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Identification, characterization, and expression of sarcomeric tropomyosin isoforms in zebrafish

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Abstract

Tropomyosin is a component of thin filaments that constitute myofibrils, the contractile apparatus of striated muscles. In vertebrates, except for fish, four TPM genes *TPM1*, *TPM2*, *TPM3*, and *TPM4* are known. In zebrafish, there are six TPM genes that include the paralogs of the *TPM1* (*TPM1-1* & *TPM1-2*), the paralogs of the *TPM4* gene (*TPM4-1* & *TPM4-2*), and the two single copy genes *TPM2* and *TPM3*. In this study, we have identified, cloned, and sequenced the TPM1-1 κ isoform of the *TPM1-1* gene and also discovered a new isoform TPM1-2 ν of the *TPM1-2*. Further, we have cloned and sequenced the sarcomeric isoform of the *TPM4-2* gene designated as TPM4-2 α . Using conventional RT-PCR, we have shown the expression of the sarcomeric isoforms of *TPM1-1*, *TPM1-2*, *TPM2*, *TPM3*, *TPM4-1*, and *TPM4-2* in heart and skeletal muscles. By qRT-PCR using both relative expression as well as the absolute copy number, we have shown that TPM1-1 α , TPM1-2 α , and TPM1-2 ν are expressed mostly in skeletal muscle; the level of expression of TPM1-1 κ is significantly lower compared to TPM1-1 α in skeletal muscle. In addition, both TPM4-1 α and TPM4-2 α are predominantly expressed in heart. 2D Western blot analyses using anti-TPM antibody followed by Mass Spectrometry of the proteins from the antibody-stained spots show that TPM1-1 α and TPM3 α are expressed in skeletal muscle whereas TPM4-1 α and TPM3 α are expressed in zebrafish heart. To the best of our knowledge, this is by far the most comprehensive analysis of tropomyosin expression in zebrafish, one of the most popular animal models for gene expression study.

INTRODUCTION

Tropomyosins, a family of highly related actin-binding proteins, are present in cells of various organisms ranging from yeast to humans. In vertebrates, it has been known for some time that tropomyosins are encoded by four different genes, designated as *TPM1*, *TPM2*, *TPM3*, and *TPM4*. Each of the four TPM genes can form a multitude of isoforms via alternate splicing and/or using an alternate promoter [Gunning et al. 2008; Lees-Miller and

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Helfman, 1991; Schevzov et al. 2011]. However, in the last ten years, our knowledge about the tropomyosin gene number and structure in various organisms has been changed significantly. Unlike in avian [Fleenor et al., 1992], amphibian [Hardy et al., 1995; Thomas et al., 2010] or mammalian systems [Pieples and Wieczorek, 2000; Perry 2001], there are six TPM genes in fish. This was first reported in Fugu fish [Ikeda et al., 2003; Toramoto et al., 2004] followed by Zebrafish (*Danio rerio*) [Schevzov et al. 2011]. The fish tropomyosin genes are designated as *TPM1-1*, *TPM1-2*, *TPM2*, *TPM3*, *TPM4-1*, and *TPM4-2*. Of the six, *TPM1-1* & *TPM1-2* as well as *TPM4-1* & *TPM4-2* are duplicated paralogs. In fish, *TPM2* and *TPM3* are single copy genes equivalent to mammalian *TPM2* and *TPM3*, respectively. The organization of *TPM1-1* gene is highly conserved in vertebrates with exons 1a, 2a, 1b, 3, 4, 5, 6a, 6b, 7, 8, 9a, 9b, 9c, 9d, whereas the duplicated paralog *TPM1-2* does not have exon 2a and 6a. Fish *TPM2*, unlike its other vertebrate counterparts, does not have exon 6a whereas fish *TPM3* differs from other vertebrates by not having a second promoter nor exon 6a. The exon compositions in the duplicated paralog of *TPM4* are very similar to the *TPM4* gene in amphibians, avian, and humans.

Toramoto et al [2004] reported the *TPM* gene organization and the expression of various isoforms encoded by the six *TPM* genes in Fugu fish. However, the expression analysis of various TPM transcripts was carried out by conventional RT-PCR using gene and isoform specific primer-pairs with no Southern hybridization. Currently, there is no report on the quantification of various TPM transcripts in Fugu fish. On the basis of the ethidium bromide staining of the amplified DNA band on agarose gel, Toramoto et al (2004) inferred that the high molecular wt *TPM1-1α* and *TPM1-2α* are expressed in fast and slow skeletal muscle but very little in heart. As in amphibians [Spinner et al. 2002] and avian [Forry-Schaudies et al. 1990], *TPM4-1α* is expressed mostly in the heart. On the contrary, *TPM4-2α* is mostly expressed in fast and slow skeletal muscle but very little, if not at all, in heart. As in other vertebrates, *TPM2α* and *TPM3α* are expressed in both fast and slow skeletal muscle in Fugu fish.

It has been reported that the *TPM4* gene is essential for cardiac contractility/function in zebrafish [Zhao et al., 2008]. Also, cDNAs of *TPM1-1α* [Accession # NM_001024467.1] and *TPM1-2α* [Accession # M24635.1] have been cloned and sequenced from skeletal muscles. To the best of our knowledge, there is no report on comparative analyses of the expression of transcripts of various isoforms encoded by different TPM genes in zebrafish striated muscles. Therefore, we have undertaken a comprehensive study on tropomyosin RNA and protein isoform expression in zebrafish. As *TPM2* and *TPM3* are the two single copy genes in fish similar to other vertebrates we have focused mostly on *TPM1-1*, *TPM1-2*, *TPM4-1*, and *TPM4-2*.

In this report, we have employed conventional as well as qRT-PCR for determining the expression level of transcripts of sarcomeric isoforms of *TPM1-1α*, *TPM1-2α*, *TPM4-1α*, and *TPM4-2α* as well as the new *TPM1-2ν* isoform that we discovered in heart and skeletal muscles. Protein expression was analyzed by conventional and 2D Western blot analyses using anti-tropomyosin antibodies. Amino acid sequences of isolated peptides were determined by Mass Spectrometry. Expression constructs containing known *TPM1-2α* or a

novel zebrafish isoform of TPM1-2 ν were transfected into quail myotubes to determine whether they are incorporated into organized myofibrils *in situ*.

RESULTS

Identification of sarcomeric TPM1-1 κ expressed in zebrafish striated muscles

TPM1-1 and *TPM1-2* are two paralogs, however, their exon compositions are somewhat different. *TPM1-1* contains both exon 2a and 2b (Figure 1A) whereas *TPM1-2* has only exon 2b (Figure 1B). The TPM1 gene in other vertebrates is known to generate two sarcomeric isoforms – TPM1 α and TPM1 κ . TPM1-1 α consists of exon 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a/b whereas the TPM1 κ contains all the same exons except that exon 2a replaces exon 2b (Figure 1A). Since *TPM1-2* gene has only exon 2b, it cannot generate TPM1-2 κ . To date, it is not known whether TPM1-1 κ is expressed in zebrafish (Figure 1). We have addressed this issue using conventional RT-PCR with RNA from heart and skeletal muscle. The primer-pair for identification of TPM1-1 κ was designed from published exon-specific nucleotides sequences of TPM1-1 as given in Table 1. The results depicted in Figure 2 show the ethidium bromide stained DNA bands amplified by the generic primer-pair both in heart (lane 1, panel A) and skeletal muscle (lane 2). No amplification was detected in lane 3, the negative control. When amplification was carried out with the TPM1-1 κ specific primer-pair (see Table 1), TPM1-1 κ expression was also evident in heart (lane 4, panel A) and in skeletal muscle (lane 5, panel A). No DNA was detected in primer-control (lane 6, panel A). The results were further confirmed by Southern blot hybridization with TPM1-1 κ probe (panel B). As the generic primer-pair amplified both TPM1-1 α and TPM1-1 κ , the hybridization was positive with TPM1 α probe (lanes 1 and 2 in panel C). However, TPM1-1 α probe did not give any positive signal when the amplification was done with a TPM1-1 κ specific primer-pair (lanes 4 and 5 in panel C). Our results strongly indicate that TPM1 κ was expressed in heart and skeletal muscle in zebrafish as in axolotl [Thomas et al. 2010]. The expression of TPM1-1 κ was further confirmed by determining the nucleotide sequences of the gel extracted DNA amplified by TPM1-1 κ specific primer-pairs. The nucleotide as well as deduced amino acid sequences are given in Figure 3.

Expression of TPM4-1 in zebrafish heart and skeletal muscles

The *TPM4* gene in zebrafish is known to express a high molecular wt sarcomeric isoform designated as TPM4-1 α and a low molecular wt isoform with exon 1b instead of exon 1a using a different promoter. We carried out conventional RT-PCR with RNA from heart and skeletal muscle using primer-pair specific for TPM4-1 α and primer-pair that can amplify RNA for both short and long isoforms representing the total TPM4-1 transcripts. The results illustrated in Figure 4 show that TPM4-1 α is expressed in heart (lane 1, panels A & B) but not in skeletal muscle (lane 2 in panels A & B). However, the total TPM4-1 transcripts are expressed in both heart and skeletal muscle (lanes 4 & 5 in panels A & B).

