國 立 清 華 大 學
碩 士 論 文

利用斑馬魚模式研究原肌球蛋白 3 的新生突變造成先天性肌病

The investigation of a *de novo* mutation in human *tropomyosin 3* of congenital myopathy in transgenic zebrafish

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博碩士論文電子檔著作權授權書

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本授權書所授權之學位論文，為本人於國立清華大學生物科技研究所_____組， 107 學年度第____學期取得碩士學位之論文。

論文題目：利用斑馬魚模式研究原肌球蛋白3的新生突變造成先天性肌病
指導教授：喻秋華, 汪宏達

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National Tsing Hua University Master Thesis
Advisor Approval Form

生物科技研究所
許博睿 君（學號106080522）所提之論文

利用斑馬魚模式研究原肌球蛋白3的新生突變造成先天性肌病

經由本人指導撰述，同意提付審查。

Institute of Biotechnology
許博睿 君 (Student ID: 106080522) who has submitted the thesis

The investigation of a de novo mutation in human tropomyosin 3 of congenital myopathy in transgenic zebrafish

under my guidance, I approve for its submission to the oral defense committee.

指導教授 (Advisor) 喻秋華 (簽章) (Signature)

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Institute of Biotechnology 許博睿 君（Student ID: 106080522）who has submitted the thesis

The investigation of a de novo mutation in human tropomyosin 3 of congenital myopathy in transgenic zebrafish

has passed the oral defense and has met the qualifications to be awarded the degree of Master of Science.

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委員 (Members) 董庸哲

王宏達

喻秋華

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中文摘要

先天性肌病是一種具有遺傳性和臨床異質性的肌肉疾病，其主要特徵為肌肉無力、肌張力減弱、運動發育遲緩。先天性肌病以肌肉切片所見的特徵進行命名。大部分的先天性肌病患者病情是進行性且罕見的，但目前的醫療對於先天性肌病沒有明確的治療方式。有名七歲的病童自嬰幼兒時期就患有先天性肌病，透過血清 DNA 定序發現病童原肌球蛋白 3 (Tropomyosin 3, TPM3)基因在核苷酸第 452 位點單一點從 A 突變為 G，導致第 151 個氨基酸從麩氨酸(Glutamic acid, E)改變為甘氨酸(Glycine, G)。先前文獻已報導過 TPM3 基因在核苷酸第 452 位點單一點從 A 突變為 C，導致第 151 個氨基酸從麩氨酸(Glutamic acid, E)改變為丙氨酸(Alanine, A)會造成先天性肌病。本研究建立了轉基因斑馬魚動物模型，探討 TPM3(E151G)和先天性肌病之間的病理關係，研究致病機轉和開發藥物篩選平台。我利用 Tol2-Gateway 轉基因技術，分別產生三種轉基因斑馬魚，將野生型 TPM3、兩種突變型 TPM3(E151A)、TPM3(E151G)表現在斑馬魚肌肉組織。我觀察了三種不同基因型斑馬魚的形態特徵，游泳速度和肌肉耐力，以及肌肉染色。首先，TPM3(WT)在外表呈現正常的外觀，一些 TPM3(E151G)在 F0 及 F1 成魚具有沒有尾巴、彎曲的軀幹、異常的骨骼等外型上之變異。其次，我利用 DanioVision 測量幼魚游動能力，我發現 TPM3(E151G)轉基因斑馬魚游動速度明顯比 TPM3(WT)或 TPM3(E151A)轉基因斑馬魚緩慢。我利用 T-maze 迷宮測量成魚游動能力之行為測試，與幼魚的結果相符，TPM3(E151G)轉基因斑馬魚游動速度明顯比 TPM3(WT)或 TPM3(E151A)轉基因斑馬魚緩慢。此外，透過游泳隧道測量肌耐力，TPM3(E151G)成魚明顯比 TPM3(WT)或 TPM3(E151A)表現出較弱的肌耐力。再者，我利用冷凍切片技術作為診斷肌肉疾病的工具，通過蘇木精 - 伊紅染色我觀察到 TPM3(E151G)轉基因斑馬魚肌肉纖維排列不整、大小不成比例呈現先天性肌不均(congenital fiber type disproportion)的病變，TPM3(E151A)則呈現類似桿狀體肌病(Nemaline myopathy)的病變。 最後，我使用 TPM3 轉基因斑馬魚幼魚作為作為篩選某些天然物質的藥物篩選平台，我發現餵食 TPM3(E151G)左旋肉鹼(L-carnitine）可以改善 TPM3(E151G)幼魚的游動能力。左旋肉鹼可以貯存並調節能量 ATP 的供給，達到增強肌耐力。我還進行了肌肉標本的下一代測序以鑑定失調的基因/途徑。透過使用此斑馬魚模型作為臨床前模型，我不僅可以研究 TPM3(E151G)突變引起之先天性肌病的致病機制，並為這種罕見的人類遺傳疾病開發個人化的藥物篩選。

