent non-specific binding. Primary antibody (for example CH1 or anti GAPDH) with appropriate dilution in 5% milk was incubated at 4ºC overnight with constant shaking. The membrane paper was washed three times in TBST solution. Secondary antibody was then applied to the membrane. Secondary antibody used for GAPDH, CH1 was anti-Mouse IgG. After the application of secondary antibody the nitrocellulose membrane was again washed three times with TBST. Electrochemiluminescence (ECL) was applied by using equal amounts of BioRad ClarityTM Western ECL substrates. Imaging was done using BioRad chemi MP Imaging system. In the case that the signal from the

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ECL process was too weak the blot was exposed to x-ray film.

Statistical Analysis

The means, standard deviations, and comparative analyses of each data set for statistical significance were done using paired Student's *t*-test. Western Blot analyses for protein expression with extracts of various mammalian systems (Yuan, *et al*., 2006).

Results

### Evaluation of expression of TPM1α and TPM1κ in horse cardiac and skeletal muscles using conventional as well as nested RT-PCR.

Figure 1 shows the exon composition of TPM1α and TPM1κ. The only difference between these two TPM1 isoforms is in exon 2. For identification, we first used conventional RT-PCR with a primer-pair that amplifies both TPM1α and TPM1κ.
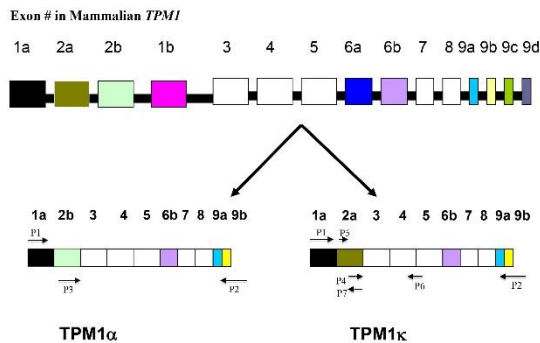
Fig. 1. Alternatively spliced two sarcomeric *TPM1* isoforms.

The results presented in Figure 2A (panel a-b) show the amplified DNA in heart (lane 1) and skeletal muscle (lane 2). In the next step, we amplified the previous amplicons with isoform specific primer-pairs as described under methods section. The results in Figure 2B (panel a-b) show that TPM1α is expressed in both heart (lane 1) and skeletal muscle (lane 2). Similarly, the results in panel Figure 2B (panel c-d) show that TPM1κ is also expressed in both cardiac and skeletal muscle. The expression patterns of the two isoforms were further confirmed by determining the nucleotide sequences of the gel extracted DNA.

Further, we cloned the DNA amplified by the generic primer-pair into T/A cloning vector and identified the particular isoform by PCR amplification of the DNA of each colony directly with an isoform specific primer-pair. We identified colonies (3 from each isoform), isolated DNA from each colony, and determined nucleotide sequences. The cloned DNAs for each isoform were subsequently used as the template for determining the absolute copy number of the particular transcript expressed in cardiac and skeletal muscle cells by qRT-PCR.
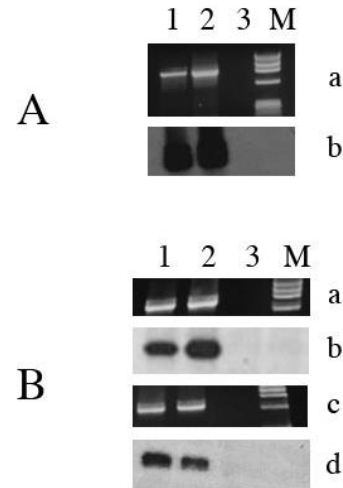
Fig. 2. Agarose gel electrophoresis of amplified TPM1α and TPM1κ DNAs by nested RT-PCR. **(A)**: The PCR products generated by the generic primer-pair MTPM1-1A(+)/MTPM1-9A(-) contains both TPM1α and TPM1κ. (Panel a): Ethidium bromide staining of the amplified DNA ran onto agarose gel. Panel b. Hybridization with [^{32}P] labeled TPM1exon 3 probe. **(B):** The final amplified DNA (Fig. 2A) was diluted 200 fold and 2 μl of diluted DNA was used for amplification with TPM1α and TPM1κ specific primer-pairs. (Panel a): The PCR amplified DNA with TPM1α – specific primer –pair MTPM1-2B (+)/MTPM1-9A(-) were separated by agarose gel electrophoresis and finally stained with ethidium bromide. (Panel b): Southern hybridization with [^{32}P] labeled TPM1exon 3 probe. (Panel c): Amplified DNA with TPM1κ specific primer pair [MTPM1-2A(+)/MTPM1-9A(-)] were separated by agarose gel electrophoresis and stained subsequently with ethidium bromide. (Panel d): Southern hybridization with [^{32}P] labeled TPM1exon 3 probe. For (A) and (B): (Lane 1): RNA from 3 horse heart; (Lane 2): RNA from horse skeletal muscle; (Lane 3): Primer control.