Identification, characterization, and expression of a new isoform of the TPM1-2 gene

We designed a primer-pair to amplify the full length sarcomeric isoform TPM1-2 α from the total skeletal muscle RNA using conventional RT-PCR. The nucleotide sequences of the primer-pairs are given in Table 1. The PCR-amplified DNA was cloned into a T/A cloning

vector using our standard protocol [Denz et al. 2011]. Ten clones were randomly selected and the isolated DNA from the selected clones were sequenced. The nucleotide sequence from most of the clones were with full-length TPM1-2 α but a few of the clones designated TPM1-2 ν (Figure 5) had a shorter nucleotide sequence with a shorter open reading frame, which contains exons 1a, 2b, 3, 4, 8, & 9a/b and encodes 186 amino acid long polypeptide without altering the open reading frame (Figure 1B & Figure 5). Figure 6 demonstrates the comparison of the protein sequences encoded by TPM1-2 α and TPM1-2 ν . Next, we designed TPM1-2 ν specific primer-pair and evaluated the expression of TPM1-2 α and TPM1-2 ν in heart and skeletal muscle using conventional RT-PCR. The level of expression of TPM1-2 α and TPM1-2 ν transcripts in zebrafish heart are practically undetectable. On the contrary, both TPM1-2 α and TPM1-2 ν are expressed in skeletal muscle, which was further confirmed by the subsequent quantification of expression of various TPM isoforms including TPM1-2 ν in zebrafish striated **muscle** as stated in the following section.

Expression analyses of TPM2 α and TPM3 α in heart and skeletal muscle using conventional RT-PCR

The data in Figure 7 indicate that both TPM2 α and TPM3 α transcripts are expressed in heart and skeletal muscle from adult zebrafish.

Identification and characterization of TPM4-2 α , the sarcomeric isoform of the TPM4-2 gene

Recently, the predicted TPM4-2 α nucleotide as well as deduced amino acid sequences have been posted in GenBank (accession # XM_005168448). We designed primer pairs (Table 1) and performed conventional RT-PCR with the cDNAs made of RNA from zebrafish heart and skeletal muscle. The mRNA copy numbers of TPM4-2 α in striated muscle is most likely very low. Hence, hardly any discrete band in the agarose gel after staining with ethidium bromide was visible. We diluted the original PCR product (1:200) and used for second PCR amplification with a second set of nested primers (Table 1). A clear and distinct visible band was seen in the sample from heart RNA, whereas the signal was quite lighter in skeletal muscle. DNA was eluted from the band and was ligated to the T/A cloning vector. DNA was isolated from 10 colonies with positive identification and sequenced from both sides. The nucleotide as well as deduced amino acid sequences are shown in Figure 8. The nucleotide as well as deduced amino acid sequences of TPM4-2 α from heart and skeletal muscle are identical. They match with the predicted sequence available in the GenBank (Accession # XM_005168448) with the exception of 4 nucleotides, which are shown in bold larger font and underlined. These four nucleotide changes cause only silent mutation and no changes in the amino acids.

qRT-PCR for determining relative expression of TPM1-1 α , TPM1-1 κ , TPM1-2 α , TPM1-2 ν , TPM4-1 α , and TPM4-2 α in heart and skeletal muscle

We analyzed the qRT-PCR results by 2^{-ΔΔCT} method using zebrafish 18S rRNA as the reference gene to determine the relative expression of TPM1-1 α and TPM1-1 κ . It is to be noted that TPM1-1 α and TPM1-1 κ have the identical N- and C- terminal ends. Hence, both isoforms contains exon 9b at the C-terminus. In order to eliminate the interference of other isoforms, we used an exon 9 specific reverse primer to make the 1st strand cDNA with total

RNA from heart and skeletal muscle. Melt curve data show different unique melting temperatures for TPM1-1 α and TPM1-1 κ (data not shown). Also, agarose gel electrophoresis of the PCR amplified DNA for TPM1-1 α or TPM1-1 κ shows a single band for each of them. Hence, each primer pair recognizes only TPM1-1 α or TPM1-1 κ amplified DNA. The results presented in Figure 9 indicate that the level of expression of TPM1-1 α in zebrafish skeletal muscle is significantly higher compared to its expression in cardiac muscle. On the other hand, the level of expression of TPM1-1 κ is comparable in heart and skeletal muscle (Figure 9).

Next, we determined the relative expression of TPM1-2 α and the new variant TPM1-2 ν in heart and skeletal muscle. Again, both isoforms contain identical N and C-terminal ends. As stated above we used a TPM1-2 specific oligonucleotide from exon 9 to make the cDNA. The nucleotide sequences of different oligonucleotides used are given in Table 1 and Table 2. The results presented in Figure 10 indicate that the relative expression of both the isoforms is significantly higher in skeletal muscle. On the contrary, the relative expression of TPM4-1 α and TPM4-2 α is significantly higher in cardiac muscle (Figure 11). In case of TPM4-1 α , the cDNA was made with an oligo dT primer and exon 9A specific oligonucleotide (as presented in Table 2) was used for cDNA of TPM4-2 α .

Determination of absolute copy number of various TPM isoforms in zebrafish heart and skeletal muscle (Table 3)

The quantification of copy number of different TPM isoforms was carried out using our published protocol [Nan et al., 2015; Dube et al., 2014]. The standard curves for different isoforms are not shown. The results presented in Table 3 showed the copy number for each isoform in heart and skeletal muscle. Of all the isoforms, the copy number of TPM1-2 α per μg of total RNA is highest in skeletal muscle (5.14×10^8), followed by the expression of TPM4-1 α in zebrafish cardiac muscle, which is 1.56×10^8 . Again, as was found with relative expression results, the expression of TPM4-1 α is much higher in heart compared to skeletal muscle. The expression pattern of TPM1-1 α is higher in skeletal muscle. The results of the expression of various isoforms using two different methods are very similar. It is important to point out that the expression of TPM1-2 α is about 10^4 fold higher than the expression of TPM1-2 ν isoform in skeletal muscle. Again, the expression of both isoforms is significantly higher in skeletal muscle (Table 3).

Transfection of pYFPN1.TPM1-2 α and pYFPN1.TPM1-2 ν in quail myotubes

The newly discovered isoform TPM1-2 ν , like the sarcomeric isoform TPM1-2 α (Figure 2A), contains exon 1a and exon 9a/b at the N-terminus and C-terminus ends, respectively. However, exons 5, 6b, 7, and 8 are missing in TPM1-2 ν . In order to examine whether this new isoform can localize in organized myofibrils *in vivo*, we made fusion protein constructs of pYFPN1.TPM1-2 α and pYFPN1.TPM1-2 ν . Each expression construct was transfected into quail skeletal muscle myotubes and cells were then fixed after three days and stained with anti-alpha-actinin monoclonal antibody (Figure 12). In the peripheral areas of transfected myotubes, especially at the spreading ends and sides (Figure 12a and 12b), the full length YFP.TPM1-2 α was incorporated in a non-striated pattern in the premyofibrils and nascent myofibrils as defined by White et al [2014]. Distal to the ends of the myotubes and

in the shafts of myotubes, YFP.TPM1-2 α was most prominently localized in banded patterns (Figure 12c) reflecting the aligned thin filaments of the I-bands of mature myofibrils [White et al 2014; Sanger et al. 2017]. On the other hand, YFP.TPM1-2 ν fusion protein exhibits a mostly diffuse cytoplasmic background with weak localization in the myofibrils in both the spreading ends and the central regions of the myotubes (Figure 12 e, g). The expression of YFP.TPM1-2 α and YFP.TPM1-2 ν does not affect the myofibrils in myotubes as indicated by alpha-actinin antibody staining (Figure 12b, d, f, h).

2D Western blot analyses with protein extracts of adult zebrafish skeletal muscle and heart with CH1 monoclonal antibody that recognizes the tropomyosin isoforms with exon 9a peptide at the C-terminus

Figure 13 shows the 2D PAGE stained with Coomassie blue before western blotting and panel b depicts signals on the x-ray film exposed on the blot after being treated with ECL. Three spots were identified and marked as A, C, and X (Figure 13b). The results suggest that there are at least 3 detectable CH1 positive tropomyosin molecules present in the extract from adult zebrafish skeletal muscle. It is to be noted that each of these spots represents high molecular wt (32 kD and above) tropomyosin protein. However, we have failed to detect the presence of a low molecular wt CH1-positive tropomyosin protein with a molecular mass ~28 kDa like TPM1-2 ν in zebrafish skeletal muscle. Similarly, in adult zebrafish heart extract we have identified two CH1 positive protein spots (above 32 kDa) designated as D and E (Figure 13d). Again, we did not detect CH1 positive tropomyosin with a mass lower than 28 kDa.

