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QUALITATIVE AND QUANTITATIVE EVALUATION OF TPM TRANSCRIPTS AND PROTEINS IN DEVELOPING STRIATED CHICKEN MUSCLES INDICATE TPM4 α IS THE MAJOR SARCOMERIC CARDIAC TROPOMYOSIN ?FROM EARLY EMBRYONIC LIFE TO ADULthood

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Abstract

The chicken has been used since the 1980's as an animal model for developmental studies regarding tropomyosin isoform diversity in striated muscles, however, the pattern of expression of transcripts as well as the corresponding TPM proteins of various tropomyosin isoforms in avian hearts are not well documented. In this study, using conventional and qRT-PCR, we report the expression of transcripts for various sarcomeric TPM isoforms in striated muscles through development. Transcripts of both TPM1 α and TPM1 κ , the two sarcomeric isoforms of the *TPM1* gene, are expressed in embryonic chicken hearts but disappear in post hatch stages. TPM1 α transcripts are expressed in embryonic and adult skeletal muscle. The sarcomeric isoform of the TPM2 gene is expressed mostly in embryonic skeletal muscles. As reported earlier, TPM3 α is expressed in embryonic heart and skeletal muscle but significantly lower in adult striated muscle. TPM4 α transcripts are expressed from embryonic to adult chicken hearts but not in skeletal muscle. Our 2D Western blot analyses using CH1 monoclonal antibody followed by mass spectra evaluations found TPM4 α protein is the major sarcomeric tropomyosin expressed in embryonic chicken hearts. However, in 7-day old embryonic hearts, a minute quantity of TPM1 α or TPM1 κ is also expressed. This finding suggests that sarcomeric TPM1 protein may play some important role in cardiac contractility and/or cardiac morphogenesis during embryogenesis. Since only the transcripts of TPM4 α are expressed in adult chicken hearts, it is logical to presume that TPM4 α is the only sarcomeric TPM protein produced in adult cardiac tissues.

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INTRODUCTION

In sarcomeres, myosin (thick) filaments act as the contractile motor and actin (thin) filaments act as the scaffold (Clark et al. 2002) and the association of TPM-troponin complexes stabilize thin filaments. In vertebrates except for zebrafish, four TPM genes TPM1, TPM2, TPM3, and TPM4, are known (Dube et al. 2017a; Gunning et al. 2008; Lees-Miller and Helfman, 1991; Schevzov et al. 2011). There are six TPM genes in zebrafish (Toramoto et al. 2004; Dube et al. 2017a). Each TPM gene generates multiple isoforms through the use of alternative splicing, alternate promoters, and different polyadenylation sites. Each TPM gene produces at least one sarcomeric isoform involved in contractility in striated muscles. However, the TPM1 gene generates two sarcomeric isoforms known as TPM1 α and TPM1 κ (Denz et al. 2004; Rajan et al. 2010). The sarcomeric isoforms of TPM2, TPM3, and TPM4 are known as TPM2 α , TPM3 α , and TPM4 α . Different striated muscle isoforms generated by each TPM gene may play a distinct role in skeletal and cardio-morphogenesis and myofibrillogenesis in a developmental, organ, and/or species-specific manner. For example, TPM1 α is the major sarcomeric isoform in human hearts (Rajan et al., 2010) while lesser/minor quantities of TPM1 κ (Peng et al. 2013), TPM2 α , TPM3 α , (Marston et al. 2013) and TPM4 α are also expressed (Dube et al. 2016a). In amphibian hearts, TPM1 α , TPM1 κ and TPM4 α have been reported to be expressed (Spinner et al 2002). Upon ectopic expression, TPM1 α and TPM4 α can rescue independently mutant axolotl hearts lacking endogenous TPM proteins. The expression pattern of transcripts of each of these TPM isoforms are comparable in axolotl hearts (Nan et al. 2015; Tomas et al. 2010). The physiologic importance in the TPM isoform diversity observed during development, between organs and among different species is not completely understood.

Citing several of our published papers on the cardiac mutant axolotl, McKeown et al. (2014) suggested that, among the four TPMs, TPM1 plays the major role in cardiac myofibrillogenesis and cardio-morphogenesis. These authors, using a transgenic mouse model, found a similar role of TPM1 in murine hearts and proposed that it probably applied to all mammalian systems. In avian species, the role of sarcomeric TPM isoforms encoded by various TPM genes, is not well-defined. For a long time, it was presumed that the sarcomeric isoform of the TPM4 gene was the only sarcomeric TPM expressed in avian hearts (Fleenor et al., 1992; Forry-Schaudies et al., 1990). However, we were the first to report the expression of transcripts of TPM1 α and TPM1 κ along with TPM4 α in embryonic chicken hearts (Zajdel et al. 2003). After hatching, the expression of both TPM1 α and TPM1 κ in the heart disappear as the chicken matures. Recently we reported the cloning, sequencing, and expression of TPM3 α transcripts in chickens (Dube et al. 2018). The expression of all high molecular weight TPM3 RNA including the sarcomeric isoform TPM3 α , is very low in both adult heart and skeletal muscle but present in embryonic heart and skeletal muscle. No data have been published regarding sarcomeric TPM2 expression in chicken tissues. Further, no isoform protein data has been published regarding tropomyosin expression in chicken hearts. A complete understanding of the expression status of various sarcomeric tropomyosins in developing and adult chicken hearts will help delineate the role of each sarcomeric TPM in cardio-morphogenesis and cardiac myofibrillogenesis. We have

undertaken a comprehensive evaluation of various sarcomeric TPMs in developing and adult chicken hearts using conventional as well as qRT-PCR. Protein expression was analyzed by conventional as well as 2D Western blot analyses using CH1 monoclonal antibody, which recognizes all sarcomeric TPM isoforms (Schevzov et al., 2011). Amino acid sequences of isolated peptides were determined by mass spectrometry.