The nucleotide as well as deduced amino acid sequences of TPM1α and TPM1κ is shown in Figure 3 and Figure 4, respectively. It is to be noted that the deduced amino acid sequences of horse TPM1α is ~100% similar with human, mouse, and bovine TPM1α sequences (Table 2), whereas there are four amino acid changes in horse exon 2a (Fig. 5), which is the integral part of TPM1κ isoform. In order to determine the expression of TPM1κ protein in horse heart and skeletal muscle, we used a TPM1κ specific antibody that was raised in rabbits against a 15-mer peptide from the exon 2a of human TPM1κ (Rajan *et al*., 2010) (Fig. 6). Unfortunately, we failed to detect TPM1κ expression both in hearts and skeletal muscle of horse. It is to be noted that in horse TPM1κ, there are three changes in the 15-mer peptide from human TPM1κ exon 2A used for raising antibody in rabbits (Fig. 6). Hence, antibody against human TPM1κ protein may not recognize the horse TPM1κ protein. Alternatively, the expression level of TPM1κ protein is extremely low in equine striated muscle used in this study.
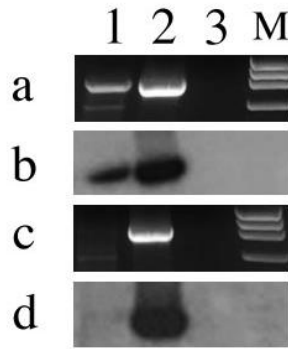
Fig. 3. Agarose gel electrophoresis of amplified TPM2α and TPM3α DNAs by conventional RT-PCR. (Panel a): The PCR amplified DNA with TPM2a specific primer-pair MTPM2-1A(+)/MTPM9A(-) were separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. (Panel b): Southern hybridization with [^{32}P] labeled TPM2 exon 3 probe Table 1). (Panel c): The PCR amplified DNA with TPM3a specific primer-pair MTPM3-1A(+)/MTPM3-9A(-) were separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. (Panel d): Southern hybridization with [^{32}P] labeled TPM 3 exon 2 probe (Table 1). (Lane 1): RNA from 3 horse heart; (Lane 2): RNA from horse skeletal muscle; (Lane 3): Primer control.

Fig. 4. Nucleotide and deduced amino acid sequences of Horse TPM1α. Nucleotide sequence of the PCR amplified DNA with the primer-pair MTPM1-1A (+)/TPM1-9A (-) as well as the cloned cDNA displayed the same nucleotide sequence as shown above. The deduced protein/amino acid sequences appear below the nucleotide sequence.

Cloning and sequencing of TPM2α and TPM3α using conventional RT-PCR

Primer-pairs used for amplification of TPM2α and TPM3α by RT-PCR with RNA from heart and skeletal muscle, designed by aligning various published mammalian and non-mammalian TPM2 and TPM3 sequences, are listed in Table 1.

Fig. 5. Nucleotide and deduced amino acid sequences of Horse TPM1κ. The deduced protein/amino acid sequences appear below the nucleotide sequences. It is to be noted that the copy number of TPM1κ compared to the copy number of TPM1α is significantly lower. Hence, using the T/A cloning system, it was extremely difficult to get the full length TPM1κ cDNA clone from the amplified products using generic primer-pair. Instead, we cloned the partial TPM1κ DNA (amplified by P1/P2 primer-pair as shown in Fig. 1) into T/A cloning vector and sequenced it. The rest of the N-terminus sequencing was done as follows: cDNAs were made with RNA from horse heart and skeletal muscle made using P2 primer, which is located in exon 9a/b (Fig. 1) and the amplification was done with the two sets of primer-pairs – P1/P7 and P4/P6. The amplified DNAs were separated by agarose gel electrophoresis and stained with ethidium bromide. DNA was extracted from the desired band using gel extraction kit as described under the Methods section. Isolated DNAs were sequenced from both sides with appropriate oligonucleotides.

Fig. 6. Alignment of the peptide sequences encoded by exon 2a of the TPM1 gene from human, horse, and mouse. Double underlined 15 mer peptide of human TPM1κ is the peptide used for raising antibody (Rajan *et al*., 2010). Please note the differences in the amino acid residues between different species. For comparison, we have included the TPM1.exon 2b sequences (part of TPM1α) also at the end. (*) Position refers to the location in leucinezipper coiled-coil motif; *a* and *d* are the interface residues; *e* and *g* are outer residues that may interact across the coiled-coil; *b*, *c* and *f* are on the far side from the coiled-coil interface (Rajan *et al*., 2010).