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 method section) for LC-MS/MS analyses using NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Ultima API MS (Micromass/Waters, Milford, MA). 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 51 peptide sequences with 2309 Mascot Score identified TPM1-1 α (gi|18859505) with MW 32774. Peptide sequences cover 83% of the identified zebrafish protein TPM1-1 α (Figure 14). Subsets of these peptides also identify the presence of zebrafish TPM3 α protein in zebrafish skeletal muscle extract (Spot A2 in Figure 14) covering 62% of the protein sequence. The peptide LDKENALDQAEQAETDRKAAEER, a signature peptide of TPM3 in exon 1a, was detected. Again, the peptide KAADAEAEVASLNR is also a typical TPM3 specific peptide that was also being sequenced.

As mentioned earlier the 2D Western blot was also carried out with extract from adult heart tissue and CH1 monoclonal antibody. Two spots with a positive signal were marked D and E in (panel c in Figure 13d). From Spot D, 33 peptides were extracted. Twenty four of them with 875 Mascot Score were aligned with TPM4-1 α of MW 32846 (gi|66792936) and the remaining 9 peptide sequences with Mascot Score 349 aligned with TPM3 α of MW 32941

(gi|45387763) with 21% protein sequence coverage (Figure 15). The results strongly suggest that TPM4-1 α is the major sarcomeric TPM isoform expressed in adult hearts. In addition, adult zebrafish hearts may also express TPM3 α protein. The expression of a lower quantity of TPM3 α protein in human cardiac tissues has also been recently reported [Marston et al. 2013].

DISCUSSION

An ancient segmental duplication or whole genome duplication in fish lineage [Ikeda et al., 2003; Smith et al., 2002; Amores et al., 1998] resulted the creation of the paralogs of TPM1 and TPM4 genes in Fugufish and zebrafish. Both paralogs *TPM1-1* & *TPM1-2* as well as *TPM4-1* & *TPM4-2* co-exist in zebrafish. Interestingly, the protein sequences of TPM1-1 α and TPM1-2 α are ~98% identical suggesting that both copies of TPM-1 paralogs are capable of performing the same/identical function within the cells. This is also true for TPM4-1 α and TPM4-2 α [Toramoto et al., 2004]. The retention of both copies of these paralogs implies that each is under strong selective pressure such that there is a very little change in the coding regions. The non-coding regions and/or the regulatory region of one or both members of the paralog may undergo mutational alterations. As a result, the transcriptional efficiency of both copies of the gene decrease significantly and each of them produces fewer copy numbers of transcripts, which are sufficient enough for maintaining an effective concentration of the protein(s) *in vivo* [Toramoto et al. 2004]. Hence, both copies of genes are essential for cellular function(s). Higher intracellular concentration of TPM1 protein(s) does not necessarily mean that it requires a large number of corresponding transcripts. Two different groups independently reported that the ablation of the *TPM1* gene resulting in a single copy of the gene in mice reduced TPM1 α transcript levels to half but the protein concentration of the sarcomeric TPM1 α in the ablated mice was the same, if not identical to wild type [Blanchard et al. 1997; Rethinasamy et al., 1998]. This is due to a variable translational efficiency of TPM1 α transcripts, which in turn, regulate the expression level of the gene product [Dube et al., 2014]. In order to comprehend the reason for the co-existence of both members of the *TPM1* and/or *TPM4* paralogs in zebrafish, one would need more information about the quantity of transcripts as well as the corresponding protein(s) for each isoform of the *TPM1-1* and *TPM1-2* genes. This is true also for *TPM4-1* and *TPM4-2* paralogs. As *TPM1* has more than 15 different isoforms, it would be a huge task to quantify all the different transcripts as well as the corresponding TPM1 proteins. Lack of isoform specific anti-tropomyosin antibodies would make protein quantification extremely difficult, if not impossible. Various isoforms of tropomyosin are expressed in striated muscles, smooth muscles, and non-muscle cells thereby performing a variety of functions [Lees-Miller and Helfman, 1991]. In this study, we have analyzed only the isoforms expressed in striated muscles involved in muscle contraction in zebrafish. Thus, we have quantified the expression of the transcripts of sarcomeric isoforms only. Also, for protein expression, we have performed 2D western blot analyses using CH1 monoclonal antibody followed by Mass spectrometry that allowed us to assess the expression of the various tropomyosin proteins in cardiac and skeletal muscles in zebrafish.

TPM1 in other vertebrates generates two alternatively spliced sarcomeric isoforms- TPM1 α and TPM1 κ [Thomas et al. 2010]. Using conventional RT-PCR with isoform specific-primer

pairs we have found that TPM1-1 κ is expressed in zebrafish heart and skeletal muscle (Figure 2). We have cloned and sequenced the PCR amplified cDNA of TPM1-1 κ (Figure 3). Our qRT-PCR, using isoform specific primer-pairs show that the relative expression of TPM1-1 α compared to TPM1-1 κ is higher in skeletal muscle. However, the expression of TPM1-1 κ is comparable in heart and skeletal muscle (Figure 9). We have also performed qRT-PCR to determine the relative expression of TPM1-2 α and TPM1-2 ν in heart and skeletal muscle. The relative expression of TPM1-2 α like TPM1-1 α is significantly higher in skeletal muscle (Figure 10). Finally, the relative expression of both TPM4-1 α and TPM4-2 α is significantly higher in zebrafish heart compared to skeletal muscle (Figure 11). It is to be noted that our finding on the higher expression level of TPM4-2 α in zebrafish heart compared to skeletal muscles is quite different from its expression in Fugu fish. TPM4-2 α was found to be expressed mostly in skeletal muscles [Toramoto et al. 2004]. Further, we have determined the absolute copy number of the transcripts of TPM1-1 α , TPM1-2 α , TPM1-2 ν , and TPM4-1 α in heart and skeletal muscle (Table 3). The expression levels of TPM1-1 α and TPM1-2 α are practically identical – about 600–1000 fold higher in skeletal muscle compared to heart. On the contrary, the expression level of TPM4-1 α is much higher in heart (>3333 fold). The transcription pattern as well as transcriptional efficiency of TPM1-1 α and TPM1-2 α are comparable (Figures 9 & 10; Table 3); whereas TPM4-1 α expression is very cardiac-specific, which is true for TPM4 α expression in avian as well as amphibian species.

From transcript analyses, one can argue TPM1-1 α and TPM1-2 α may be involved in myofibrillogenesis primarily in skeletal muscles. So, for the effective concentration of sarcomeric TPM1 protein within the cell both gene products are required. In cardiac myofibrillogenesis a lower amount of TPM1 protein is required so the transcriptional efficiency or splicing event or both are significantly lower. This means that transcripts of both paralog genes are lower in quantity. Protein analyses will provide further information about the magnitude of the expressed TPM molecules encoded by the each of the paralog genes. We performed western blot analyses of the extracts from heart and skeletal muscles with CH1 monoclonal antibodies that recognize all sarcomeric tropomyosin with exon 9a peptide at the C-terminus ends. We found strong signals at the ~32–40 kD region suggesting that sarcomeric TPM proteins are expressed in heart and skeletal muscle. As isoform specific anti-TPM antibodies are not available, therefore we performed 2D western blot analyses with CH1 monoclonal antibodies. Protein was extracted from the gel spots (with positive signal) and amino acid sequences were determined using Mass spectrometry. In hearts, the expression of TPM4-1 α and TPM3 α were detected. A large number of extracted peptide are being sequenced that led to the sequencing of 83% of TPM4-1 α isoform. This most likely suggests that a relatively high concentration of TPM4-1 α protein is present in zebrafish heart. Our finding is consistent with the report that TPM4-1 is essential for cardiac contractility in zebrafish [Zhao et al, 2008]. Although TPM3 α is known to be expressed mostly in slow muscles in mammalian systems, recently **its** low level of expression has been reported in human hearts [Marston et al., 2013]. In fact, our own unpublished results also indicate a low level of expression of TPM3 α in human cardiac tissues. Also, TPM2 α has long been known to be expressed in mammalian hearts including humans at a very low level (~5% of the total sarcomeric TPM) [Rajan et al. 2010]. Interestingly, transcripts of both

TPM1-1 α and TPM1-2 α are expressed in zebrafish heart, however, we were unable to detect the expression any of the corresponding proteins in zebrafish heart. Our inability to detect TPM1-1 α and TPM1-2 α in zebrafish heart could be due to extremely low levels of expression.