RESULTS

Conventional RT-PCR analyses for expression of various sarcomeric TPM in adult chicken hearts and skeletal muscle –

Total RNA extracted from heart and skeletal muscle from adult chicken was used for conventional RT-PCR with gene and/or isoform specific primer-pairs designed to amplify the corresponding gene-specific full length coding sequences (852 bp for each gene). Southern hybridization was carried out after running the amplicons onto an agarose gel followed by hybridization with a radiolabeled isoform-specific oligonucleotide probe (Table 1). The results show a single ethidium bromide band in skeletal muscle (lane 2, panel a, Figure 1) when amplified with a TPM1 α specific primer-pair indicating the expression of TPM1 α is in adult skeletal muscle but not in adult heart tissues (lane 1, panel a). Southern hybridization with a radiolabeled TPM1 α specific probe (in panel b) confirms the results depicted in panel a. For TPM2 α , ethidium bromide staining of the PCR amplified DNA in adult chicken skeletal muscle shows the correct size band (~ 870 bp) in lane 2, panel C, Figure 1), and possibly a smaller band. In adult heart (lane 1), however, a little smaller band is visible. Southern hybridization with TPM2 α -specific radiolabeled oligonucleotide (designed from exon 9b of the *TPM2* gene) shows a very strong signal in lane 2, panel d. No hybridization signal is detected in lane 1. It is to be noted that the negative primer for amplification of TPM2 α is from exon 9b and the probe is from exon 9b. Hence, the amplified product that does not hybridize with the probe cannot be TPM2 α . The ethidium band may represent an unusual spliced isoform of the chicken *TPM2* gene or a non-specific amplified DNA. Hence, one can conclude that TPM2 α is expressed in adult skeletal muscle but not in adult chicken heart. Panel e in Figure 1 depicts the ethidium staining of the DNA products amplified with full length TPM4 α specific f primer-pair (Table 1). A ~870 bp band is visible in lane 1 (or AH) and to a lesser extent also in lane 2 (or ASK). Subsequent Southern hybridization with a TPM4 α -specific radiolabeled probe (designed from exon 3 of the *TPM4* gene) show a strong signal in lane 1 in panel f and no signal is detected in lane 2 in panel f. The results display that TPM4 α , unlike TPM1 α and TPM2 α , is expressed in adult heart (lane 1) and not in adult skeletal muscle (lane 2, panel f, Figure 1).

As a control/reference transcript, we amplified slow myosin heavy chain 3 (slow MyHC3) using a gene-specific primer-pair. The amplified DNA was subsequently subjected to Southern hybridization with a gene specific oligonucleotide probe (Table 1). Our results show that the slow MyHC3 is expressed in adult heart (lane 1, panel h), but not in adult skeletal muscle (lane 2, panel h).

Amplification of TPM1 α and TPM1 κ transcripts in embryonic and post embryonic chicken hearts —We have previously reported earlier the expression of two

sarcomeric TPM isoform transcripts designated as TPM1 α and TPM1 κ in embryonic chicken hearts (Zajdel et al.2003). The only difference in the two isoforms is in exon 2. TPM1 α contains exon 2b whereas TPM1 κ has exon 2a. The primer pairs for TPM1 α and TPM1 κ are TPM1 α (F) / TPM1 α & TPM1 κ (R) and TPM1 κ (F) / TPM1 α & TPM1 κ (R) (Table 1), respectively. The results depicted in Panel A of Figure 2 show the ethidium bromide staining of TPM1 α expression in embryonic hearts (lanes 1–3), which is practically absent in post embryonic heart (lane 4). This is more evident in Southern hybridization results with a TPM1 α specific probe (Table 1) as shown in Panel B, Figure 2. Similarly, TPM1 κ expression as shown in lanes 6 – 9 (both panels A and panel B) [a TPM1 specific probe (Table 2) was used for the Southern hybridization] is stronger in embryonic chicken hearts. The highest expression is found in 5-day old embryonic heart (lane 6) whereas the weakest expression is in 15- day old embryonic heart (lane 8 in both panels). In 15-day old post hatch chicken heart the expression is undetectable (lane 9; panel B). The relative expression of TPM1 κ would appear to be greater than TPM1 α in the embryonic hearts (Fig. 2).

Relative expression of sarcomeric TPM1 (both TPM1 α & TPM1 κ), TPM2 α and TPM4 α isoforms in embryonic, 15 days post hatch, and adult chicken hearts –

We performed qRT-PCR for determining the relative expression of total sarcomeric TPM1 (includes both TPM1 α and TPM1 κ) and TPM4 α . We analyzed our results by $2^{-\Delta\Delta CT}$ method using 18S rRNA as the reference gene to determine the relative expression of sarcomeric isoforms of TPM1 and TPM4 α . (Figure 3). Melt curve data presented in Figure 4 show different unique melting temperatures for TPM1 (Figure 4A) and TPM4 α (Figure 4B). Also, agarose gel electrophoresis of the PCR amplified DNA for TPM1(α & κ) or TPM4 α shows a single band for both (Figure 4C). The data depicted in Figure 3 show that TPM1 transcript expression is highest in 10-day old embryonic heart and then declines (TOP, Figure 3). We have compared the relative expression of TPM1 α in developing heart and skeletal muscles by using $2^{-\Delta\Delta CT}$ methods. The results presented in Figure 5 show that TPM1 α expression level in developing and adult chicken hearts are significantly lower than in developing and adult chicken skeletal muscle. It is maximum in skeletal muscles in 15-day post hatch chicken.