Abstract

Congenital myopathies (CM) are genetically and clinically heterogeneous muscle diseases, characterized by muscle weakness and hypotonia since birth, and pathologically defined by morphological features seen on muscle biopsy. Currently no definite treatment for CM. A 7-year-old boy has suffered from CM since infancy. His serum DNA sequencing revealed a *de novo* mutation in *tropomyosin 3* (TPM3) at nucleotide 452 changed from A to G, resulting in amino acid 151 changing from glutamic acid (E) to glycine (G). The same position changing from A to C, and resulting TPM3 amino acid 151 changing from glutamic acid (E) to alanine (A) was previously reported causing CM. We have generated transgenic zebrafish for studying the pathological causality between TPM3(E151G) and CM, also as potential drug screening platform. Using Tol2-Gateway transgenesis technology, we established three transgenic zebrafish expressing TPM3(WT), TPM3(E151A) and TPM3(E151G) mutants in muscles, and observed the morphological features, swimming speed, and muscular endurance, as well as muscle staining. While TPM3(WT) showed normal appearance, some TPM3(E151G) fishes displayed either no tail, crooked body or abnormal skeletal in both F0 and F1 adults. TPM3(E151G) larvae exhibited dramatically slower swimming speed than TPM3(WT) and TPM3(E151A) mutant measured by DanioVision. TPM3(E151G) adults swam significantly slower than TPM3(WT) and TPM3(E151A) by T-maze. Furthermore, TPM3(E151G) transgenic fish exhibited weaker muscular endurance measured by swim tunnel. Moreover, we used cryosection technology as a tool for diagnosing muscle disease, TPM3(E151G) exhibited muscle fiber disproportion similar to congenital fiber type disproportion, and TPM3(E151A) seemed like nemaline myopathy by hematoxylin-eosin staining. Finally, using TPM3(E151G) transgenic zebrafish larvae as a drug screening platform to screen some natural substances, we identified L-carnitine specifically improved the swimming speed of TPM3(E151G) larvae. L-carnitine can stored and regulated the supply of energy ATP to enhance muscle endurance. Next generation sequencing for the muscle specimens were also performed to identify the dysregulated genes/pathways. By using the TPM3(E151G) transgenic zebrafish as a preclinical model, we not only can uncover the molecular mechanisms of TPM3(E151G) mutation mediated CM, and develop a personalized drug screening for this rare human genetic disease.

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Chapter 1 Introduction

1.1 Congenital myopathy as a rare disease

1.1.1 Congenital myopathy

Congenital myopathy (CM) is a general term for a group of rare muscle disorders that is present at birth, with estimated prevalence of about 1:25,000. Muscle weakness and lack of muscle tone are the most common symptoms associated with CM (North, 2008; Wang et al., 2012). Due to the genetic heterogeneity, the clinical phenotypes and histopathology are very diverse. Some CM patients may have difficulty breathing or feeding, and even affect the brain development and some with skeletal problems such as scoliosis. The most common types of CM were central core myopathy, myotubular myopathy, centronuclear myopathy, nemaline myopathy and congenital fiber type disproportion. The type of CM was distinguished according to the electromyogram (EMG), muscle biopsy (North et al., 2014) and the symptoms of the patients.

1.1.2 Treatment for congenital myopathy

Currently, there are no known cure exist for any of CM, only supportive therapy. Some animal study has attempted to find the effective drugs to treat muscle disease. Spinal muscular atrophy (SMA) zebrafish model treated with Terazosin, a FAD approved small molecule, significantly rescued the MN axonal in smn morphant (Boyd et al., 2017). L-carnitine, is an amino acid naturally produced in the body, reduces skeletal muscle atrophy in rats (Jang et al., 2016). Five patients with nemaline myopathy have good initial after treated with L-tyrosine which is an amino acid precursor for dopamine,

epinephrine, and norepinephrine (Ryan et al., 2008). In this study, we generated transgenic zebrafish for CM and hope to serve as drug screening platform to uncover the therapeutic for this rare disease.

1.1.3 A patient with congenital myopathy

Dr. Yuh-Jyh Jong has a seven-year-old boy who has suffered from generalized muscle weakness and atrophy with hyporeflexia, poor feeding, high-arched palate and motor delay since early infancy. This boy has manifested motor delay with head control at 7-8 months, sit alone at 1 year of age, walk alone at 1-year-8-month old, and cannot run till 7 years old. However, his parents refused to take invasive clinical examination. By sequencing his serum DNA, Dr. Jong revealed a *de novo* mutation of *tropomyosin 3* (TPM3) at nucleotide 452 changed from A to G, resulting in amino acid 151 changing from glutamic acid (E) to glycine (G). Previously, TPM3 mutation changing nucleotide 452 from A to C resulting amino acid 151 changing from glutamic acid (E) to alanine (A) was reported in a congenital myopathy patient (Marttila et al., 2014). To study the pathological causality between TPM3(E151G) and congenital myopathy, we generated transgenic zebrafish overexpressing human TPM3 gene (both wild-type and mutants) in the muscle of zebrafish for functional analysis and potential drug screening.

1.1.4 Tropomyosin family

Tropomyosin is a family of actin binding protein found in muscle cell and non-muscle cell, and is dimers of alpha-helical coiled-coil proteins that polymerize head-to-tail along the major groove of actin filament (Bailey, 1948). There are more than 40 isoforms of mammalian tropomyosin (Cohen and

Cohen, 1972). Tropomyosin is an important component of most actin filaments in animals. Muscle is formed by various arrangements of myofilaments. There are three different types of myofilaments: thick, thin, and elastic filaments. Each thin myofilament is wrapped by a tropomyosin protein which connect to troponin complex, and bind to actin and myosin. Tropomyosin regulates calcium-sensitive interaction of actin and myosin in the muscle.

Tropomyosin alpha-3 chain is a protein that is encoded by human TPM3 gene located on human chromosome 1. Mutations of TPM3 are commonly associated with congenital fiber type disproportion (Lawlor et al., 2010), and other diseases include nemaline myopathy and cap myopathy (De Paula et al., 2009; Marttila et al., 2014).