TPM protein expression in zebrafish skeletal muscle is rather interesting. The results depicted in Figure 14 show the expression level of TPM1-1 α in skeletal muscle. As the number of peptides and percent of protein sequences are high one can assume that the expression level of TPM1-1 α is very high in skeletal muscle. The protein expression is consistent with results on the expression of TPM1-1 α transcripts (Figure 9). The next most abundant TPM protein in zebrafish skeletal muscle is TPM3 α , which is also consistent with fact that TPM3 α is expressed primarily in slow muscles in other species. Our conventional RT-PCR also show that the level of expression of TPM3 α is much higher in skeletal muscle compared to its expression in heart (Figure 7). The most striking finding is the absence of the TPM1-2 α protein in skeletal muscle. The expression of transcripts of TPM1-1 α and TPM1-2 α are high and comparable and the expression of TPM1-1 α protein in skeletal muscle is high still we were unable to detect TPM1-2 α specific protein. One of the reasons could be due to a very low level of expression of TPM1-2 α protein compared to TPM1-1 α protein. As a result, we were unable to detect peptide(s) specific for TPM1-2 α prepared from the CH1 positive spot “A” (Figures 14 and 15). Another possibility is that although both paralogs can make a similar quantity of transcripts, the translational efficiency of TPM1-2 α is very poor or may undergo translational repression due the mutation at the regulatory sequences at the UTRs regions. Additionally, the newly discovered alternatively spliced isoform TPM1-2 ν may compete with TPM1-2 α transcripts and thereby exert an inhibitory effect. It is to be noted that both 5' UTR and 3' UTR of the two transcripts are identical. If it is true that TPM1-2 α transcripts undergo translational repression in skeletal muscle this finding itself justifies the co-existence of the TPM1 paralogs in zebrafish.

Zhao et al [2008] reported that a heart-specific TM isoform of TPM4-1 gene is essential for myofibril assembly and heartbeat in zebrafish. McKeown et al [2014], however, pointed out that a lack of confirmed association of TPM4 α protein with thin filaments makes it unclear whether the myofibril assembly defects observed in TPM4-deficient zebrafish represent primary or indirect events. These authors also suggest that the primary direct evidence for a role of TM in myofibril assembly comes from the Mexican axolotl, in which the naturally occurring cardiac mutation eliminates TM expression and disrupts cardiac myofibril assembly via aberrant intracellular targeting of a host of thin filament and sarcomere-associated proteins and lack of coalescence of organized sarcomeres [Lemanski 1979; Zajdel et al., 1998, 1999, 2003; McLean et al., 2006]. It is important to point out that more than one isoform of sarcomeric TPM proteins for example, TPM1 α , TPM1 κ , and TPM4 α , are expressed in axolotl hearts. In **tropomyosin** deficient cardiac mutant axolotl hearts, however, all these sarcomeric tropomyosin proteins are practically absent. One can promote myofibrillogenesis in mutant axolotl hearts *in situ* either by lipofecting total tropomyosin protein or by ectopically expressing axolotl TPM1 α or murine TPM1 α or axolotl TPM1 κ or axolotl TPM4 α protein [Denz et al., 2004; Zajdel et al., 1998,1999, 2007; Spinner et al. 2002]. Conversely, ectopic expression of murine TPM2 α protein fails to promote

myofibrillogenesis in cardiac mutant axolotl hearts [Zajdel et al. 1998]. Although the transcript analyses show that TPM1-1 α , TPM1-2 α , and TPM4-1 α are expressed in zebrafish heart, we were unable to detect the presence of TPM1-1 α and TPM1-2 α protein there [Figure 15]. On the contrary, Mass Spectrometry results indicate that TPM4-1 α is the major sarcomeric TPM protein present in zebrafish heart. We believe, our finding is consistent with the reported finding that *TPM4* is essential for myofibril assembly and heartbeat in zebrafish [Zhao et al. 2008]. Comparison of the α TM1 $^{-/-}$ phenotype in mice with the phenotype of cardiac mutant axolotls is instructive for elucidating the mechanisms of α TM1 (TPM1) function in cardiac myofibril assembly in mice. However, it is an oversimplification to state that absence of only α TM1 leads to widespread abnormalities leading to failure of myofibril assembly and a lack of contraction and beating in cardiac mutant axolotl hearts. In mouse hearts, while TPM1 α is the major (~95%) component, TPM2 α is only ~5% of the total sarcomeric tropomyosin [Rajan et al., 2010]. TPM3 α is not expressed in mouse heart [Pieples and Wieczorek, 2000]. As *TPM4* gene is truncated, TPM4 α is not expressed in rodent hearts [Lees-Miller & Helfman, 1991; Perry 2001]. Hence, it is not surprising that embryonic hearts in TPM1 $^{-/-}$ mice also shows the phenotype [McKeown et al., 2014] mimicking the cardiac mutant axolotl hearts devoid of sarcomeric isoforms encoded by several *TPM* genes [Lemanski 1973; Zajdel et al., 1998, 2007; Spinner et al., 2002].

Two of our observations deserve special attention. First, we have cloned and sequenced TPM4-2 α from zebrafish heart. Our results as depicted in Figure 8 show that the deduced amino acid sequences are exactly same as in the predicted sequences available in the GenBank (XM_005168448) except for four silent mutations as pointed out in Figure 8. This could be due to the difference in the strain of zebrafish. As far as the transcript expression is concerned, TPM4-2 α is expressed more in zebrafish heart compared to skeletal muscle (Figure 11). We do not know whether the expression pattern is somewhat different in various stages of development. It is worth mentioning that the expression of TPM4-2 α in Fugu fish is primarily in skeletal muscle [Toramoto et al. 2004]. To our defense, we would like to point out that Singh et al. [2016] reported the expression TPM4-2 in bulbus arteriosus of zebrafish. In their study, these researchers using RNA sequencing data generation and subsequent alignment technology showed that Tpm-1 and TPM4b (TPM4-2) are expressed primarily in bulbus arteriosus but not in other heart chambers like atrium and ventricle. Singh et al [2016] using one of the most modern technologies, however, failed to detect the expression of a cardiac specific tropomyosin isoform like TPM4-1 α in any of the zebrafish heart chambers, which is puzzling since TPM4-1 α has been reported to be an essential tropomyosin isoform for beating zebrafish heart [Zhao et al. 2008].

Our other novel observation is the finding of an alternatively spliced isoform TPM1-2 ν of the *TPM1-2* gene. As shown in Figures 1B and 5, this novel isoform has exon 1a, 2b, 3, 4, 8, and 9a/b with an open reading frame. Although its mRNA is expressed in skeletal muscle like TPM1-2 α , we could not detect a ~24 kD protein in our western blot with CH1 monoclonal antibody the epitope of which is at the C-terminus end encoded by exon 9a. However, when it was ectopically This property may reflect GFP-TPM1-2 ν decreased ability to bind to actin filaments in the presence of native tropomyosin competition (Dome et al., 1988), a property it does not share with the zebrafish GFP-TPM1-2 α . expressed as a

GFP fusion protein in quail myotubes, the fusion protein, unlike TPM1-2 α , was mostly diffused in cells with very weak localization in myofibrils (Figure 12 e, g).

One can wonder what other role TPM1-2 ν may play in skeletal muscle. TPM1-2 ν may somehow control/influence the translation of the sarcomeric isoform TPM1-2 α in skeletal muscle. In support, one may argue that, although mRNA level is high, hardly any TPM1-2 α protein could be detected in zebrafish skeletal muscle. In order to address this issue, we are planning to create transgenic zebrafish ectopically expressing GFP.TPM1-2 ν or GFP.TPM1-2 α in skeletal muscle using an expression construct with a zebrafish skeletal muscle-specific promoter.

In summary, this is the first report that reveals the characterization and expression of transcripts of various sarcomeric TPM isoforms like TPM1-1 κ , TPM4-2 α in striated muscles that has never been done before. Also, our discovery of the new TPM1-2 ν isoform and its localization in myotubes by confocal microscopy deserves further investigations. Finally, our 2D Western blot analyses followed by Mass Spectrometric determination of protein expression divulge the sarcomeric TPM protein expression in zebrafish heart and skeletal muscles.

Materials and Methods

RNA isolation from heart and skeletal muscle of adult zebrafish

Zebrafish were obtained from the zebrafish colony of Dr. Joseph Sanger here at the Upstate Medical University. The animals were cooled in ice-cold solution and then killed instantly by decapitation prior to dissecting the heart and skeletal muscle samples for protein and nucleic acid analyses. All operations were carried out following the institutional CHUA guidelines. Specific tissues were dissected and placed in liquid nitrogen. The frozen tissues were homogenized with an RNAase-Free disposable pestle (Fischer Scientific) in Tri Reagent included with the Ribo-pure RNA extraction kit (Ambion, Thermo Fischer) following the manufacturer's instruction. The concentration of the isolated RNA was determined by measuring the absorbance in a spectrophotometer. The quality of RNA was determined by Capillary Electrophoresis using 2100 Bio analyzer, Agilent Technologies. The RIN (RNA Integrity Number) of RNA samples of heart and skeletal muscles are 9.2 and 8.90, respectively, suggesting the RNA we used were of good quality.