The expression of TPM2 α is relatively higher in skeletal muscle compared to heart in most of the embryonic stages and also in adult animals. However, it is much higher in 15-day old embryonic skeletal muscle (Figure 6). Regarding the expression of the sarcomeric TPM3 α in heart and skeletal muscles in developing chicken, we have recently reported that TPM3 α is expressed in mostly embryonic skeletal muscles (Dube et al. 2018). Sarcomeric TPM4 α is expressed in hearts in all embryonic stages tested. The maximum expression is at 15-day post hatch animals. On the contrary, negligible expression of TPM4 α is observed in embryonic as well as adult skeletal muscles (Figure 7).

In brief, although transcripts of all known TPM sarcomeric isoforms are expressed in embryonic chicken hearts, only TPM4 α transcripts continue to adulthood.

Conventional as well as 2D Western blot analyses with protein extracts of embryonic chicken hearts with CH1 monoclonal antibody that recognizes all tropomyosin isoforms with exon 9a peptide -

First, we performed conventional Western blot analyses with extracts from day 9 and day 15 embryonic chicken hearts using CH1 monoclonal antibody that recognizes all sarcomeric TPM isoforms (Figure 8). One major band is visible in two lanes (Panel TPM, Figure 8) indicating sarcomeric TPM proteins are expressed in 10-day (lane 1) as well as 15-day embryonic hearts (lane 2). and skeletal muscle (not shown). As CH-1 does not differentiate gene-specific TPMs, the above data does not specifically identify which sarcomeric TPM isoform(s) are expressed in heart and skeletal muscle during embryogenesis. As gene specific sarcomeric TPM antibodies are not available, we performed 2D-western blot analyses with CH1 monoclonal antibody followed by mass spectroscopic analyses.

Figure 9 depicts results on 2D PAGE with protein extracts from 7-day (panels a & b) and 10-day (panels c & d) old embryonic chicken hearts. Panels a and c represent the Coomassie blue staining of 2D Western blots whereas panel b and d depict signals on the x-ray films exposed on blots after being treated with ECL. Spot C represents one or more sarcomeric TPM isoforms with MW 33 kDa and pI 5.0 from the blot of 7-day old embryonic chicken hearts. Similarly, the two spots designated as Spot A (MW 33 kDa with pI 5.0) and Spot B (molecular wt 33 kDa with pI 5.2) were detected from 10-day old embryonic chicken heart extracts. It is to be noted that each of the spots represent sarcomeric TPM isoform(s) because these are stained with CH1 monoclonal antibody. In the next step, each of the protein spots that were separated by 2D PAGE and stained by Coomassie dye were excised, washed and the proteins from the gel were treated/processed according to the published protocol (please see the methods section) for LC-MS/MS analyses. The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software. Mascot and PLGS database search provided a list of proteins for each gel band. To eliminate false positive results, for the proteins identified by either one peptide or a mascot score lower than 25, we verified the MS/MS spectra that led to identification of a protein. In spot A, a total of 81 peptide sequences with 2222 Mascot Score identified TPM4 α (XP_01555677.1) with MW 32774. Peptide sequences cover 83% of the identified chicken protein TPM4 α (Figure 10). Subsets of these peptides also identify the presence of TPM1 protein in 7 day-old embryonic chicken heart extract (Spot C or Sub-set in Figure 9) covering 39% of the protein sequence. The results show the presence of three unique TPM1 α and/or TPM1 κ and 37 unique TPM4 α signature peptides out of 81 total TPM homologous peptides. No unique TPM2 or TPM3 peptides were identified. This would suggest that TPM1 α and/or TPM1 κ represent 7.5% of the TPM peptides in the 7-day embryonic chicken heart. Because we have not identified any peptides from exon 2, we cannot distinguish between TPM1 α and TPM1 κ . As mentioned earlier, the 2D Western blot was also carried out with extract from 10-day old embryonic chicken heart tissue and CH1 monoclonal antibody. Two spots with a positive signal were marked A and B in (panels c and d in Figure 9). From Spot A, 11 peptide sequences were identified with TPM4 α with 517 Mascot Score that aligned with TPM4 α of MW 32846 (gi|525025765/XP_01555677.1) (Figure 9). Similarly, 16 peptides extracted from spot B aligned with the sequence of chicken TPM4 α covering 40% of the protein with Mascot score 650. In summary, although transcripts of various sarcomeric tropomyosins, for

example TPM1 α , TPM1 κ , TPM2 α , TPM3 α and TPM4 α , are expressed in embryonic chicken hearts, TPM4 α is the major tropomyosin protein expressed in embryonic hearts. However, our results indicate the expression of a low quantity of TPM1 protein in 7-day embryonic chicken heart. It is not illogical to presume that TPM4 α is the only protein expressed in adult heart as no other sarcomeric TPM transcripts can be detected.