Table 2. Percent similarity in nucleic acid as well as amino acid sequences of different sarcomeric isoforms from horse, human, bovine, and monkey

Gene/ Isoform	Comparison	% Similarity	
		N.A.	A.A.
TPM1α	Horse v Human	95.98	99.647
	Horse v Mouse	93.86	100
	Horse v Bovine	95.63	99.647
	Horse v Monkey	96.15	100
TPM1κ	Horse v Human	95.32	98.59
	Horse v Mouse	93.22	98.94
TPM2α	Horse v Human	95.07	99.647
	Horse v Mouse	94.836	100
	Horse v Bovine	95.775	100
	Horse v Monkey	95.657	99.647
TPM3α	Horse v Human	97.427	100
	Horse v Mouse	92.723	98.947
	Horse v Bovine	97.066	100
	Horse v Monkey	96.959	100

(N.A.): Nucleic acid; (A.A.): Amino acids.

The amplified DNAs were run in agarose gel as stated under TPM1α. The bands of appropriate size(s) (not shown) were gel eluted and sequenced. The nucleotide sequence data confirmed the corresponding sarcomeric tropomyosin expressed in heart and skeletal muscle. Expression analyses were carried out by qRT-PCR using relative expression and copy number determination as given in the following section. Amplified DNA was also cloned in a T/A cloning vector and a particular isoform was detected by PCR using an isoform specific primer-pair as described under TPM1α and TPM1κ. DNA for TPM2α and TPM3α were isolated from the colony carrying the plasmid with the particular amplified DNA.

Isolated DNAs were subsequently used for determining the nucleotide sequences. The nucleotide as well as deduced amino acid sequences for TPM2α and TPM3α are given in Figure 7 and Figure 8, respectively. Comparison of nucleotide as well as deduced amino acid sequences with other mammalian TPM2α and TPM3α sequence is presented in Table 2. Similarity in amino acid sequences of horse TPM2α with human and other mammals is near 100 percent and the percent similarity at the nucleotide level is ~95%. Similarly, the nucleotide as well as deduced amino acid sequences of horse TPM3α when compared to other mammalian system, are also very conserved. At the amino acid level they are about 100% similar.

Relative expression of TPM1α, TPM1κ, TPM2α, and TPM3α in horse heart and skeletal muscle

qRT-PCR analyses were carried out using the 2-(ddCt) method to determine the relative expression of TPM1α or TPM1κ and 18S rRNA was used as the reference gene in making this determination.
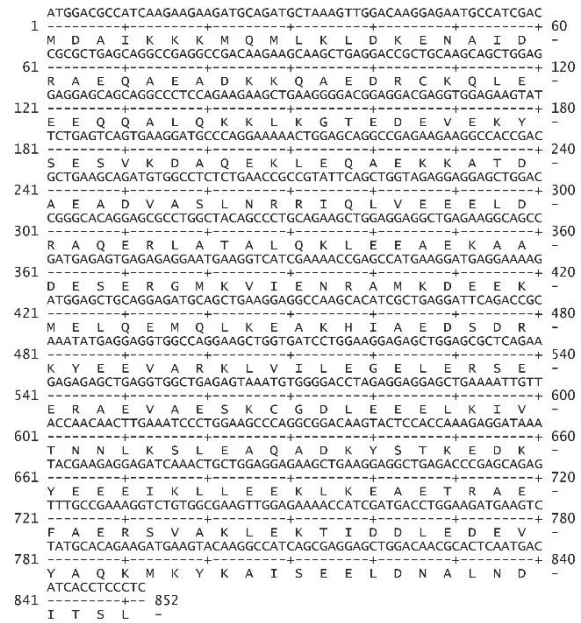
Fig. 7. Nucleotide and deduced amino acid sequences of the Horse TPM2α.
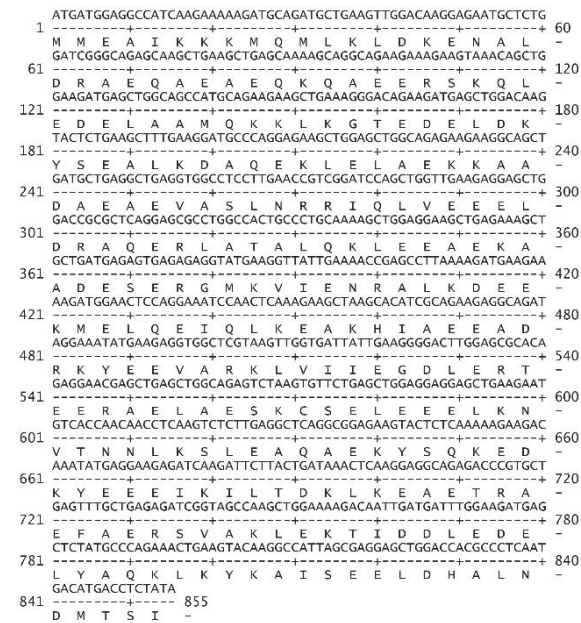
Fig. 8. Nucleotide and deduced amino acid sequences of the Horse TPM3α.