Conventional RT-PCR—All pre PCR and post PCR experiments were done by different personnel in different buildings using separate equipment and separate ventilation so as to avoid carryover contamination. For RT-PCR, 0.5 μ g of RNA in a total volume of 40 μ L was used to synthesize cDNA with SuperScriptRII (Life Technologies, Grand Island, NY) and oligo-dT primers following the manufacturer's specifications. For each PCR 3 μ L of cDNA was used and various isoform RNAs were amplified using the isoform specific primer-pairs as previously described [Denz et al., 2011; Thurston et al., 2009]. The nucleotide sequences of the primer-pairs for amplification of various isoforms are given in the legend under each figure. The PCR amplified DNAs were run in an agarose gel and stained with ethidium bromide and was subsequently used for Southern hybridization using an appropriate/isoform specific probe following the method as described earlier [Denz et al., 2011; Thurston et al.,

2009]. The nucleotide sequences of the oligonucleotide probe are stated Table 1 and also in the corresponding Figure legend.

In separate experiments, the PCR amplified DNAs were run in an agarose gel and stained with ethidium bromide and the stained bands were excised from the gel. DNA was extracted from the excised bands using a MinElute Gel extraction kit from Qiagen (Valencia, CA) following the manufacturer's direction. Part of the DNA was used for sequencing and part of the gel extracted DNAs were ligated and cloned into the T/A cloning vector (Life Technologies) following our published protocol [Denz et al., 2011; Thurston et al., 2009]. Positive clones were identified by PCR using the gene-specific as well as isoform specific primer-pairs previously described. Vectors or constructs were grown in *E. coli*, 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.

Real-Time PCR

Real-time quantitative RT-PCR—qRT-PCR analysis of cDNA template was 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 μ l reaction solution, of which 2 μ l was cDNA template and 8 μ l was SYBR green mix (5 μ l 1 $\times$ SYBR green Master mix, 2.8 μ l of PCR-grade water, and 0.2 μ l of 10 μ M primer pair). Primers for real-time PCR for various TPM isoforms are listed in Table 2. In many cases, cDNA was made with an oligonucleotide specific for exon 9a/b that allowed us to make a gene specific cDNA corresponding to the mRNA containing exon 9/b as in various sarcomeric isoforms. We were able to avoid any subsequent PCR amplification of non-sarcomeric TPM 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 [Livak and Schmittgen, 2001; Pfaffl, 2007; Yuan et al. 2006]. A comparative value was calculated using the formula $X^{-\text{delta delta CT}}$, where “X” 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 zebrafish cDNAs generated with specific primer.

For determination of absolute copy number, optical density was taken of various zebrafish isoform specific clones (for example, TPM1-1 α , TPM1-1 κ , TPM1-2 α , TPM1-2 ν , TPM4-1 α , TPM4-2 α) 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 $\times 6.02 \times 10^{23}$)/(bp length of plasmid $\times 1 \times 10^9 \times 650$), which was simplified by Andrew Staroscik at the URI Genomics and Sequencing Center. A dilution series of each clone was done for 1×10^1 – 1×10^4 copies of template, which was used to create a standard curve after amplification (Table 3) [Thomas et al., 2010; Thurston et al., 2009]. For better accuracy, each sample in the dilution series was run in triplicate.

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

Preparation of YFP Plasmids for transfection—The cDNAs of TPM1-2 α and TPM1-2 ν were PCR amplified with the following primer-pair:

1. ZB.YFP.TPM1-2.EcoR1 (+): 5'-TGGAATTCCTATGCCATTAAGAAGAA-3'
2. ZB.YFP.TPM1-2.Sal1 (-): 5'-TAGTCGACGGTATTGAAGTCATGTCATTGAGAGC-3'.

The amplified DNA was cloned into pYFP-N1 (Clontech) plasmid using the techniques previously described [Sanger et al., 2009; Wang et al., 2005].

Quail cultures, transfection, and imaging

Skeletal myoblasts were isolated from the breast muscles of 9-day old quail embryos and plated on collagen-coated MatTek glass bottom culture dishes (MatTekCorp., Ashland, MA) [Dabiri et al., 1999]. The cells were transfected after 2 days of culture with plasmids encoding YFP-fused tropomyosin isoforms using FuGene HD transfection reagent according to previous methods [Wang et al., 2007, 2008]. Cells intended for immunostaining were fixed, permeabilized and stained with anti-alpha-actinin antibody, Alexa Fluor594 secondary antibody. Fluorescence images were acquired on a Leica SP5 confocal microscope.

2D (Two-dimensional) Western blot analyses

Two-dimensional western blot analyses of the extracts of heart and skeletal muscle of adult zebrafish were performed for us by Kendrick Labs, Inc. (Madison, WI). Zebrafish were obtained from the zebrafish colony of Dr. Joseph Sanger at Upstate Medical University. The frozen heart and skeletal muscle tissues were lysed in 150–350 μ l of osmotic lysis buffer (containing 10 $\times$ nuclease stock, phosphatase inhibitor stocks [I and II], and protease inhibitor stock) and 50–150 μ l of SDS boiling buffer without reducing agents. The samples were homogenized with a micropipette, pulled through a small bore needle, and heated in a boiling water bath for five minutes. The protein concentrations of the samples were determined using the BCA Assay [Smith et. al., 1985 and Pierce Chemical Co., Rockford, IL]. Samples were then lyophilized and redissolved as indicated in 1:1 diluted SDS Boiling Buffer: Urea sample Buffer with reducing agents before loading. Gel loading and sample preparation are given in Table 4. 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. The developed x-ray film when superimposed on the coomassie stained protein gel, 6 spots were exhibited (as in Figure 13). 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.

LC-MS/MS—The peptides 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 $\times$ 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 LC-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 LC-MS/MS ion counts reached 10,000 or for up to 6 seconds each. The entire procedure used was previously described. 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.

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 [2011].

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TPM1-1

TPM1-1 α

TPM1-1 β

TPM1-1 κ

Figure 1A

TPM1-2

TPM1-2 α

TPM1-2 β

TPM1-2 (?)

TPM1-2 New Variant

Figure 1B

TPM4-1 **TPM 4-1 α** **TPM4-2** **TPM 4-2 α** **Figure 1C****Figure 1. Alternative splicing pattern of *TPM* genes in zebrafish**

Exon composition was of the TPM1-1 gene is derived from the published documents, the recently submitted data in Gen Bank (Accession #), published documents on Fugue fish (Toramotou et al. 2004), and results from this study.

- A. TPM1-1
- B. TPM1-2
- C. TPM4-1 & TPM4-2

Figure 2. Conventional RT-PCR amplification of TPM1α and TPM1κ with RNA from heart and skeletal muscle of Zebrafish

- A.** Ethidium bromide staining of the gel
- B.** Hybridization with TPM1-1κ specific probe, the sequences of which are in Table 1.
- C.** Hybridization with TPM1α specific probe. The nucleotide sequences are given in Table 1

Lane 1. Amplification of both TPM1α and TPM1κ by generic primer-pair (Gen TPM1-1α&κ(+)/Gen TPM1-1α&κ(-) as given in Table 1) in heart; lane 2. Amplification of both TPM1α and TPM1κ by generic primer-pair in sk muscle; lane 3. Primer control; lane M. Marker; lane 4. Amplification of TPM1κ by isoform specific primer pair (TPM1-1κ Primer (+)/Gen TPM1-1α&κ(-) as given in Table 1) in heart; lane 5. Amplification of TPM1κ in skeletal muscle; lane 6. Primer control.

```

catggatgccattaagaagaagatgcagatgctcaagctcgacaaggagaacgccttgg
1  -----+-----+-----+-----+-----+-----+-----+ 60
   M D A I K K K M Q M L K L D K E N A L D
cagagctgagcaggctgagggagacaaaaaggcagcggaggacaggagcaaacagttaga
61  -----+-----+-----+-----+-----+-----+-----+ 120
   R A E Q A E G D K K A A E D R S K Q L E
agacgaactaattcagttggaaaagaggttgcgctgaccgaggacgagcgcgataaagt
121  -----+-----+-----+-----+-----+-----+-----+ 180
   D E L I Q L E K R L R V T E D E R D K V
gctcgaggagtaccagagcgtcgaggagaagctcctgaccgccgaggaggtcgccaccaa
181  -----+-----+-----+-----+-----+-----+-----+ 240
   L E E Y Q S V E E K L L T A E E V A T K
ggctgaaggtgatgtggcatccctgaacagacgtatccagctggttgaggaggagctgga
241  -----+-----+-----+-----+-----+-----+-----+ 300
   A E G D V A S L N R R I Q L V E E E L D
cagggcacaggagcgtttggccactgccctgcagaagctggaggaggccgagaaggcagc
301  -----+-----+-----+-----+-----+-----+-----+ 360
   R A Q E R L A T A L Q K L E E A E K A A
cgatgagagcgagagaggcatgaaggtgattgagaacagagctctgaaggacgaggagaa
361  -----+-----+-----+-----+-----+-----+-----+ 420
   D E S E R G M K V I E N R A L K D E E K
gatggagattcaggagatccagctgaaagaggccaagcacatcgctgaggaggccgaccg
421  -----+-----+-----+-----+-----+-----+-----+ 480
   M E I Q E I Q L K E A K H I A E E A D R
caaatacaggaggtggcccgttaagctggtgattgttgagggtgagctggagcgcacaga
481  -----+-----+-----+-----+-----+-----+-----+ 540
   K Y E E V A R K L V I V E G E L E R T E
ggagcgtgcagagctcaacgagagcaaatgctcagagcttgaggaagagttgaaaactgt
541  -----+-----+-----+-----+-----+-----+-----+ 600
   E R A E L N E S K C S E L E E E L K T V
gaccaacaacctgaagtctctggaagcccaggctgagaagtactcacagaaagaagacaa
601  -----+-----+-----+-----+-----+-----+-----+ 660
   T N N L K S L E A Q A E K Y S Q K E D K
gtatgaggaggagatcaaggtgctcactgacaaactgaaggaggtgagactcgcgctga
661  -----+-----+-----+-----+-----+-----+-----+ 720
   Y E E E I K V L T D K L K E A E T R A E
gttcgctgaaaggtccgtcgccaagcttgagaagaccatcgatgatcttgaagatgagtt
721  -----+-----+-----+-----+-----+-----+-----+ 780
   F A E R S V A K L E K T I D D L E D E L
atattcccagaaactcaagtacaaagccatcagcgaggagctggaccacgctctcaatga
781  -----+-----+-----+-----+-----+-----+-----+ 840
   Y S Q K L K Y K A I S E E L D H A L N D
catgacttcaatataa
841  -----+-----+-----+-----+-----+-----+-----+
      M T S I * -