DISCUSSION

The exon/intron composition of the four known TPM genes are comparable and the coding sequences of their sarcomeric isoforms are also very similar (Lees-Miller JP and Helfman, 1991; Gunning et al., 2008). However, the expression pattern of various sarcomeric isoforms in different vertebrates varies considerably. Avian species are thought to specifically express TPM4 isoforms in cardiac muscles. TPM1 α is known to be expressed in adult chicken skeletal muscle but not in cardiac tissues (Forry-Scaudies et al., 1990). TPM1 α and TPM2 α transcripts are expressed both in embryonic and adult skeletal muscles but expressed only in embryonic cardiac tissues (Figure 1). We were the first to publish the complete nucleotide sequence of chicken TPM3 α (Dube et al. 2018). TPM3 α transcripts are expressed in embryonic heart and skeletal muscle but are barely detectable in adult animals (Dube et al. 2017). TPM4 α , the sarcomeric isoform of the *TPM4* gene, was reported to be expressed in cardiac but not in skeletal muscles (Forry-Schaudies et al. 1990; Fleenor et al. 1992). The expression of sarcomeric tropomyosin in chicken heart was thought to be only TPM4 α . We first reported the expression of TPM1 α and TPM1 κ transcripts in embryonic but not in adult chicken cardiac tissue. All our previous published results were based on conventional RT-PCR that generated only qualitative data. Current expression analyses by qRT-PCR show that sarcomeric TPM1 α and/or TPM1 κ are expressed in embryonic hearts but not in 15-day post hatch and/or adult chicken hearts (Figure 3). TPM2 α is expressed more in embryonic skeletal muscle. TPM2 α expression in skeletal muscle is 13700 fold higher compared to its expression in 15-day old cardiac muscle (Figure 6). TPM3 expression is limited to only embryonic hearts. TPM4 α is expressed in both embryonic and adult cardiac tissues (Figure 1; Figure 7). Throughout all life stages, it is the overwhelmingly predominant TPM transcript expressed in cardiac muscle.

The expression pattern of slow MyHC is interesting. It is known that slow MyHC3 is expressed in embryonic chicken skeletal muscle but not in adult skeletal muscle. It is also known that two alternatively spliced transcripts are expressed in adult cardiac tissues in avian species (Sacks et al. 2003). Consistent with these reported findings, we also find two different PCR products in adult heart. Both bands hybridize with the gene-specific oligonucleotide probe. We have determined the nucleotide sequences of the DNA extracted from each of the two bands separately. Our sequence data indicate that both bands are consistent with those predicted from published sequences of slow MyHC3 gene available in GenBank (Accession # S78540). Interestingly, the putative alternatively spliced product is expressed only in adult heart and not in adult skeletal muscle (lane 1, panel h, Figure 1).

Currently, precise protein expression data on sarcomeric TPM4 in chicken is not available primarily due to a paucity of gene and/or isoform specific TPM antibodies. Forry-Schaudies et al.(1990) by 2-D PAGE analysis and using radiolabeled in vitro transcription/translation

products of a cardiac high molecular weight TPM4 clone showed that TPM4 protein is expressed in chicken cardiac tissues. Our 2D Western blot analyses followed by LC-MS/MS analyses show that TPM4 α is the major tropomyosin isoform expressed in 7-day embryonic chicken heart. However, our results also indicate the expression of TPM1 protein in 7-day embryonic heart. Out of 81 TPM peptides, we found only 3 unique to TPM1 while 37 are unique to TPM4 α (Figure 10). All others are common to both. We did not find such TPM1 signature peptide(s) in either of the spots from 10-day embryonic hearts. One can infer that TPM4 α is the major sarcomeric isoform in chicken hearts in all stages of development, but that TPM1 protein is expressed in small amounts during embryonic life. It is unclear whether this small amount of TPM1 protein is essential for cardiac development in a chicken.

Recently, McKeown et al. (2014) have proposed a major role of TPM1 in cardiac-morphogenesis and myofibrillogenesis in mammalian hearts. They failed to produce live TPM1 $-/-$ progeny by inter crossing TPM1 $+/-$ mice. TPM1 $-/-$ embryos develop non-beating abnormal hearts and die at E9.5. The loss of TPM1 resulted in defects in cardiac morphogenesis and function during embryogenesis. These authors compared TPM1 $-/-$ mice embryos with cardiac mutants of Mexican axolotl with abnormal morphogenesis and non-beating heart (Lemanski et al. 1979; Zajdel et al. 1998) and implied a universal role of sarcomeric TPM1 α in the process. In their discussion, they also proposed that some level of TPM1 expression may be critical to cardiac myofibrillogenesis as well as cardiac contractility in all vertebrates. To date, It is the common belief that TPM4 α is the only sarcomeric isoform expressed in avian hearts (Fleenor et al. 1992; Schevzov et al. 2011). Our findings of the expression of TPM1 α and TPM1 κ transcripts in embryonic hearts (Zajdel et al. 2003 & this study) and the expression of TPM1 protein in embryonic chicken hearts raise the possibility of the involvement of TPM1 protein in cardiac function at the onset and during early cardiac myofibrillogenesis in chickens.

4. MATERIALS AND METHODS

4.1 | Isolation of RNA from embryonic chicken hearts & skeletal muscles and adult chicken hearts

Fertile eggs (White Leghorn) were incubated at 37°C for 7–15 days. RNA was isolated from specific tissues, from 3–5 embryos, which were frozen in liquid nitrogen. The details have been described elsewhere (Zajdel et al., 2003; Dube et al. 2017). Tissues from adult leghorn chickens were procured from a local farm (Cobblestone Valley Farm, Preble, NY13141); also tissues from 15-day post hatch leghorn chickens were obtained from another local farm (Treasures of Joy Farm, Nedrow, NY 13120). RNA was isolated from tissues as described before (Zajdel et al., 2003; Dube et al. 2017). The RIN (RNA Integrity Number) of RNA samples of heart and skeletal muscles are between 8.90 to 9.2, suggesting the RNA we used were of good quality.