In order to eliminate the effect of other TPM isoforms, the cDNA was made with a gene and isoform specific primers.

The results show the unique melting temperatures for TPM1α (top, Fig. 9A) relative to TPM1κ (bottom). Agarose gel electrophoresis was also performed with the qRT-PCR amplified DNA of both these isoforms (Fig. 9B). Ethidium bromide staining shows a single band for each amplified DNA.
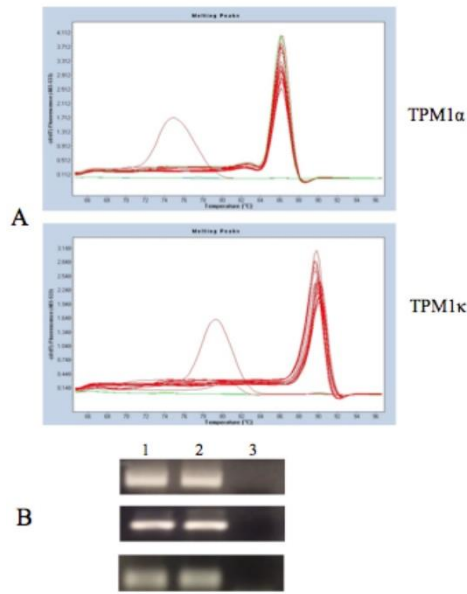
Fig. 9. Melt curves of qRT-PCR and the agarose gel electrophoresis of PCR amplified DNA stained with ethidium bromide. **(A):** Melt curves for amplified horse TPM1α and TPM1κ DNA. The melting points of TPM1α and TPM1κ are different. The multiple curves represent the products from multiple replicates of the RT-PCR assay. **(B):** PCR products separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. The results demonstrate amplification of a single product for TPM1α (top panel) or TPM1κ (middle panel) or 18S rRNA amplification (bottom panel). (Lane 1): Heart; (Lane 2): Skeletal muscle; (Lane 3): Primer control.

The results indicate that the primer-pairs used for amplification for TPM1α or TPM1κ are specific. Panel A in Figure 10 depicts that TPM1α expression in horse skeletal muscle is ~1.65 fold higher compared to cardiac muscle (p=0.02578). On the contrary, the expression of TPM1κ is a little higher in heart when compared to skeletal muscle (Fig. 10, Panel B). However, the small difference is statistically significant as determined by Student's T-Test (p = 0.01859).

We also performed qRT-PCR for determining the relative expression level of TPM2α and TPM3 α transcripts using the 2^{DDCT} method. First we determined the efficiency of each of the primer pairs used for the amplification of TPM2α and TPM3α respectively. We then determined the melt curve for each isoform, and also analyzed the size of each amplified DNA by agarose gel electrophoresis followed by ethidium staining (Fig. 11).