```

Figure 3. Nucleotide and deduced amino acid sequences of zebrafish TPM1-1 κ

The nucleotide sequence has been submitted in the GenBank (submission Id. 1968580)

Figure 4. Conventional RT-PCR amplification of TPM4-1 α and total TPM4-1 transcripts expressed in zebrafish heart and skeletal muscle

- A.** Ethidium bromide staining of the agarose gel
- B.** Hybridization with TPM4-1-specific probe

Lane 1. TPM4 α in heart; lane 2. TPM4 α in skeletal muscle; lane 3. Primer control; lane 4. Total TPM4-1 in heart; lane 5. Total TPM4-1 in skeletal muscle; lane 6. Primer control. For lane 1 to 3 PCR amplification was carried out with TPM4-1 α specific primer-pair as shown in Table 1. The nucleotide sequences of the primer-pair used for the amplification of total TPM4-1 transcripts are also given in Table 1. Please note that the expression of TPM4-1 α is practically undetectable in skeletal muscle (lane 2 in panels A and B). However, total TPM4 transcripts are present in both heart and skeletal muscle.

```

1  ATGGATGCCATTAAGAAGAAGATGCAGATGCTCAAGCTCGACAAGGAGAACGCCTTGGAC
   -----+-----+-----+-----+-----+-----+-----+ 60
   M  D  A  I  K  K  K  M  Q  M  L  K  L  D  K  E  N  A  L  D  -
61  CGAGCAGAGCAGGCTGAGACCGACAAGAAAGCAGCTGAGGAGAGGAGCAAGCAGCTCGAG
   -----+-----+-----+-----+-----+-----+-----+ 120
   R  A  E  Q  A  E  T  D  K  K  A  A  E  E  R  S  K  Q  L  E  -
121 GATGACCTGGTCGCCCTGCAGAAGAAGCTGAAGGCAACCGAGGATGAGTTGGACAAATAC
   -----+-----+-----+-----+-----+-----+-----+ 180
   D  D  L  V  A  L  Q  K  K  L  K  A  T  E  D  E  L  D  K  Y  -
181 TCCGAGGCCCTGAAGGATGCACAGGAGAAACTGGAAGTGGCTGAGAAGAAGGCTACCGAT
   -----+-----+-----+-----+-----+-----+-----+ 240
   S  E  A  L  K  D  A  Q  E  K  L  E  L  A  E  K  K  A  T  D  -
241 GCTGAGGGCGACGTCGCTTCCCTGAACAGACGCATCCAGCTGGTTGAGGAGGAGTTGGAT
   -----+-----+-----+-----+-----+-----+-----+ 300
   A  E  G  D  V  A  S  L  N  R  R  I  Q  L  V  E  E  E  L  D  -
301 CGCGCTCAGGAGCGTCTGGCCACTGCTCTGCAGAAGCTGGAGGAGGCCGAGAAGGCTGCT
   -----+-----+-----+-----+-----+-----+-----+ 360
   R  A  Q  E  R  L  A  T  A  L  Q  K  L  E  E  A  E  K  A  A  -
361 GATGAGAGTGAGAGAGGCATGAAGGTCATTGAGAACAGGGCCCTGAAGGATGAGGAGAAG
   -----+-----+-----+-----+-----+-----+-----+ 420
   D  E  S  E  R  G  M  K  V  I  E  N  R  A  L  K  D  E  E  K  -
421 ATGGAGCTGCAGGAGATCCAGTTGAAGGAGGCCAAGCTTGAGAAGACCATTGATGATCTG
   -----+-----+-----+-----+-----+-----+-----+ 480
   M  E  L  Q  E  I  Q  L  K  E  A  K  L  E  K  T  I  D  D  L  -
481 GAGGATGAATTGTATGCCCAGAACTCAAGTACAAAGCCATCAGCGAGGAGCTGGACCAC
   -----+-----+-----+-----+-----+-----+-----+ 540
   E  D  E  L  Y  A  Q  K  L  K  Y  K  A  I  S  E  E  L  D  H  -
541 GCTCTCAATGACATGACTTCAATATAA
   -----+-----+-----+-----+-----+-----+ 567
   A  L  N  D  M  T  S  I  *  -

```

Figure 5. Nucleotide and deduced amino acid sequences of the newly identified TPM1-2v isoform from zebrafish striated muscle

The nucleotide sequence has been submitted to the GenBan (submission Id: 1968589).

TPM1-2 ν x ZBTPM1-2 α

```

      .
1  MDAIKKKMQMLKLDKENALDRAEQAETDKKAAEERSKQLEDDLVALQKKL  50
   |||||
1  MDAIKKKMQMLKLDKENALDRAEQAETDKKAAEERSKQLEDDLVALQKKL  50
   |||||
51  KATEDELDKYSEALKDAQEKLELAEKKATDAEGDVASLNRRIQLVEEELD  100
   |||||
51  KATEDELDKYSEALKDAQEKLELAEKKATDAEGDVASLNRRIQLVEEELD  100
   |||||
101 RAQERLATALQKLEEAEKAADESERGMKVIENTRALKDDEKMELQEIQLE  150
   |||||
101 RAQERLATALQKLEEAEKAADESERGMKVIENTRALKDDEKMELQEIQLE  150
   |||||
      .
      .
      .
151 .....AKLE  154
      |||||
201 TNNMKSLEAQAEKYSAKEDKYEEEIKVLTDKLKEAETRAEFAERSVAKLE  250
      |||||
155 KTIDDLEDELYAQKLKYKAISEELDHALNDMTSI  186
   |||||
251 KTIDDLEDELYAQKLKYKAISEELDHALNDMTSI  284

```

Figure 6.

Comparison of the deduced amino acid sequences of TPM1-2 ν with the published TPM1-2 α protein.

Figure 7. Conventional RT-PCR analyses on the expression of TPM 2 α and TPM 3 α in heart and skeletal muscle

Panel a: Ethidium bromide staining of the PCR products amplified with TPM3 α specific primer-pair as shown in Table 1.

Panel b: Southern hybridization of the TPM3 α amplified DNA with TPM3 α specific probe (5'-GCACATTGCTGAGGAGGCTGACCGC-3').

Panel c: Ethidium bromide staining of the PCR amplified DNA with TPM2 α specific primer-pair as given in Table 1.

Panel d: Southern hybridization of the TPM2 α amplified DNA with TPM2 α specific probe (5'-GATTCTTGAGGGTGAGCTTGAGC-3').