1.2 Zebrafish model

1.2.1 Advantages of zebrafish model

Since 1960s, the zebrafish (*Danio rerio*) has become a popular and important model to scientific research. Zebrafish is tropical fish originally from southeast asia. Zebrafish is a powerful model for research in development, regeneration, cancer research and drug screening. The genetics of zebrafish are highly conserved to human and organs including brain, immune system and musculature are similar to human (Guyon et al., 2007). About 70% of human disease genes can be found in zebrafish (Santoriello and Zon, 2012). Compared with mouse, zebrafish is easier and cheaper to maintain. Zebrafish can produce a large number of progeny. Also, FDA has approved to use zebrafish as a preclinical animal model for drug screening. Zebrafish is an ideal animal model for studying human muscle diseases and drug screening. In my thesis, transgenic fish carried patient's specific mutation can serve as a drug

screening platform for accurate personalized medicine to the patients with rare diseases.

1.2.2 Established transgenic zebrafish lines in this study

We have established three transgenic zebrafish lines to explore the relationship between TPM3 mutations and congenital myopathy.

The muscle specific Myosin Light Chain 2 (MLC2) promoter was used to drive the expression of wild type human tropomyosin 3 (TPM3) in *Tg (MLC2:TPM3(WT); myl7; EGFP)* which was used as the control.

MLC2 promoter drives the expression of E151G mutation of TPM3 in *Tg (MLC2:TPM3(E151G); myl7; EGFP)* which was used to examine the novel mutation of the patient. We also created *Tg (MLC2:TPM3(E151A); myl7; EGFP)* which the MLC2 promoter driven mutant TPM3 changed amino acid 151 from E to A, was reported previously (Marttila et al., 2014).

1.2.3 Skeletal muscle of zebrafish

The main advantage of using zebrafish in this study is the rapid development of the body muscle tissue. At 36 hours post- fertilization (hpf), adaxial cells have finished migrating from the notochord to become a layer of superficial muscle cells and the lateral presomitic cells differentiated into muscle cells remaining deep in the myotome (Devoto et al., 1996). The skeletal muscle development and differentiation are similar between zebrafish and mammals (Buckingham and Vincent, 2009). In addition, it is importance in locomotion and regulated the role in metabolic homeostasis, it also can store and generator the energy (Jackson and Ingham, 2013). Early movement behavior of zebrafish embryos and larvae can be easily observed and measured (Felsenfeld et al., 1990; Kimmel et al., 1974). The first movements

of zebrafish begin at embryo stage about 17 hpf as spontaneous contractions. At about 26 hpf, embryos exhibit swimming movements in response to touch. At about 96 hpf, larvae zebrafish was freely swimming (Maves, 2014). These motor behaviors are suitable for early observation of zebrafish with muscle defects, which help to use zebrafish as an animal model of human muscle disease.

Chapter 2 Materials and Methods

2.1 Zebrafish husbandry

The zebrafish were maintained at the Zebrafish Core Facility in National Health Research Institute. Adult fishes were maintained under an automated 14:10 hr light-dark cycle and a constant temperature of 28 °C. All experiments were approved by the Institute Animal Care and Use Committee (IACUC) of NHRI.

2.2 Zebrafish lines

AB wild type were used in this study. Other transgenic fish lines including *Tg(MLC2:TPM3-WT;myl7:EGFP)*, *Tg(MLC2:TPM3-E151A;myl7:EGFP)*, *Tg(MLC2:TPM3-E151G;myl7:EGFP)* were generated in my thesis for the study.

2.3 Amplification of TPM3 gene

I used Human Skeletal Muscle QUICK-Clone™ cDNA (TaKaRa, Japan) as template and by polymerase chain reaction to amplify the TPM3 gene with TPM3-F and TPM3-R primers listed in Table 1. The condition of PCR was given below:

PCR protocol

Initial Denaturation	94°C for 5 minutes
Denaturation	94°C for 30 seconds
Annealing	56°C for 30 seconds
Extension	72°C for 1 minutes
	Repeat 25 cycles
Final Extension	72°C for 7 minutes

2.4 Gateway cloning

To generate pME-TPM3, product of TPM3 gene was amplified with the primers attB1-TPM3-F and attB2-TPM3-R listed in Table 2, and the condition of PCR was given below:

PCR protocol

Initial Denaturation	94°C for 5 minutes
Denaturation	94°C for 30 seconds
Annealing	58°C for 30 seconds
Extension	72°C for 1 minutes
	Repeat 25 cycles
Final Extension	72°C for 7 minutes

After PCR amplification, the product was purified by MinElute PCR Purification kit (Qiagen, Germany). Using Gateway system BP reaction (Invitrogen, US) to generate pME-TPM3(WT).

The final expression construct pTol2-MLC2:TPM3(WT):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US) using p5E-MLC2, pME-TPM3(WT), p3E-polyA and pDestTol2CG2 vector. After LR recombination reaction, the DNA sample was added into TOP10 competent cells for 30 minutes on ice for the transformation. Transferred the sample to 42 °C water bath for 60 seconds for heat shock transformation. After heat shock transformation, the sample was transferred to ice for 5 minutes immediately. 1 mL of LB broth was added and incubated for 1 hour at 37 °C. Then, bacteria were spread on LB agar containing ampicillin and cultured for 16 to 18 hours at 37 °C. Finally, check the clone by using colony PCR and entrusted DNA Sequencing Core Lab in National Health Research Institute to sequence to

confirm the clone sequence. The primers used to confirm the LR recombination reaction colony PCR were listed in Table 3.

pME-TPM3(E151A) and pME-TPM3(E151G) were created by site-direct mutagenesis (section 2.5) from pME-TPM3(WT). The final expression construct pTol2-MLC2:TPM3(E151A):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US) using p5E-MLC2, pME-TPM3(E151A), p3E-polyA and pDestTol2CG2 vector. The final expression construct pTol2-MLC2:TPM3(E151G):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US) using p5E-MLC2, pME-TPM3(E151G), p3E-polyA and pDestTol2CG2 vector.