Finally, we performed the qRT-PCR amplification for determining relative expression. The results as depicted in Figure 12 show that expression of TPM2 α is ~3.6 fold higher in horse skeletal muscle compared to cardiac muscle.
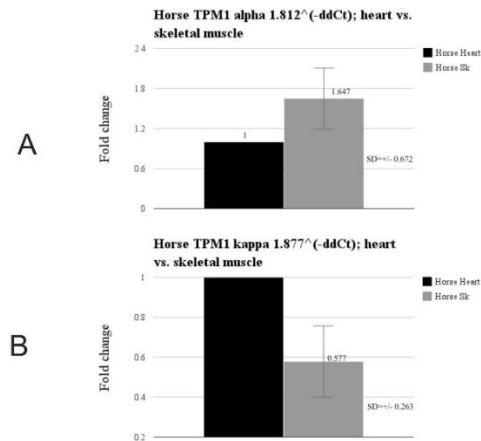
Fig. 10. Relative Expression of TPM1α and TPM1κ transcripts in heart and skeletal muscle by qRT-PCR. **(A):** Fold change of TPM1α in skeletal muscle vs heart Calculated p value as determined by Student's T-test = 0.025. **(B):** Fold change of TPM1κ in skeletal muscle vs heart Calculated p value as determined by Student's T-test = 0.0189.
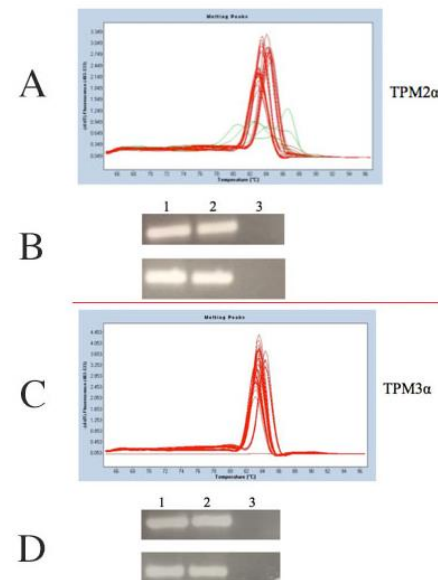
Fig. 11. Melt curves of qRT-PCR and the agarose gel electrophoresis of PCR amplified DNA stained with ethidium bromide. **(A):** Melt curves for amplified horse TPM2α DNA. The multiple curves represent the products from multiple replicates of the RT-PCR assay. **(B):** PCR products separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. The results demonstrate amplification of a single product for TPM2α (top panel) or 18S rRNA amplification (bottom panel). **(C):** Melt curves for amplified horse TPM3α DNA. The multiple curves represent the products from multiple replicates of the RT-PCR assay. **(D):** PCR products separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. The results demonstrate amplification of a single product for TPM2α (top panel) or 18S rRNA amplification (bottom panel). For (B) and (D): (Lane 1): Heart; (Lane 2): Skeletal muscle; (Lane 3): Primer control.
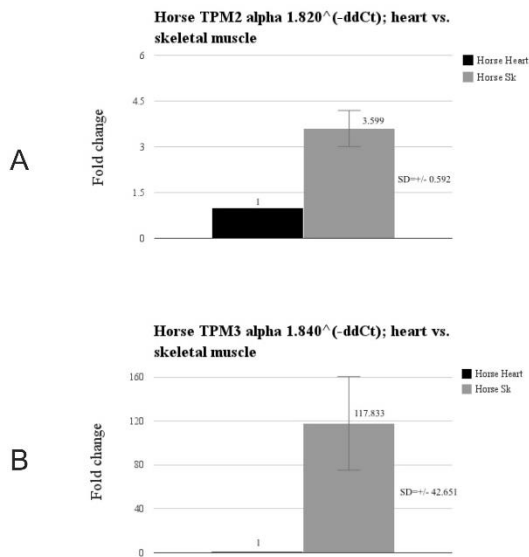
Fig. 12. Fold changes (primer efficiency (eff.)^-ddCt) of horse TPM2α and TPM3α in heart (H) vs. skeletal muscle (Sk). **(A):** Horse TPM2α eff. = 1.820 (mean value for 2 separate experiments each being in triplicate). **(B):** Horse TPM3α eff. = 1.812 (mean value for 2 separate experiments each being in triplicate).

However, the relative expression of TPM3 α is about 117 fold higher in skeletal muscle compared to heart. The relative expression experiment using 2^{DDCT} shows that the expression level of most of the sarcomeric TPM isoforms is higher in equine skeletal muscle compared to heart except for the expression of TPMκ, which is higher in cardiac muscle. In order to verify the results we determined the absolute copy number of various TPM isoforms in heart and skeletal muscles. To determine the absolute copy number of a particular isoform we had to use an isoform specific cloned cDNA as the template for calculating the standard curve. We also determined the standard curve for TPM2α and TPM3α using respective cDNA template and isoform specific primer-pair (Fig. 11).

Determination of the absolute copy number of transcripts of TPM1α, TPM1κ, TPM2α, and TPM3α in equine heart and skeletal muscle

The results presented in Table 3 show the absolute copy number of TPM1α, TPM1κ, TPM2α, and TPM3α expressed in heart and skeletal muscle. The results are in good agreement with our results on relative expression as presented in the preceding section. In brief, the copy numbers of each of three isoforms, TPM1α, TPM2α, and TPM3α are higher in skeletal muscle. On the contrary, the copy number of TPM1κ is higher in heart. It is to be noted that the expression level of TPM1κ in equine heart is very low (1.34×10^{2}/μg of total RNA) and it is even lower in skeletal muscle.

Western blot analyses for detection of sarcomeric tropomyosin expression in equine heart and skeletal muscle.