Lanes 1: Heart; Lane 2: Sk muscle; Lane 3: Primer-control

Note that TPM2 α and TPM3 α transcripts are expressed both in heart and skeletal muscle.


```

      agcacaatggaggccatcaagaagaagatgcagatgctgaagctggaTaaaggagaacgcc
1  -----+-----+-----+-----+-----+-----+-----+ 60
      M E A I K K K M Q M L K L D K E N A -
      atcgaccgcgcggagcaggcagagtctgacaagaaagcagcagaggacaaatgcaaacag
61 -----+-----+-----+-----+-----+-----+-----+ 120
      I D R A E Q A E S D K K A A E D K C K Q -
      ctagaagacgagttgctgggtctgcagaaaaagctgaaagcaacagaagatgaaTtggaac
121 -----+-----+-----+-----+-----+-----+-----+ 180
      L E D E L L G L Q K K L K A T E D E L D -
      aaatattcagaagctctgaaagacgctcaggagaagctggagctgtccgagaaaaaggct
181 -----+-----+-----+-----+-----+-----+-----+ 240
      K Y S E A L K D A Q E K L E L S E K K A -
      tcagatgctgaaggagatgtagcagcgttgaaccgcaggattcagctggtggaggaggaa
241 -----+-----+-----+-----+-----+-----+-----+ 300
      S D A E G D V A A L N R R I Q L V E E E -
      ctggaccgagctcaggagagactggccactgcgttcagaaactagaggaggctgagaaa
301 -----+-----+-----+-----+-----+-----+-----+ 360
      L D R A Q E R L A T A L Q K L E E A E K -
      gcagccgatgagagcgcagagaggcataaagggtgatcgaagaccgcgccatgaaagatgag
361 -----+-----+-----+-----+-----+-----+-----+ 420
      A A D E S E R G I K V I E N R A M K D E -
      gagaaaatggaaattcaggaaatacagctgaaagaggccaaacacatcgctgaagactcc
421 -----+-----+-----+-----+-----+-----+-----+ 480
      E K M E I Q E I Q L K E A K H I A E D S -
      gaccgcaaataatgaagaggttgcccgtaagctggtgattctggagggggaactggaaaga
481 -----+-----+-----+-----+-----+-----+-----+ 540
      D R K Y E E V A R K L V I L E G E L E R -
      gcagaagaaagagcagagatcgagaaacttaaagggtggagatctggaggaagagctgaaa
541 -----+-----+-----+-----+-----+-----+-----+ 600
      A E E R A E I A E L K G G D L E E E L K -
      aatgtaaccaacaacctcaagtccttggaaagcccaatcagaaaagtattcagagaaagag
601 -----+-----+-----+-----+-----+-----+-----+ 660
      N V T N N L K S L E A Q S E K Y S E K E -
      gacaaatatgaggatgagatcaaagttctgactgataaactAaaagaggttgaaacgcgt
661 -----+-----+-----+-----+-----+-----+-----+ 720
      D K Y E D E I K V L T D K L K E V E T R -
      gcagagtttgcaaaaaggactgtggccaaactggaaaagaccattgatgatctggaggat
721 -----+-----+-----+-----+-----+-----+-----+ 780
      A E F A E R T V A K L E K T I D D L E D -
      gaactttatgcTcagaagctgagatttaaggccattagtgaggagctggatcacgctctc
781 -----+-----+-----+-----+-----+-----+-----+ 840
      E L Y A Q K L R F K A I S E E L D H A L -
      acGgacatgtcctccttataa
841 -----+-----+-----+-----+-----+-----+-----+ 862
      T D M S S L *

```

Figure 8. Nucleotide and deduced amino acid sequences of the TPM4-2a isoform from zebrafish striated muscle

The nucleotide sequence has been submitted to the GenBan (submission Id: 1968883).

Figure 9. Relative expression of TPM1-1 α and TPM1-1 κ in zebrafish heart and skeletal muscle
 Fold changes of TPM1-1 α and TPM1-1 κ in zebrafish heart and skeletal muscle. qRT-PCR was carried out in triplicate with isoform specific primer-pairs sequences of which are given in Table 2. Zb. Zb.Fish.TPM1-1 α (+)/Zb.Fish.TPM1-1 α (-) and Zb.Fish. TPM1-1 κ (+)/Zb.Fish. TPM1-1 κ (-) were used for TPM1 α and TPM1-1 κ respectively. The primer-pair used for 16S rRNA is given in Table 2. The cDNA was made with Zb.Fish.TPM1-1Ex9A(-).

Figure 10. Relative expression of TPM1-2α and TPM1-2v in zebrafish heart and skeletal muscle qRT-PCR was carried out in triplicate with isoform specific primer-pairs sequences of which are given in Table 2. The primer used for 16S rRNA is shown in Table 2.

Figure 11. Relative expression of TPM4-1α and TPM4-2 α in zebrafish heart and skeletal muscle qRT-PCR was carried out in triplicate with isoform specific primer-pairs sequences of which are given in Table 2. The nucleotide sequences of the Primer-pair for amplification of 16 S rRNA is presented in Table 2. cDNA for TPM4-2 were made with Exon (A(-) oligonucleotide (Table 2) whereas oligo dT was used for TPM4-1α cDNA. TOP: TPM4-1α; Bottom: TPM4-2α

Figure 12. Fluorescence image of quail myotubes transfected with YFP fused zebrafish and *TPM1-2ν*

Skeletal muscle myotubes were transfected with YFP fused zebrafish TPM1-2α (a,c) and TPM1-2ν (e,g). Cells were then fixed and stained with alpha-actinin antibody to localize premyofibrils at the spreading end of myotubes (b,f) and mature myofibrils in the central regions of myotubes (d,h). Note that the YFP-*TPM1-2α* localized in both premyofibrils (a,b) and mature myofibrils (c,d), while YFP-*TPM1-2ν* is in the mostly diffused area in cells with very weak localization in the myofibrils (e-h). The expression of YFP-*TPM1-2ν* does not affect myofibrils in the myotube (f,h). Bar = 10 μm.

Figure 13. 2D western blot analyses with extracts from adult zebrafish Heart and skeletal muscle

- a.** The Coomassie stained zebrafish skeletal muscle protein across the gel.
- b.** The PVDF filter was stained with CH1 monoclonal antibody followed by treatment with secondary antibody as mentioned under materials and methods, and subsequently treated with ECL and exposed to x-ray film. Developed X-ray film was superimposed on the top of the Coomassie stained second gel as well as on the Coomassie stained PVDF filter. Three spots A, C, X were marked, excised and was used for extraction of protein for subsequent Mass spectrometric analyses.
- c.** The Coomassie stained zebrafish cardiac muscle protein across the gel.
- d.** Same as in b. However, in this gel 2 spots D & E were marked for subsequent Mass Spectrometric analyses.

Spot A: TPM1-1 α (Gi/1885905; 83% Protein sequence coverage)

1	MDAIKKKMQM	LKLDKENALD	RAEQAETDKK	AAEERSKQLE	DDLVALQKKL
51	KATEDELDKY	SEALKDAQEK	LELAEKKATD	AEGDVASLNR	RIQLVEEELD
101	RAQERLATAL	QKLEEAEEKAA	DESERGMKVI	ENRALKDEEK	MELQEIQLE
151	AKHIAEEADR	KYEEVARKLV	IVEGELERTE	ERAELNEGKC	SELEELKTV
201	TNNMKSLEAQ	AEKYSAKEDK	YEEIIVLTD	KLKEAETRAE	FAERSVAKLE
251	KTIDDLEDEL	YAQKLKYKAI	SEELDHALND	MTSI	

TPM3 α (gi|45387763; 62% Protein sequence coverage)

1	MEAVKKKMMM	LKLDKENALD	QAEQAETDRK	AAEERSKQHE	DELIQMOKKL
51	KGTEDELDKY	SEALKDAQEK	LEISEKKA <u>AD</u>	AE <u>AE</u> VASLNR	RIQLVEEELD
101	RAQERLATAL	QKLEEAEEKAA	DESERGMKVI	ENRALKDEEK	MELQEIQLE
151	AKHIAEEADR	KYEEVARKLV	IVEGELERTE	ERAELAESKC	AELEELKNV
201	TNNLSLEAQ	AEKYSQKEDK	YEEIIVLTD	KLKEAETRAE	FAERSVAKLE
251	KTIDDLEDEL	YAQKLKYKAI	SEELDHALND	MTSI	

Figure 14. Identification of amino acid sequences from the peptides extracted from spot A after 2D western blot analyses of zebrafish skeletal muscle extracts with CH1 antibodies

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

Top: TPM1-1 α

Bottom: TPM3 α . Note that peptide from amino acid residue 13 to 35 in exon 1 α is specific form TPM3 and also the peptide LEISEKKAADAEAEVASLNR from 176 to 190 in TPM3 α is different from TPM1-1 α isoform. The altered amino acid residues marked in larger font and underlined.

The results indicate that both TPM1-1 α and TPM3 α are expressed in zebrafish skeletal muscle.