The sequence of pTol2-MLC2:TPM3(WT):pA/CG2 construct was listed in Supplementary Data 1. The sequence of pTol2-MLC2:TPM3(E151A):pA/CG2 construct was listed in Supplementary Data 2. The sequence of pTol2-MLC2:TPM3(E151G):pA/CG2 construct was listed in Supplementary Data 3.

2.5 Site-directed mutagenesis

I used QuickChange II site-directed mutagenesis (Agilent Technologies, US) to create the mutation of TPM3 nucleotide 452 mutated from A to C and G. Using TPM3(WT) as template, and TPM3 A452S-F and TPM3 A452S-R as primers, I changed the nucleotide 452 from A to C. Using TPM3(WT) as template, and TPM3 A452G-F and TPM3 A452G-R as primers, I changed the nucleotide 452 from A to G. The sequence of those primers were listed in Table 4. The sample reaction and condition of the kit was given below:

Sample reaction mix

10X reaction buffer	5 µL
DNA template (5-50 ng)	X µL

Forward primer (10 μM)	1.25 μL
Reverse primer (10 μM)	1.25 μL
dNTP mix	1 μL
PfuUltra polymerase (2.5 U/μL)	1 μL
Nuclease-free water	40.5-X μL

PCR protocol

Segment 1	95°C for 30 seconds
Segment 2	95°C for 30 seconds
	68°C for 1 minute
	68°C for 3.5 minutes
	Repeat 12 cycles
Segment 3	4°C for 2 minutes

After polymerase chain reaction, 1 μL of *Dpn* I was added and incubated for 1 hour at 37 °C to digestion the amplification products. Then, 1 μL of *Dpn* I -treated DNA sample was added into XL1-Blue supercompetent cells and incubated on ice for 30 minutes. The sample was transferred to 42 °C water bath for 60 seconds for heat shock, then immediately transferred to ice for 5 minutes. 1 mL of LB broth was added and incubated at 37 °C for 1 hour. Then, bacteria were spread on LB agar containing kanamycin and cultured at 37 °C for 16 to 18 hour. Finally, the clones were checked by colony PCR and the DNA was extracted and sent to Sequencing Core Lab in National Health Research Institute for sequencing to confirm the clone.

2.6 Embryos collection

Embryos can be collected once a week with best results on a regular schedule. The night before embryo collection, adult male and female zebrafish

were placed into mating tank with a clapboard after the last feeding. Next morning, the clapboard was removed to start mating. Embryos were collected after 1 hour, removed the dead and unfertilized embryos. Transfer healthy embryos to a clean petri dish containing E3 medium. Keep the embryos in the incubator at 28 °C.

E3 medium:

NaCl (AMRESCO, US)	5 mM
KCl (AMRESCO, US)	0.17 mM
$CaCl_2$ (J.T.Baker, US)	0.33 mM
$MgSO_4$ (J.T.Baker, US)	0.33 mM

2.7 Microinjection

Preparation the needle with a micropipette puller, pull a glass capillary into two needles. Inserted the needle into the Nanoject II™ Nanoliter injector (Drummond Scientific, US). For DNA and RNA co-injection, injected 2.3 nL contained 0.05% phenol red into an embryo at one-cell stage. Ensured the embryos did not develop past the two-cell stage. After injection, embryos were placed in E3 medium and incubated at 28°C.

2.8 Selection and confirmation of transgenic zebrafish

Two days after microinjection, used fluorescent microscope to screen the heart of zebrafish embryo have green fluorescent or not. When the transgenic zebrafish were 3 months old, DNA was extracted by cutting the fins. After amplification the target fragment by polymerase chain reaction, we entrusted DNA Sequencing Core Lab in National Health Research Institute to sequence

to confirm the target sequence. The primers used for sequencing were listed in Table 5.

2.9 Fish measurement

Before examination, the fish was anesthesia by 0.0125% buffered MS-222 solution (Ethyl 3-aminobenzoate methanesulfonic acid salt, Sigma-Aldrich, US). Total length and standard length were measured by vernier calipers with 0.1 mm accuracy. Fish body weigh was determined to an accuracy of 0.01g.

2.10 Morphological analysis

Before examination, the fish was anesthesia by 0.0125% buffered MS-222 solution (Ethyl 3-aminobenzoate methanesulfonic acid salt, Sigma-Aldrich, US). A digital CMOS X-ray detector (Model 2315, Dexela, UK) with a custom set at 45 kV / 120 mA with 2.5 sec of exposure, was used to view for vertebrae.

2.11 Tracking of larvae fish swimming behavior

The zebrafish larvae swimming behavior data were tracking by DanioVision equipment (Noldus, Wageningen, Netherlands). Before the indicated times of development, zebrafish larvae placed individually in 48-well plate with 1200 µL E3 medium for overnight. According to DanioVision analysis, we used light-induced visual motor response. The swimming behavior test for zebrafish larvae were as following condition: the first 30 minutes was required for their adaptation, then use the light switch (20 minutes: 10 minutes in dark and 10 minutes in light) for 2 cycles (Gao et al., 2014; Liu et al., 2017). The water temperature during the experiment was maintained at 28 ± 0.5 °C.

2.12 Analysis of larvae fish swimming behavior

The zebrafish larvae swimming behavior test were analyzed by EthoVision XT 13 (Noldus, Wageningen, Netherlands). We evaluate the velocity and distance movement of individual zebrafish.