Our Western blot results with extracts from horse hearts and skeletal muscle using the CH-1 monoclonal antibody that recognizes all sarcomeric tropomyosin proteins, shows the expression in hearts (lane 1, panel b, Fig. 13).
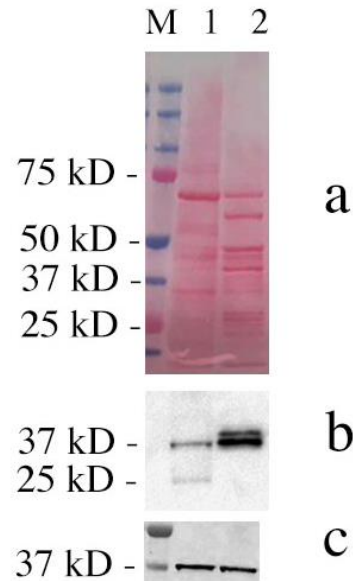
Fig. 13. Western blot analyses of sarcomeric tropomyosin in horse heart and skeletal muscle extracts. (Panel a): Ponceu staining of the blot. (Panel b): Staining with CH1 monoclonall antibody. (Panel c): staining with anti-GAPDH antibody. (Lane M): Molecular weight marker; (Lane 1): Heart ; (Lane 2): Skeletal muscle.

Because CH1 recognizes all sarcomeric isoforms for example, TPM1α, TPM1κ, TPM2α, and TPM4α, the results do not help determine expression of a particular TPM isoform. As mammalian hearts are known to generate overwhelming amount of TPM1a (~95% of the total sarcomeric TPMs) (Rajan *et al.*, 2010), one can assume that the ~37 kD band represents TPM1α. At this point it is extremely difficult to assign an identity to the `25 kD band.

To the best of our knowledge, sarcomeric tropomyosin is not low molecular weight TPM. However, there are reports in the literature and also our unpublished results that strongly support that the low molecular weight TPM with exon 9a are expressed in mammalian striated muscles. As a matter fact, the predicted sequence of tropomyosin alpha-1 chain isoform X1 with exon 9a of horse is available in the data base (NCBI Reference Sequence: XP_005603060.1). It is possible that the low molecular band may represent such an isoform.

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Table 3. Quantitative RT-PCR of horse TPM1α, TPM1κ, TPM2α, and TPM3α by determining absolute copy number.

Tissue / organ	TPM1α	TPM1κ	TPM2α	TPM3κ	Ratio TPM1α : TPM2α	Ratio TPM1α : TPM3α
Heart	$4.25 \times 10^4 + 9.1 \times 10^2$	1.34×10^2	$2.43 \times 10^5 + 2.2 \times 10^3$	$1.93 \times 10^3 + 1.1 \times 10^2$	0.17	22
Sk muscle	$1.86 \times 10^4 + 8.4 \times 10^2$	0.46×10^2	$2.47 \times 10^6 + 1.9 \times 10^4$	1.54×10^6 2.1×10^4	0.007	0.012
Ratio H : Sk	2.28	2.9	0.098	0.001		

Sarcomeric tropomyosin expression in equine skeletal muscle (lane 2, panel b, Fig. 13) is relatively higher compared to heart. Multiple bands are also visible in the skeletal muscle – the higher band is ~39 kD and the lower band ~37 kD. The broadness of the lower band (~37 kD) suggests the presence of more than one protein/iosform. It is to be noted that the higher band in the skeletal muscle may represent TPM2α or TPM1κ, each of which run slower compared to TPM1α or TPM3α. The lower band may represent TPM1α and TPM3α because the electrophoretic mobility of the two are similar (Pieples and Wieczorek, 2000; Rajan *et al.*, 2010). The results with CH1 monoclonal antibody show that the overall sarcomeric TPM protein expression in equine skeletal muscle is higher compared to heart. while GAPDH expression is similar in heart and skeletal muscle. (compare lane 1 and 2, panel c, Fig. 13).

Discussion

Tropomyosin (TPM) belongs to a multigene family of actin binding proteins. In vertebrates, tropomyosins are encoded by four genes designated as *TPM1, TPM2, TPM3,* and *TPM4*, which generate more than 50 isoforms via alternate splicing and/or using different promoters. To the best of our knowledge no sarcomeric tropomyosin has been cloned and sequenced from an equine species. Nor has the full isoform expression repertoires been defined. Hence, we undertook a comprehensive study on the characterization of all sarcomeric tropomyosins from horse hearts and skeletal muscle.

Sarcomeric tropomyosin includes TPM1α and TPM1κ, which are the alternatively spliced products of the *TPM1* gene. The difference between these two isoforms is in exon 2. TPM1α contains exons 1a, 2b, 3, 4, 5, 6b, 7, 8, 9a/b whereas TPM1κ includes all the exons as in TPM1α except for exon 2b; instead it has exon 2a. The sarcomeric isoform of TPM2 and TPM3 are designated as TPM2α and TPM3α, respectively.