Spot D TPM4-1 α (gi|66792936; 56% sequence coverage]

1	MEAIKKKMQM	LKLDKENAID	RAEQAETEQK	AAEDKCKQLD	DELVGLQKKL
51	RQTEDELDKY	SEALKDAQEK	LELSEKKAAD	AEGDVAALNR	RIQLVEEELD
101	RAQERLGTAL	QKLEEAEEKAA	DESERGMKVI	ENRAMDEEK	MEIQEMQLKE
151	AKHIAEEADR	KYEEVARKLV	ILEGELERA	ERAEVAECKA	SDLEEEELKNV
201	TNNLKSLEAQ	AEKYSEKEDK	YEEEIKVLSD	KLKEAETRAE	FAERTVAKLE
251	KSIDDLEDEL	YAQKLKYKAI	SEELDHALND	TTSL	

TPM3 α (gi|45387763; 21% sequence coverage]

1	MEAVKKKMMM	LKLDKENALD	QAEQAETDRK	AAEERSKQHE	DELIQMOKKL
51	KGTEDELDKY	SEALKDAQEK	LEISEKKAAD	AAAEVASLNR	RIQLVEEELD
101	RAQERLATAL	QKLEEAEEKAA	DESERGMKVI	ENRALKDEEK	MELQEIQLKE
151	AKHIAEEADR	KYEEVARKLV	IVEGELERTE	ERAELAESKC	AELEEEELKNV
201	TNNLKSLEAQ	AEKYSQKEDK	YEEEIKILT	KLKEAETRAE	FAERSVAKLE
251	KTIDDLEDEL	YAQKLKYKAI	SEELDHALND	MTSI	

Figure 15. Identification of amino acid sequences from the peptides extracted from spot D after 2D western blot analyses of zebrafish heart extracts with CH1 antibodies

Top: TPM4-1 α

Bottom: TPM3 α

Table 1

Nucleotide sequences of the primer-pairs used for conventional RT-PCR

Primer Name	Gene	Isoform	Primer/Probe	Sequence	Reference
Gen TPM1-1a&κ(+)	TPM1-1	TPM1-1a & κ	Primer	5'-CATGGATGCCATTAAAGAA-3'	
Gen TPM1-1a&κ(-)	TPM1-1	TPM1-1a & κ	Primer	5'-AAGTCATGTCATTGAGAGCG-3'	
TPM1-1a.Pro(+)	TPM1-1	TPM1-1a	Probe	5'-CTTGAAGATGATCTGCTCGCAC-3'	
TPM1-1κ Primer (+)	TPM1-1	TPM1-1κ	Prime	5'-TAGAAGACGAACTAATTCAG-3'	
TPM1-1κ Pro (+)	TPM1-1	TPM1-1κ	Probe	5'-GGAAAAAGAGTTGGCGGTG-3'	
TPM1-2a (+)	TPM1-2	TPM1-2a	Primer	5'-ACTCACACAGCCATGGAT-3'	M24635.1
TPM1-2a (-)	TPM1-2	TPM1-2a	Primer	5'-GCCCCGGAGCCGAGATGAGATGT-3'	M24635.1
TPM1-2v(-)	TPM1-2	TPM1-2v	Primer	5'-TGGTCTTCTCAAGCTTGGCCCTCCT-3'	This study
TPM2a(+)	TPM2	TPM2a	Primer	5'-ATGGAGGCCATCAAGAAGAA-3'	
TPM2a(-)	TPM2	TPM2a	Primer	5'-AGACAGAGGAGAGCCGAAAGAGT-3'	
TPM3a (+)	TPM3	TPM3a	Primer	5'-GTAACACACATTAAAAATGGCCGGAT-3'	
TPM3a(-)	TPM3	TPM3a	Primer	5'-TTAGAAACTATTGAGCTCGCTCA-3'	
TPM4-2(+)	TPM4-2	TPM4-2a	Primer	5'-TCCCTCACAGCAGCACAA-3'	
TPM4-2(-)	TPM4-2	TPM4-2a	Primer for Nested PCR	5'-CACAGATTCTGATTCAGTTATA-3'	
TPM4-3(+)	TPM4-2	TPM4-2a		5'-ATGGAGGCCATCAAGAAGAA	

Name	Gene	Isoform	Primer/Probe	Sequence	Reference
TPM4-1a (+)	TPM4-1	TPM4-1a	Primer +ve	5'-CCATGGAGGCCATCAAGAA-3'	NM001024467
TPM4-1a (-)	TPM4-1	TPM4-1a	Primer -ve	5-CAGGGATGTCGTGTCATTGAGAGCATGA-3'	NM_001024467
TPM4-1.Total(+)	TPM4-1	Total transcripts	Primer +ve	5'-GCCCTGAACCCGAGGATT-3'	NM_001024467
TPM4-1.Total(-)	TPM4-1	Total transcripts	Primer -ve	5'-ATTTTCCAGTTTGGCCACTGT-3'	NM_001024467
TPM4-1.Probe	TPM4-1		Probe	5'-AGAAGACAAAGTATGAAGAAAG-3'	NM_001024467

Table 2

Nucleotide sequences of the primer-pairs used for qRT-PCR

Name	Gene	Isoform	Primer/cDNA	Sequence	Reference
Zb.Fish.TPM4-1a (+)	TPM4-1	TPM4-1a	Primer	5'-AGAAAGACAAAGTATGAAGAAG-3'	BC085518
Zb.Fish.TPM4-1a (-)	TPM4-1	TPM4-1a	Primer	5'-GTAGAGCGAGACAGATGGAT-3'	BC085518
Zb.Fish.TPM4-1Ex9A(-)	TPM4-1	TPM4-1	cDNA	5' CAGGGATGTCGTGTCAATTGAGAGCATGA-3	BC085518
Zb.Fish. TPM4-2a (+)	TPM4-2	TPM4-2a	Primer	5'-AAGAGGACAAATATGAGGAT-3'	
ZB.Fish. TPM4-2a(-)	TPM4-2	TPM4-2a	Primer	5'-ATACCACATATAGGCACCTCAT-3'	
ZB.Fish.TPM4-2 Ex9A(-)	TPM4-2	TPM4-2	cDNA	5'-CACAGTTCGTATTCAGTTATA-3'	
Zb.Fish.TPM1-1a(+)	TPM1-1	TPM-1a	Primer	5'-AGAAAAGCTGCGGGAGGACAAAT-3'	NM_001024467.1
Zb.Fish.TPM1-1a(-)	TPM1-1	TPM-1a	Primer	5'-TCCTCCACCCAGCTGAATCCTG-3'	NM_001024467.1
Zb.Fish. TPM1-1κ(+)	TPM1-1	TPM-1κ	Primer	5'-CAGTTAGAAAGACGAACTAA-3'	This study
Zb.Fish. TPM1-1κ(-)	TPM1-1	TPM-1κ	Primer	5'-ACATCAGCTTCAGCCTTG-3'	This study
Zb.Fish.TPM1-1Ex9A(-)	TPM1-1	TPM-1a&κ	cDNA	5'-AAGTCATGTTCATTGAGAGCG-3'	NM_001024467.1
Zb.Fish.TPM1-2a(+)	TPM1-2	TPM1-2a	Primer	5'-TGACTGACAAAGCTGAAAGGAGG-3'	M24635.1
Zb.Fish.TPM1-2a(-)	TPM1-2	TPM1-2a	Primer	5'-GCCCCGGAGCCGAGATGAGATGT-3'	M24635.1
Zb.Fish.TPM1-2ν (+)	TPM1-2	TPM1-2NeW	Primer	5'-CCCTGAACAGACGCGATCCAGC-3'	This study
Zb.Fish.TPM1-2ν (-)	TPM1-2	TPM1-2New	Primer	5'-TGGTCTTCTCAAGCTTGGCCTCCT-3'	M24635.1
Zb.Fish. 18S.rRNA (+)	18s rRNA	-	Primer	5'-CTGGAGCGATTTCGTCTGGTT-3'	FJ915075.1
Zb.Fish. 18S.rRNA (-)	18s rRNA	-	Primer	5'-TTATGACCTGCGCTTACTGG-3'	FJ915075.1

Table 3Copy number of TPM1-1 α , TPM1-2 α , TPM1-2New variant, & TPM4-1 α

TPM isoform	Copy number (number of copies per μ gm of total RNA		
	Heart	Skeletal Muscle	Sk muscle:Heart
TPM1-1 α	$2.36 \times 10^4 \pm 2.05 \times 10^3$	$2.24 \times 10^7 \pm 1.15 \times 10^5$	949
TPM1-2 α	$7.72 \times 10^5 \pm 1.2 \times 10^4$	$5.14 \times 10^8 + 8.5 \times 10^6$	665
New TPM1-2 variant	$8.82 \times 10^2 + 10^1$	$1.31 \times 10^5 + 9.2 \times 10^2$	148
TPM4-1 α	$1.56 \times 1.75^8 \pm 1.75 \times 10^6$	$5.39 \times 10^4 \pm 1.42 \times 10^3$	0.0003

Table 4

Table 1. Key to 2D Gel Loading and Sample Preparation.

Gel ID #	Sample	µl loaded	µg loaded	Treatment	BCA Total µg
2938 #1	Skeletal muscle from zebrafish	50	200	Coomassie	6,410
2938 #2	Skeletal muscle from zebrafish	50	200	Anti-Tropomyosin	
2938 #3	Heart from zebrafish	50	165	Coomassie	330
2938 #4	Heart from zebrafish	50	165	Anti-Tropomyosin	