2.13 T-maze apparatus

A three armed T-maze was used for our experiment. The stem of maze (length 36 cm × width 11 cm × height 20 cm) included the start box (11 cm × 11 cm × 20 cm). Each arm of the maze (25 cm × 11 cm × 20 cm). Blue or red cellophane was attached to the end of the arm (Supplementary figure 1). The T-maze was filled with system water and the temperature was maintained at 28 ± 1 °C.

2.14 Adult fish T-maze behavior test

The zebrafish adult behavior test used T-maze for memory testing and velocity testing. T-maze behavior test divided into three phases: pre-training, training and testing. Pre-training phase: adult zebrafish were placed in a 3 L fish tank when they were 3 months old. Placed a white sponge which filled with food in the fish tank to induce the fish to find the white sponge twice a day (10 am and 3 pm) for one week. Training phase: adult zebrafish were moved to the behavioral room and placed into an individual tank. All fishes were stand for 1 hour before placed into T-maze. During each trial, a zebrafish was placed in the start box for 1 minute with door closed. Then, the door was raised and closed after the fish had left. A stopwatch was used to measure the time it took for a fish to find a white sponge which filled with food. If the zebrafish did not leave the start box or find the target within 3 minutes, the experiment will stop. In

training phase, each fish training once time a day for one week. Testing phase: adult zebrafish were moved to the behavioral room and placed into an individual tank. All fishes were stand for 1 hour before placed into T-maze. During each trial, a zebrafish was placed in the start box for 1 minute with door closed. Then, the door was raised and closed after the fish had left. A stopwatch was used to measure the time it took for a fish to found a white sponge. If the zebrafish did not leave the start box or find the target within 3 minutes, the experiment will stop. In testing phase, each fish testing once time a day for one week.

2.15 Analysis of adult fish T-maze behavior test

The zebrafish adult behavior test were analyzed by EthoVision XT 13 (Noldus, Wageningen, Netherlands). We evaluate the velocity, latency and distance movement of individual zebrafish.

2.16 Adult fish muscle endurance swimming performance

Critical swimming speed *(Ucrit)* was defined as the maximum speed an adult fish can sustain over a period of time (Brett, 1964). Critical swimming speed was measured by swim tunnel (Loligo Systems, Denmark). Fish were not fed for 24 hours before measurements. One fish was selected by net from the fish tank, placed in the swim tunnel and let the fish recover for 5 minutes. Water velocity was increased in step of 10 cm s^{-1} at intervals of 5 minutes until the fish was tired, unable to hold the position in swim tunnel and retreat to the downstream screen. The water temperature during experiments was 28 ± 1 °C. Critical swimming speed *(Ucrit)* was calculated using the equation (Brett, 1964):

$$Ucrit = Ui + [Uii(Ti/Tii)]$$

*U*i is the highest velocity the fish maintained for the whole 5 minutes (cm s^{-1}), Uii is the velocity of increment, *T*i is the time elapsed at tired velocity and *T*ii is the time between velocity changes (5 minutes).

2.17 Analysis of adult fish muscle endurance swimming performance

Principal component analysis (PCA) was performed using XLSTAT® 2014 to identify the main cause of induced responses and the relationship between these parameters, and a biplot was graphed with both the measured parameters and observations.

2.18 Chemicals treatment for larvae fish

To test the rescued effect of swimming behavior of TPM3 transgenic zebrafish model. We used five chemicals to test the effect, L-tyrosine (All Lines Technology, US) was dissolved in water at a concentration of 10 μM, Taurine (NIPPON SHINYAKU, Japan) was dissolved in water at a concentration of 1 mM, L-carnitine (Sigma-Aldrich, US) was dissolved in water at a concentration of 10 μM, and creatine (Sigma-Aldrich, US) was dissolved in water at a concentration of 100 μM. Those four chemicals were following the previous paper for the dosage analysis (Sztal et al., 2018). In addition, Terazosin (Selleckchem, US) was dissolved in DMSO at a concentration of 2.5 μM were following the previous paper for the dosage analysis (Boyd et al., 2017).

For treatment of the TPM3 transgenic zebrafish model, TPM3 transgenic adult zebrafish F2 were in-crossed to get the embryos. Following the previous method (Sztal et al., 2018), embryos were placed in different chemical at 28 hpf. Changed the chemical every day until 7 dpf. Then, zebrafish larvae transferred

into 48-well plate with 1200 µL E3 medium for larvae fish swimming behavior test.

2.19 Chemicals treatment for adult fish

To test the rescued effect of swimming behavior of TPM3 transgenic zebrafish model. Terazosin and L-carnitine were oral gavage to TPM3 transgenic adult zebrafish. The dosage of Terazosin was 0.075 µg/fish which was converted from the mice dosage 0.05 mg/kg (Papay et al., 2002). And the dosage of L-carnitine was 0.065 mg/fish which was converted from the clinical used for human 4 g/day. Before oral gavage, the fish was anesthesia by 0.0125% buffered MS-222 solution (Ethyl 3-aminobenzoate methanesulfonic acid salt, Sigma-Aldrich, US). The fish were put on a wet sponge and 5 µL chemical solution was fed under the microscope by using a MICROLITER syringes (HAMITON, US). The fish were put back to the fish tank immediately. Oral gavage the chemicals twice a week for 1 month. All the fish were measured the body weight and body length before and after oral gavage. To test the rescued effect of swimming behavior of TPM3 transgenic zebrafish model. Using swim tunnel (Loligo Systems, Denmark) to measured critical swimming speed.