4.2 | Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNAs were made from various RNAs using oligo dT as the primer following our published protocols (Denz et al., 2004; Zajdel et al., 2003). Subsequently, PCR amplifications for

various genes were carried out using gene-specific and/or isoform specific-primer-pairs as shown in Table 1. The PCR amplified DNAs were run in an agarose gel, stained with ethidium bromide and subsequently analyzed by Southern hybridization using an appropriate, isoform-specific probe following the method as described earlier (Denz et al., 2011; Thurston et al., 2009). The sequences of various gene specific oligonucleotides are presented in Table 1 and also in the corresponding figure legend.

Also, the PCR amplified DNAs were run in an agarose gel and stained with ethidium bromide; the stained bands were excised from the gel and DNA was extracted from using a MinElute Gel extraction kit from Qiagen (Valencia, CA) as stated earlier (Dube et al. 2017). Part of the DNA was used for sequencing and part was ligated and cloned into the T/A cloning vector (Life Technologies) following our published protocol (Dube et al. 2017). Positive clones were identified by PCR as stated by Dube et al (2017). Positive clones were grown in appropriate media over-night and the DNA was extracted using Qiagen mini-prep kit (Valencia, CA). The isolated DNA was sequenced (Cornell University Life Science Core Laboratories Center, Ithaca, NY). Each clone was sequenced in both directions.

4.3 | Real-time quantitative RT-PCR

qRT-PCR was carried out using the LightCycler 480 Real-Time PCR System as described before (Dube et al. 2014; Nan et al., 2015; Dube et al. 2017). Reactions were carried out in a 384-well plate using the LightCycler 480 SYBR Green I Master kit (Roche). Relative quantification of qRT-PCR data was performed using the $2^{-\Delta\Delta CT}$ (sample CT minus 18S rRNA CT) and $2^{-\Delta CT}$ (sample CT minus comparator CT) methods (Livak & Schmittgen, 2001; Pfaffl, 2001; Yuan et al. 2006). A comparative value was calculated using the formula $X = 2^{-\Delta\Delta CT}$, where X equals the efficiency of specific primer pairs. This is similar to the $2^{-\Delta CT}$ method but corrects for the assumption that the reaction is occurring with 100% efficiency.

Efficiencies (X) were determined using a 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 corresponding chicken cDNAs generated either with oligo dT or with gene specific and/or isoform-specific oligonucleotide.

Statistical Analyses.—The means, standard deviations, and comparative analyses of each data set for statistical significance were done using paired Student's *t*-test.

4.4 | Conventional Western blot analyses

Protein was extracted from embryonic heart and skeletal muscles with Tissue Extraction Reagent (Invitrogen) containing 50 mM Tris, pH 7.4; 250 mM NaCl; 5mM EDTA; 1 mM EGTA; 1 mM NaF; 20 mM $\text{Na}_4\text{P}_2\text{O}_7$; 2 mM Na_3VO_4 ; and proprietary detergent (name and composition not given by the company). The tissue extraction buffer was supplemented with 1 mM PMSF and protease inhibitor cocktail (Roche) following manufacturer specifications. Tissue lysates were centrifuged at 10000 rpm at 4°C for ten minutes. The pellets were discarded and supernatant from each sample was used for subsequent Western blot analyses following our published protocol (Dube et al. 2017b). ~20 µg protein was used for loading

each lane. Protein was determined by using Bio-Rad reagent following the protocol supplied by the company (Bio-Rad Laboratories, Richmond, CA). Primary antibodies included CH1 anti-sarcomeric tropomyosin (Hybridoma Bank, DHSB, University of Iowa, Iowa) and a monoclonal anti-GAPDH antibody (Aviva Systems Biology, Corp, San Diego, CA). Secondary antibody was goat anti-mouse immunoglobulin HRP (GE Healthcare).

4.5 | 2D (Two-dimensional) Western blot analysis

2D Western blot analyses of the extracts of 7-day and 10-day old embryonic chicken hearts were performed for us by Kendrick Labs, Inc., Madison, WI. Embryonic chicken hearts were obtained as stated above. It is to be noted that extracts were made with at least 3 hearts for each embryonic stage. The frozen heart muscles were homogenized in 150 μ l osmotic lysis buffer containing 10x nuclease stock, phosphatase inhibitor stocks [I and II], and protease inhibitor stock. 150 μ l of SDS boiling buffer without reducing agents was added and the samples were heated in a dry bath at 95°C for ten minutes before the protein concentration (4.12 mg/ml) was determined using the BCA Assay (Smith et. al. 1985 and Pierce Chemical Co., Rockford, IL). Samples were then lyophilized and re-dissolved to 3.33 mg/ml in 1:1 diluted SDS boiling buffer: urea sample buffer before loading. Gel loading and sample preparation are given in Table 2. The Coomassie blue-stained PVDF membrane was desktop scanned, destained in 100% methanol, rinsed briefly in Tween-20 tris buffered saline (TTBS), and blocked for two hours in 5% non fat dry milk (NFDM) in TTBS. The blot was incubated in primary antibody (anti Tropomyosin-Clone CH1 [Developmental Studies Hybridoma Bank] diluted 1:10,000 in 2% NFDM TTBS) overnight and rinsed 3 $\times$ 10 minutes in TTBS. The blot was placed in secondary antibody (anti-mouse IgG-HRP [GE Healthcare, Cat # NA931V, Lot # 9621358] diluted 1:2,000 in 2% NFDM TTBS) for two hours, rinsed as above, treated with ECL, and exposed to x-ray film. When developed x-ray film was superimposed on the coomassie stained protein gel, 3 spots (1 in 10-day and 2 in 7-day old embryonic chicken hearts) were exhibited (as in Figure 7). The gel spots were excised, washed, and trypsinized following the published protocols [Shevchenko et al.1996; Darie et al.,2011; Sokolowska et al., 2012]. The resulting peptides were extracted twice with 5% formic acid/50 mM ammonium bicarbonate/50% acetonitrile and once with 100% acetonitrile under moderate shaking. The peptide mixture was then dried in a speed-vac, solubilized in 20 μ L of 0.1% formic acid/2% acetonitrile.