In this study, we have not included *TPM4* because the role(s) of TPM4α in muscle contraction is not well defined in mammals as in the case of avian (Fleenor *et al.*, 1992), amphibians (Hardy *et al.*, 1995; Spinner *et al.*, 2002), and aquatic animals like fish (Schevzov *et al.*, 2011). In some mammals for example in rodents, TPM4α is not expressed. In humans, however, we have recently reported the expression of TPM4α transcripts in hearts and skeletal muscle. In fact, we have also reported the potential of TPM4α protein expression in human hearts (Spinner *et al.*, 2002). However, we do not know yet the function it may perform in cardiac contractility of human striated muscles.

For the first time, we have cloned and sequenced TPM1α and TPM1κ from horse heart and skeletal muscle. Interestingly, the amino acid sequences of horse TPM1α is ~100% similar with human TPM1α isoform (Fig. 6). As there are some differences in exon 2a sequences of horse and human TPM1 gene, there are some differences in TPM1κ from human and horse. Unfortunately, the nucleotide as well as amino acid sequences of TPM1α from *Equus caballus* (the horse species that includes all domestic horses) has not been reported in GenBank. Recently predicted sequences of two TPM1 isoforms from E caballus have been posted in the GenBank; neither of them can encode a high molecular wt tropomyosin like TPM1α.

However, the amino acid sequences of the Predicted TPM1isoform X 1 (XP_008520892.1; submitted in July 2014) from *Equus przewalskii* (another horse species that still lives in the wild of Central Asia) shows 100% similarity with our TPM1α sequence from *Equus caballus*. Seven predicted TPM1 isoform sequences from *Equus przewalskii* (known as Przewalski horse) are available in GenBank. Unfortunately none of the seven isoforms have exon 2a like sequences. So, we cannot compare our TPM1κ sequence from *Equus caballus* with predicted TPM1κ from Przewalski horse. Tropomyosin is an alpha helical coiled-coil dimeric protein. The coiled-coil structure is characterized by a heptapeptide repeat motif (a-b-c-d-e-f-g). The amino acid residues in the hydrophobic core positions a and *d* are the primary determinants of folding and stability as explained by Rajan *et al.* (2010).

The difference between TPM1α and TPM1κ is only in exon2. TPM1α contains exon 2b where as TPM1κ contains exon 2a. Hence, 26 out of 40 amino acid residues are different between human TPM1α and TPM1κ. As pointed out earlier, 284 amino acid

residues are identical in human and horse TPM1α. Although the TPM1α from horse and human are identical and exon 2a in horse contain four additional substitutions, the stability of the coiled-coil structure for TPM1α-TPM1κ hetero dimer as well as TPM1κ-TPM1κ homodimer may be altered. For example, L43I substitution in human TPM1κ in position *a* of the heptad may slightly destabilize of the κ/κ coiled coil relative to the α/α coiled coil. Also, this substitution causes the loss of hydrophobicity.

In horse, the same substitution will have the similar effect forming α−κ coiled coil heterodimer. L46M in position *d* unlike in humans, there will be lesser loss of the hydrophobicity and the destabilization will also be less. No SB (salt bridge) will be added like in the case of human. In the case of L50M in position "*a*" may cause some loss of hydrophobicity. V52A in a position of *c* may not affect significantly the coiled coil structure in horse TPM1κ. E75D in position *e* may not change the coiled coil structure. K77T in position *g* may stabilize the structure may still lose the hydrophobicity. At this point it's worth mentioning that the different 26 out of 40 amino acids in exon 2 changes the biding affinity of TPM1κ with F-actin.

In the absence of troponins, the binding is significantly lower compared to the binding of F-actin with TPM1α. In fact, no biding between TPM1κ and F-actin was observed in the absence of troponins. However, no differences were observed for the binding of TPM1α or TPM1κ with F-actin in the presence of troponins in vitro (Rajan *et al*., 2010). We are planning to perform similar experiments with bacterially expressed TPM1α and TPM1κ with F-actin in vitro in future.

At this juncture one of our observations deserves added attention. The 41-mer peptide encoded by exon 2b, which is the integral part of TPM1α is highly conserved not only among mammals but most likely in all vertebrates from fish to humans (Fig. 6). On the contrary, the 4-mer peptide sequences encoded by exon 2a, which is a component of TPM1κ, have not been conserved throughout vertebrate evolution (Fig. 6). Twenty five years ago David Helfman had the same observation although at that time exon 2a was known to be part of TPM1β and exon 2b as part of TPM1α (Lees-Miller and Helfman, 1991). Currently, exon 2a is also known to be an integral part of the sarcomeric isoform TPM1κ. Although functional involvement of the tropomyosin isoforms containing the exon 2a peptide has been broadened, actin is still the only protein that is known to bind to this region of tropomyosin.