2.20 Tissue collection and frozen section

Before examination, the fish was anesthesia by 0.025% buffered MS-222 solution (Ethyl 3-aminobenzoate methanesulfonic acid salt, Sigma-Aldrich, US). Following the previous method (Sztal et al., 2017). Cut the muscle tissue between anal and caudal fins (Supplementary figure 2). Placed the muscle tissue in wet gauze. Placed 10% Tragacanth gum (Wako, Japan) on the wood,

which was stirred evenly. Make it to the small hill shape. Placed the muscle tissue into the small hill. Then, a beaker filled with 100 mL 2-Methylbutane (Sigma-Aldrich, US) and placed in liquid nitrogen. Submerge the wood with muscle tissue completely into 2-Methylbutane for 1 minute. Transfer muscle tissue to a -80 °C freezer immediately. The muscle tissue was sectioned to 8 μm by using Cryostat Microtome CM3050S (Leica, Germany). Tissue sections were stored at -80 °C freezer.

2.21 Hematoxylin and eosin stain (H&E stain)

Hematoxylin is a natural dye that was used since 1863, it combination with aluminum, iron, copper, chromium and tungsten. Hematoxylin and eosin stain (H&E stain) is one of the most commonly used histochemistry stains in pathology. H&E stain can identify the nucleus and cytoplasm. The slides were taken out from the -80 °C freezer and returned to the room temperature and washed with tap water for 10 times. For hematoxylin stain, the slides were incubated with Mayer's hematoxylin solution (Sigma-Aldrich, US) for 10 minutes to stain the nuclei to blue. Then, the slides were washed under running tap water for 5 minutes. For eosin stain, the slides were incubated with 0.5% eosin solution (MUTO PURE CHEMICALS, Japan) for 50 seconds to stain the cytoplasm, collagen and muscle fiber to red. Dehydrate the sections with two changes of 95% alcohol and two changes of 100% alcohol for 10 times each. Extract the alcohol with two changes of Non-Xylene (MUTO PURE CHEMICALS, Japan) for 10 minutes each. Finally, add two or three drops of xylene-based mounting medium and covered with cover slides. The slides were stored at room temperature.

2.22 Gömöri's trichrome stain

Gomori's trichrome stain is George Gömöri developed in 1950 (Gomori, 1950). Gomori's one step trichrome stain combined chromotrope 2R (plasma stain) and fast green FCF (connective fiber stain) in a phosphotungstic acid solution with acetic acid, and it used on muscle tissue for histological stain. The result will show that muscle fibers is blue, collagen is green, neuromyelin is red and nuclei is blue black. Gomori's reagent was given below:

Gomori's solution

Chromotrope 2R (Sigma-Aldrich, US)	0.3 g
Fast green FCF (Sigma-Aldrich, US)	0.15 g
Phosphotungstic acid (Sigma-Aldrich, US)	0.3 g
Glacial acetic acid (J.T.Baker, US)	0.5 mL
Distilled water to 50 mL	
Adjust to pH 3.4	

The slides were taken out from the -80 °C freezer and returned to the room temperature and washed with tap water for 10 times. Then, the slides were incubated with Mayer's hematoxylin solution (Sigma-Aldrich, US) for 10 minutes. The slides were washed under running tap water for 5 minutes. After wash, the slides were incubated with Gomori's solution for 30 minutes. Put the slides in 0.2% acetic acid (J.T.Baker, US) rinse for 3 times. Dehydrate the sections with 50% alcohol, 75% alcohol, two changes of 95% alcohol and two changes of 100% alcohol for 10 times each. Extract the alcohol with two changes of Non-Xylene (MUTO PURE CHEMICALS, Japan) for 10 minutes each. Finally, add two or three drops of xylene-based mounting medium and covered with cover slides. The slides were stored at room temperature.

2.23 Nicotinamide adenine dinucleotide tetrazolium reductase stain (NADH-TR stain)

Nicotinamide adenine dinucleotide tetrazolium reductase stain (NADH-TR stain) was used as identified the types of muscle fibers that were oxidative or glycolytic in muscle tissue. The electrons were transferred from NADH to the colorless soluble tetrazolium salt, which is converted to an insoluble blue. This reaction is catalyzed by an enzyme in the endoplasmic reticulum (NADH cytochrome b5 reductase). NADH-TR reactivity indicated activation of enzymes in mitochondria or endoplasmic reticulum (Charles-Schoeman and Verity, 2012).

NADH-TR solution

0.05 M Tris (pH7.4) (J.T.Baker, US)	30 mL
Nitro Blue Tetrazolium (Sigma-Aldrich, US)	30 mg
NADH (Sigma-Aldrich, US)	24 mg

The slides were taken out from the -80 °C freezer and returned to the room temperature and washed with tap water for 10 times. Then, the slides were incubated with NADH-TR solution for 30 minutes at 37 °C. Rinse the sections with 60% acetone, 90% acetone and 60% acetone for 10 times each. After rinse the section, the slides were washed with tap water for 10 times. Finally, add one or two drops of pre-warm (58 °C) glycerin jelly mounting medium (Sigma-Aldrich, US) and covered with cover slides. The slides were stored at room temperature.

2.24 ATPase stain

The calcium method used for ATPase demonstration, used solutions of different pH values, ATPase mainly used to distinguish muscle fiber types.

Muscle fibers can be roughly classified into type 1 (slow muscle, red muscle, oxidative muscle) and type 2 (fast muscle, white muscle, glycolysis muscle). Type 2 muscle fibers are further divided into 2a (glycolysis), 2b (glycolytic/oxidative) and 2c which changed type due to disease or damaged (Pestronk et al., 1992).