2.5. LC-MS/MS.

The peptide mixture was analyzed by reverse phase liquid chromatography (LC) and MS (LC-MS/MS) using a NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Ultima API MS (Micromass/Waters, Milford, MA), according to published procedures [Darie et al., 2011; Sokolowska et al., 2012; Shevchenko et al., 1996]. Briefly, the peptides were loaded onto a 100 μ m x 10 mm NanoAcuity BEH130 C18 1.7 μ m UPLC column (Waters, Milford, MA) and eluted over a 150 minute gradient of 2–80% organic solvent (ACN containing 0.1% FA) at a flow rate of 400 nL/min. The aqueous solvent was 0.1% FA in HPLC water. The column was coupled to a Picotip Emitter Silicatip nano-electrospray needle (New Objective, Woburn, MA). MS data acquisition involved survey MS scans and automatic data dependent analysis (DDA) of the top three ions with the highest intensity ions with the charge of 2+, 3+ or 4+ ions. The MS/MS was triggered when the MS signal

intensity exceeded 10 counts/second. In survey MS scans, the three most intense peaks were selected for collision-induced dissociation (CID) and fragmented until the total MS/MS ion counts reached 10,000 or for up to 6 seconds each. The entire procedure used was previously described (Dube et al. 2015). Calibration was performed for both precursor and product ions using 1 pmol GluFib (Glu1-Fibrinopeptide B) standard peptide with the sequence EGVNDNEEGFFSAR and the monoisotopic doubly-charged peak with m/z of 785.84.

2.6. Data processing and protein identification.

The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software as previously described by Darie et al [2010].

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Figure 1. RT- PCR and Southern hybridization of TPM1, TPM2, TPM4, and slow myosin heavy chain RNA expressed in heart and skeletal muscle of adult chicken.

Panel a. Ethidium bromide staining of TPM1 amplified DNA.

Panel b. Southern hybridization with TPM1 probe.

Panel c. Ethidium bromide staining of TPM2 amplified DNA.

Panel d. Southern hybridization with TPM2 probe.

Panel e. Ethidium bromide staining of TPM4 amplified DNA.

Panel f. Southern hybridization with TPM4 probe.

Panel g. Ethidium bromide staining of slow myosin heavy chain amplified DNA.

Panel h. Southern hybridization with slow myosin heavy chain probe.

Lane M: Molecular marker; Lane AH: Adult Heart; Lane ASK: Adult skeletal muscle; Lane PC: Primer control.

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Figure 2. RT-PCR and Southern hybridization of TPM1 α and TPM1 κ in developing chicken hearts.

Panel A. Ethidium bromide staining of the RT-PCR products separated on agarose gel

Panel B: Southern hybridization with TPM1 α or TPM1 κ specific probe.

Lanes 1 to 5: Amplified with TPM1 α primer-pair

Lanes 6 to 9: Amplified with TPM1 κ primer-pair

Lanes 1 & 6: 5 day embryonic heart; Lanes 2 & 7: 10 day embryonic heart; Lanes 3 & 8: 15 day embryonic heart; Lanes 4 & 9: 15 day post hatch heart; Lane 5 primer control. Lanes 1–5 probed with TPM1 α –specific probe; lanes 6–9 probed with TPM1 κ -specific probe.

Figure 3. Expression of sarcomeric TPM1 and TPM4α transcripts relative to 18S rRNA in developing and adult chicken hearts using 2^{-ddCt} method.

The results were normalized with those for E7=1.

Top: qRT-PCR of TPM1 including both TPM1α and TPM1κ

Bottom: : qRT-PCR of TPM4α

E7: 7-day old embryo; E10: 10-day old embryo; E15: 15-day old embryo; P15: 15-day post hatch chicken; Ad: Adult chicken.

cDNAs were made with oligo dT. Chicken TPM1 α (F)/Chicken TPM1 α (R) primer-pair was used for amplification of TPM1 and Chicken TPM4(F) / Chicken TPM4(R) was used for amplification of TPM4 α .

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Figure 4. Melt curves of SYBER green PCR and the agarose gel electrophoresis of PCR amplified DNA stained with ethidium bromide

A. Melt curves for amplified chicken TPM1. The multiple curves represent the products from multiple replicates of the qRT-PCR assay performed on transcripts from all stages of chicken heart development shown in Figure 3.

B. Melt curves for amplified TPM4α DNA in developing chicken from the same samples.

C. PCR products separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. The results demonstrate amplification of a single product for TPM1 or TPM4 α or 18S rRNA amplification.

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Figure 5. Relative expression of TPM1α in embryonic and adult chicken heart and skeletal muscle using both 2^{-Ct} method and $2^{-(\Delta Ct)}$ methods.

Top: Ct method

Bottom: - Ct method. Fold changes in developing heart v skeletal

Red bar: Heart and Blue bar: skeletal muscle. The results for the heart were considered to equal one for each developmental stage tested.

Figure 6. Relative expression of TPM2α in embryonic and adult chicken using both 2^{-Ct} method and 2^(-ddCt) methods.

Top: Ct method

Bottom: - Ct method. Fold changes in developing heart v skeletal muscles. The results for each developmental stage are normalized to the heart results being one.