The fundamental implication and the reasons for the large divergence in amino acid residues 39-80 of TPM1κ and /or TPM1β remains a mystery as stated by

David Helfman (Lees-Miller and Helfman, 1991). TPM2α protein sequences from *Equus caballus* are identical with the predicted TPM2 isoform X 1 published in GenBank (XP_003364171). Our TPM2α protein sequence from horse is almost identical with human and other mammalian TPM2α (Table 2). Finally, our TPM3α protein sequence is identical with Predicted tropomyosin alpha-3 chain isoform X1 [*Equus caballus*] (XP_005610140 submitted in November 2015) and also ~97-98% similar to human and other mammalian TPM3α sequences (Table 2).

We performed expression analyses of the transcripts of each of these isoforms with RNA from heart and skeletal muscle by qRT-PCR using two different approaches − relative expression and by determining absolute copy number using a standard curve of the corresponding cloned cDNA as a template. The results from both assays are comparable. Our results indicate that the relative expression of TPM1α is higher in skeletal muscle compared to heart.

On the other hand the relative expression of TPM1κ is higher in the heart. The results are consistent with our reported findings in mice (Dube *et al*., 2014), and axolotl (Thomas *et al*., 2010). However, when we compared the absolute copy number expression in heart and skeletal muscle, the expression of TPM1α compared to TPM1κ is much higher (~300 fold) in heart. In skeletal muscle, the expression of TPM1α compared to TPM1κ is ~4000 fold higher. In other words the copy number of TPM1κ both in heart and skeletal muscle is very low compared to TPM1α. However, the expression of TPM1κ is ~2.9 fold higher in heart whereas the expression of TPM1α in skeletal muscle compared to heart is ~4.5 fold higher. This is consistent with published results in other systems (Booth *et al*., 1998).

Interestingly, the expression level of transcripts of TPM1α and TPM1κ is comparable in human hearts unlike in Equine system. However, at the protein level, TPM1α constitutes ~90% of the total sarcomeric trpomyosin whereas TPM1κ is ~5%. There is a sense that a control mechanism is operative at the translation level that determines the efficiency and/or priority of the various TPM transcripts (Denz *et al*., 2004; Dube *et al*., 2016). It's worth mentioning at this point that Rajan *et al*. (2010) generated transgenic mice overexpressing TPM1κ in a cardiac-specific manner that led to a concomitant decrease in TPM1α protein without affecting the level of total sarcomericTPM1 proteins. Incorporation of increased levels of TPM1κ protein in myofilaments leads to dilated cardiomyopathy (DCM). Physiological alterations include decreased fractional shortening, systolic and diastolic dysfunction, and decreased myofilament calcium sensitivity with no

change in maximum developed tension. Also, they found that the level of expression of TPM1κ is increased in human dilated cardiomyopathy and heart failure.

In order to illustrate biological significance for expressing the TPM1k isoform these authors suggested that in terminally differentiated cells such as cardiomyocytes, there is a need for adaptation to changing environments. Increasing protein isoform diversity through processes such as alternative splicing meets this need. The TPM1κ isoform, one of the several products of the *TPM1* gene (Dube *et al*., 2014), might provide the opportunity to modulate sarcomeric performance during changing conditions such as exercise, stress, or cardiac disease.

We now speculate that it is most likely beneficial for the athletic activity of Equines to having a lower level of TPM1κ in heart. However, we must admit that we do not know whether TPM1κ protein is expressed in equine striated muscle. Also, it is worth trying to create a transgenic mouse that will overexpress TPM1κ protein in skeletal muscle. This may also give some evidence whether TPM1κ protein is somehow associated with animal athletic activity. However, the functional role of TPM1κ is yet to be established. It is known that TPM1κ is ~5% of the total sarcomeric tropomyosin protein in human hearts, which is also true for axolotl (Thomas *et al*., 2010).

We already reported that an anti-sense mediated down regulation of TPM1κ inhibited the cardiac contractility in axolotl in situ suggesting a critical role of TPM1κ protein in cardiac contractility. However, we do not know whether this is true for other vertebrates like humans. The expression of TPM2α and TPM3α in horse skeletal muscle is higher compared to cardiac muscle. Our findings are consistent with the published information in other mammalian systems. It is long been known that TPM3α transcripts are expressed in hearts and skeletal muscle.

It was not known that TPM3α protein is expressed in human hearts. Recently, Marston *et al*. (2013) showed that TPM3α protein is expressed in human hearts in lower quantity (< 5% of the total sarcomeric TPM protein).

Our unpublished data also support this observation. TPM3α transcripts have not been detected in mouse hearts. Although TPM3α transcripts are expressed in equine hearts and skeletal muscle, we do not know the expression patterns of the corresponding protein.

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Conflict of interest
The authors declare that there is no conflict of interests.

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