Barbital acetate solution

Sodium acetate (MUTO PURE CHEMICALS, Japan)	0.97 g
Sodium barbital (Sigma-Aldrich, US)	1.47 g
2 N Hydrochloric acid (J.T.Baker, US)	4.5 mL
Distilled water	175 mL
Adjust to different pH (4.3, 4.4, 4.5, 4.6, 4.7)	

ATP solution

0.1 M Sodium barbital (Sigma-Aldrich, US)	40 mL
0.18 M Calcium chloride (Wako, Japan)	20 mL
ATP (Wako, Japan)	500 mg
Distilled water to 200 mL	
Adjust to pH 9.6	

Solution I

0.1 M Sodium barbital (Sigma-Aldrich, US)	20 mL
0.18 M Calcium chloride (Wako, Japan)	10 mL
Distilled water	70 mL

The slides were taken out from the -80 °C freezer and returned to the room temperature. Then, the slides were incubated with different pH (4.3~4.7) Barbital acetate solution for 5 minutes at room temperature. Rinse the sections with Solution I for 1 minutes. After rinse the section, the slides were incubated with ATP solution for 45 minutes at room temperature. The slides placed into

1% Calcium chloride solution and shaking for 2 minutes and 3 minutes each. After shaking, stand the slides into 1% Calcium chloride solution for 5 minutes. The slides placed into 2% Cobalt chloride solution and shaking for 3 minutes. The slides placed into 0.01 M Sodium barbital solution and shaking for 8 times. The slides were washed under running tap water for 3 minutes. The slides placed into 1% Ammonium sulfide solution for 1 minute. Finally, wash the slides under running tap water for 5 minutes, dehydrate with alcohol and covered with cover slides. The procedure of ATPase stain was finished by Department of Pathology in Kaohsiung Medical University Chung-Ho Memorial Hospital.

2.25 Total RNA isolation

Total RNA from muscle tissue were extracted by using traditional method. Muscle tissue used Microtube Pellet Pestle Rods (Violet BioScience, Taiwan) to grinding. 800 µL of TRI Reagent® (Sigma-Aldrich, US) was mixed with sample and homogenize using a homogenizer. 160 µL of chloroform (Honeywell, US) was added. Vertex the sample and centrifuged at 13,000g at 4 °C for 15 minutes. Transfer the supernatant containing RNA to a new eppendorf. To precipitate the RNA, 500 µL of isopropyl alcohol (Sigma-Aldrich, US) was added and mixed gently, incubated for 10 minutes at room temperature, centrifuged at 13,000g at 4 °C for 10 minutes. Then, removed the supernatant. To wash the RNA, 600 µL of 75% ethanol was added to resuspend the pellet and centrifuged at 13,000g at 4 °C for 5 minutes. After removed the supernatant, dry the RNA sample for 10 minutes. To solubilize the RNA, 100 µL of nuclease-free water was added to resuspend the pellet for 15 minutes at room temperature. RNA sample were stored at -80 °C freezer.

2.26 RNA clean up

After using traditional method to extracted the total RNA from muscle tissue, total RNA used NucleoSpin® RNA kit (MACHEREY-NAGEL, US) for the cleanup. For each sample, added nuclease-free water to final volume was 100 µL. To adjust the RNA binding condition, 600 µL of Buffer RA1 premix with ethanol (Buffer RA1 300 µL and Absolute ethanol 300 µL) was added. The lysate was added to NucleoSpin® RNA column (Blue) and centrifuged at 11000xg for 30 seconds. Next, to desalt the silica membrane, 350 µL of MDB (Membrane Desalting Buffer) was added to NucleoSpin® RNA column and centrifuged at 11000xg for 1 minute. To digest the DNA, 95 µL of DNase reaction mixture (rDNase 10 µL and Reaction Buffer for rDNase 90 µL) was added to NucleoSpin® RNA column and incubated at room temperature for 30 minutes. To wash the silica membrane, 200 µL of Buffer RA2 was added to NucleoSpin® RNA column and centrifuged at 11000xg for 30 seconds, 600 µL of Buffer RA3 was added to NucleoSpin® RNA column and centrifuged at 11000xg for 30 seconds, 250 µL of Buffer RA3 was added to NucleoSpin® RNA column and centrifuged at 11000xg for 2 minutes. To dry the silica membrane, transfer the NucleoSpin® RNA column into a new 1.5 mL nuclease-free Eppendorf and dry at the room temperature for 5 minutes. To elute the RNA, 30 µL of nuclease-free water was added to NucleoSpin® RNA column for 30 minutes and then centrifuged at 11000xg for 2 minute. Finally, all the RNA sample measurement the concentration by using NanoDrop ND-1000 (Thermo Fisher Scientific, US) and then stored at -80 °C freezer.

2.27 Reverse transcription polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was reverse transcribed by using iScript™ cDNA synthesis kit (BioRad, US). The reverse transcription reagents and condition of the kit was given below:

Reverse transcription reaction mix

5X iScript Reaction Mix	4 µL
iScript Reverse Transcriptase	1 µL
RNA template	1 µg
Nuclease-free water	add to 20 µL

RT protocol

Priming	25°C for 5 minutes
Reverse transcription	46°C for 20 minutes
RT inactivation	95°C for 1 minutes
Optional step	hold at 4°C

2.28 Real time quantitative polymerase chain reaction (QPCR)

Complementary DNA (cDNA) were 100-fold diluted to final concentration was 0.5 µg/µL for QPCR analysis. The QPCR reagents mixture was given below:

QPCR reaction mix

cDNA	3.8 µL
Primer mix (2.5µM of forward and reverse primer)	1.2 µL
2X Fast SYBR Green (Thermo Fisher Scientific, US)	5 µL

Primers used for QPCR analysis listed in Table 6. For loading the reagents, cDNA were loading to 384-well plate at first. Then, the primers and Fast SYBR green were mixed together and added to 384-well plate.