Red bar: Heart and Blue bar: skeletal muscle.

E7: 7-day old embryo; E10: 10-day old embryo; E15: 15-day old embryo; P15: 15-day post hatch chicken; Adult: Adult chicken.

Figure 7. Relative expression of TPM4α in embryonic and adult chicken heart and skeletal muscle using both 2^Δ-Ct method and 2^Δ(-Ct) methods.

Top: Ct method

Bottom: - Ct method. Fold changes in developing heart v skeletal muscle. The results for skeletal muscle were normalized to equal one for each development stage tested.

Red bar: Heart and Blue bar: skeletal muscle

E7: 7-day old embryo; E10: 10-day old embryo; E15: 15-day old embryo; P15: 15-day post hatch chicken; Adult: Adult chicken.

cDNA was made with gene specific oligonucleotide from exon 9a/b region :5'-GAGGTGCAGACTACAGGGAG-3'. Amplification of TPM4 α was carried out with TPM4 α (F) / TPM4 α (R) primer pair.

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Figure 8. Western blot analyses of sarcomeric tropomyosin in embryonic chicken hearts.
(Top): Ponceu staining of the blot. (Middle): Staining with CH1 monoclonal antibody.
(Bottom): staining with a monoclonal anti-GAPDH antibody.
Lane 1: 10-day embryonic heart; lane 2: 15-day embryonic heart; and lane M: molecular weight marker

Figure 9. 2D western blot analyses with extracts from 7-day and 10-day old chicken embryonic hearts.

a. The Coomassie stained gel of 7-day old embryonic chicken heart muscle protein after 2D electrophoresis

b. The PVDF filter was stained with CH1 monoclonal antibody followed by treatment with secondary antibody as mentioned under materials and methods. One spot “C” was marked, excised and used for extraction of protein for subsequent Mass spectrometric analyses. The corresponding spot was also marked as “C” in panel A.

c The Coomassie stained gel of 10-day old embryonic chicken heart muscle protein after 2D electrophoresis.

d. Same as in b. However, in this gel 2 spots “a” & “b” were marked for subsequent Mass Spectrometric analyses. The corresponding spots were also marked in panel C.

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MS Analysis for protein

SPOT #C FROM THE EXTRACT OF 7-DAY OLD CHICKEN HEART

TPM4 ISOFORM X2 (Gallus gallus) (XP_015155677.1)

Protein sequence coverage : 83%

```

1 MEAIKKKMQM LKLDKENAID RAEQAETDKK AAEDCKQVE DELVALQKKL KGTEDELDKY
61 SEALKDAQEK LEQAEEKATD AEGEVAALNR RIQLVEEELD RAQERLATAL QKLEEAEEKAA
121 DESERGMKVI ENRAMKDEEK MEIQEMQLKE AKHIAEEADR KYEEVARKLV ILEGELERAE
181 ERAEVSEVKC SDLEELKNV TNNLKSLEAQ SEKYSKEDK YEEIKILSD KLKEAETRAE
241 FAERTVAKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI

```

PEPTIDE SEQUENCES IDENTIFIED IN SPOT C : 78

TPM1 ISOFORM (Gallus gallus) (UniProtKB/Swiss-Prot: P04268.2)

Protein sequence coverage: 39%

```

1 MDAIKKKMQM LKLDKENALd RAEQAEDDKK AAERSKQLE DELVALQKKL KGTEDELDKY
61 SESLKDAQEK LELADKKATD AESEVASLNR RIQLVEEELD RAQERLATAL QKLEEAEEKAA
121 DESERGMKVI ENRAQKDEEK MEIQEIQLKE AKHIAEEADR KYEEVARKLV IIEGDLERAE
181 ERAELSESKC AELEELKTV TNNLKSLEAQ AEKYSQKEDK YEEIKIVLTD KLKEAETRAE
241 FAERSVTKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI

```

SPOT #A FROM THE EXTRACT OF 10-DAY OLD CHICKEN HEART

TPM4 ISOFORM X2 (Gallus gallus) (XP_015155677.1)

Protein sequence coverage: 33%

```

1 MEAIKKKMQM LKLDKENAID RAEQAETDKK AAEDCKQVE DELVALQKKL KGTEDELDKY
61 SEALKDAQEK LEQAEEKATD AEGEVAALNR RIQLVEEELD RAQERLATAL QKLEEAEEKAA
121 DESERGMKVI ENRAMKDEEK MEIQEMQLKE AKHIAEEADR KYEEVARKLV ILEGELERAE
181 ERAEVSEVKC SDLEELKNV TNNLKSLEAQ SEKYSKEDK YEEIKILSD KLKEAETRAE
241 FAERTVAKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI

```

PEPTIDE SEQUENCES IDENTIFIED : 11

SPOT #B FROM THE EXTRACT OF 10-DAY OLD CHICKEN HEART

TPM4 ISOFORM X2 (Gallus gallus) (XP_015155677.1)

Protein sequence coverage: 40%

```

1 MEAIKKKMQM LKLDKENAID RAEQAETDKK AAEDCKQVE DELVALQKKL KGTEDELDKY
61 SEALKDAQEK LEQAEEKATD AEGEVAALNR RIQLVEEELD RAQERLATAL QKLEEAEEKAA
121 DESERGMKVI ENRAMKDEEK MEIQEMQLKE AKHIAEEADR KYEEVARKLV ILEGELERAE
181 ERAEVSEVKC SDLEELKNV TNNLKSLEAQ SEKYSKEDK YEEIKILSD KLKEAETRAE
241 FAERTVAKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI

```

PEPTIDE SEQUENCES IDENTIFIED : 16

Figure 10. Identification of amino acid sequences from the peptides extracted from spot a, b, and c after 2D western blot analyses of embryonic chicken hearts.

Red color letters indicate the isolated peptides that have been sequenced by Mass spectrometry.

Bold, underlined, and larger font letters indicate the signature peptides for TPM1 that are different from TPM4α.

Both TPM4α and TPM1α are identified in spot “c” from 7-day embryonic chicken heart.

TPM4 α is the only TPM isoform detected in both Spots “a” and “b” in 10-day old embryonic chicken heart.

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Table 1.

Primers and probes utilized for amplification of various TPM isoforms and control RNAs

Gene	Isoform amplified	Primer/Detector	Type of PCR	Sequence
TPM1	TPM1 α & TPM1 κ (F)	Forward Primer	Conventional	5'-ATGGATGCCATCAAGAAAAAGATG-3'
	TPM1 α & TPM1 κ (R)	Reverse Primer	Conventional	5'-AAATTATATGGAAGTCATATCG-3'
	TPM1 α & TPM1 κ (P)	Probe	Conventional	5'-TGTGCTGAGCTTGAAGAGGAG-3'
	TPM1 α (F)	Forward Primer	Conventional	5'-GGAGGACGAGCTGGTGGCCT-3'
	TPM1 α (P)	Probe	Conventional	5'-ACTGAGGATGAGCTGGACAA-3'
	TPM1 κ (F)	Forward primer	Conventional	5'-GATGACATTGTGCAATTGGAA-3'
	TPM1 κ (P)	TPM1 κ Sp Probe	Conventional	5'-CTGGAAGAGCTGCACAAG-3'
	Chicken TPM1(F)	Forward Primer	qRT-PCR	5'-CTGAGACCCGTGCTGAATTT-3'
	Chicken TPM1(R)	Reverse Primer	qRT-PCR	5'-AAGTCATATCGTTGAGAGC-3'
	TPM1 α (F)	Forward Prime	qRT-PCR	5'-GGAGGACGAGCTGGTGGCCT-3'
	TPM1 α (R)	Reverse Primer	qRT-PCR	5'-CTGCAGGGCAGTAGCCAAGC-3'
TPM2	TPM2A ENTIRE (F)	Forward Primer	Conventional	5'-CACCGCCACCATGGAGGC-3'
	TPM2A ENTIRE (R)	Reverse Primer	Conventional	5'-GGTTCAGAGGGAGGTGAT-3'
	TPM2A ENTIRE (P)	Probe	Conventional	5'-GCGCTTAACGACATCACCTC-3'
	Chicken TPM2 (F)	Forward Primer	qRT-PCR	5'-AAGGAGGACAAGTACGAGGA-3'
	Chicken TPM2 (R)	Reverse Primer	qRT-PCR	5'-GGTGCCGGTGCCAGTGT-3'
TPM3	Chicken TPM3 (F)	Forward Primer	qRT-PCR	5'-5'-ATGGAGGCCATCAAGAAGGAT-3'
	Chicken TPM3 (R)	Reverse Primer	qRT-PCR	5'-GTGGCGAGGCGCTCCTGGGC-3'
TPM4	TPM4 α (F)	Forward Primer	Conventional	5'-ATGGATGCCATCAAGAAAAAGATG-3'
	TPM4 α (R)	Reverse Primer	Conventional	5'-AGACTACAGGGAGGTCATATCATT-3'
	TPM4 α (P)	Probe	Conventional	5'-GCAGAAGGTGAGGTGGCGGCG-3'
	Chicken TPM4(F)	Forward primer	qRT-PCR	5'-CCCAGGAGGTGCAGACTACA-3'
	Chicken TPM4 (R)	Reverse Primer	qRT-PCR	5'-CGTGCTGAGTTTGCTGAGAG-3'
	Chicken TPM4a (F)	Forward Primer	qRT-PCR	5'-TTGATAGAGCAGAGCAGGCTG-3'
	Chicken TPM4a (R)	Reverse Primer	qRT-PCR	5'-ATGCGTCTGTTGAGCGCC-3'
18S rRNA	Chicken 18S (F)	Forward Primer	qRT-PCR	5'-GAACGCCACTTGTCCTCTA-3'
	Chicken 18S (R)	Reverse Primer	qRT-PCR	5'-ATGGCCGTTCTTAGTTGGTG-3'

Gene	Isoform amplified	Primer/Detector	Type of PCR	Sequence
GAPDH	GAPDH (F)	Forward Primer	qRT-PCR	5'-GACGTGCAGCAGGAACACTA-3'
	GAPDH (R)	Reverse Primer	qRT-PCR	5'-CTTGGACTTTGCCAGAGAGG-3'
Slow MyHC-3	sMyHC (F)	Forward Primer	Conventional	5'-TACCAGACAGAGGAAGACCG-3'
	SMyHC (R)	Reverse primer	Conventional	5'-TTGCTGAAAGCAGAGAGATCC-3'
	SMyHC (P)	Probe		5'-GCTGCGGGCACGGAGCCGTG-3'

Table 2.

Key to 2D Gel Loading Sample Preparation.

Gel ID #	Sample	μl loaded	μg loaded	Treatment
LF1175 #1	7 Day Chicken Heart	150	500	Coomassie
LF1175 #2	7 Day Chicken Heart	150	500	Anti-Tropomyosin
LF1161 #1	10 Day Chicken Heart	150	500	Coomassie
LF1161 #2	10 Day Chicken Heart	150	500	Anti-Tropomyosin