After all the reagents loading to 384-well plate, the plate must be covered with “optical adhesive cover” (Life technologies, US). And the plate was centrifuged at 3000 rpm for 1 minute to spin down the reaction mixture. The QPCR was using ViiA^{TM} 7 Real-Time PCR machine (Thermo Fisher Scientific, US) and set as following program:

QPCR protocol

Hold stage	95°C for 3 minutes
PCR stage	95°C for 1 second
	95°C for 20 seconds
	Repeat 40 cycles
Melt curve stage	95°C for 15 seconds
	60°C for 1 minute
	95°C for 15 seconds

All the experiments were performed in triplicate for QPCR. And relative quantification were used for QPCR analysis. All the Ct value of target genes were normalized with internal control 18S rRNA: $\Delta Ct = (Ct_{target} - Ct_{18S\ rRNA})$. The relative quantification expression ratio was calculated based on ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{control}$, and the fold change are $2^{-\Delta\Delta Ct}$.

2.29 Statistical analysis

Data were collected and analyzed by using Microsoft Excel and GraphPad Prism 7 software. Individual tests are description in the main text. For all the statistical analysis was performed using two-tailed Student’s t-test. All data are expressed as mean ± SEM. P-value less than 0.05 was considered statistically significant. For all figures: NS: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****: P≤0.0001

Chapter 3 Results

3.1 Establishment of TPM3 transgenic zebrafish

To generate TPM3 transgenic zebrafish model, we generated three constructs. We used three pair of primers in different position to confirm the constructs by polymerase chain reaction (Figure 1A). First pair of primers was used to confirm the fusion of MLC2 promoter with TPM3, and the expected product was 397 bp. Second pair of primers was used to confirm the fusion of TPM3 with polyA, and the expected product was 340 bp. Third pair of primers was used to confirm the fusion of MLC2 promoter to the polyA, and the expected product was 1172 bp. After polymerase chain reaction, we checked the amplicon size by agarose gel electrophoresis. All of the 12 colonies had the right sizes as we expected (Figure 1B). After DNA sequencing confirmation of the constructs, we microinjected those constructs into AB wildtype zebrafish embryos to generate TPM3 transgenic zebrafish, myl7 is heart specific promoter driving GFP expression which can be observed at 48 hpf after injection by fluorescent microscope.

Three TPM3 transgenic zebrafish were generated, *Tg (MLC2:TPM3(WT); myl7; EGFP)*, *Tg (MLC2:TPM3(E151A); myl7; EGFP)* and *Tg (MLC2:TPM3(E151G); myl7; EGFP)* which expressed TPM3(WT), TPM3(E151A) and TPM3(E151G) under the muscle promoter, MLC2. We raised the F0 TPM3 transgenic zebrafish to 3 months old, and extracted the DNA from the fins. After DNA sequencing, we confirmed that *Tg (MLC2:TPM3(WT); myl7; EGFP)* at nucleotide 452 was A, *Tg (MLC2:TPM3(E151A); myl7; EGFP)* at nucleotide 452 was C and *Tg*

(MLC2:TPM3(E151G); myl7; EGFP) at nucleotide 452 was G (Figure 2A and 2B).

We also examined the mRNA level of TPM3 transgenic zebrafish, and confirmed all three TPM3 transgenic zebrafish expressed human TPM3 gene (Supplementary Figure 3).

3.2 TPM3(E151G) mutant displayed abnormal appearance

We observed adult TPM3(E151G) fishes with abnormalities in appearance. F0 adult TPM3(WT) and TPM3(E151A) fishes displayed normal appearance. Among the F0 adult TPM3(E151G) fish, 3 of the 36 (8%) without tail, 2 of the 36 (6%) displayed crooked body (Figure 3A). In their next generation F1 adult, TPM3(WT) and TPM3(E151A) fishes also display normal appearance. Among the F1 adult TPM3(E151G) fish, 3 of the 42 fish (7%) without tail, 5 of the 42 fish (12%) displayed crooked body (Figure 3B). Since in the patient the mutation is de novo dominant, both the F0 and F1 TPM3(E151G) displayed abnormal appearance.

We also found multiple independent lines of the F1 larvae TPM3(E151G) and TPM3(E151A) fish had higher proportion of abnormalities. Among F1 larvae TPM3(E151G) TG1, TG2 and TG3, 6 of the 61 fish (9.8%), 2 of the 34 fish (5.9%) and 13 of the 15 fish (86.7%) respectively displayed crooked body appearance. Even among F1 larvae TPM3(E151A) TG1 and TG2, 3 of the 71 fish (4.2%) and 17 of the 25 fish (68%) displayed crooked body appearance. 1 of the 26 fish (3.8%) F1 larvae TPM3(WT) TG1 also displayed crooked body appearance (Figure 4A and 4B).

3.3 TPM3 mutants' adult displayed abnormal skeleton

Some congenital myopathy associated with oculo-facial and skeletal abnormalities. We examined the skeleton of the TPM3 transgenic zebrafish by a digital X-ray. The control fish AB(WT) have 31 normal alignment vertebrate (Figure 5A), four independent lines of F1 adult TPM3(WT) also had normal skeleton. All four independent lines of F1 adult TPM3(E151G) exhibited skeleton abnormalities. Among the four independent lines of F1 TPM3(E151G), TG1, TG2, TG3 and TG4, the malformations in the vertebrate were 2 out of 8 (25%), 4 out of 7 (57.1%), 2 out of 4 fish (50%), and 2 out of 8 fish (25%) respectively. Among the two independent lines of F1 TPM3(E151A), TG2 and TG4, the malformations in the vertebrate were 4 out of 7 (57.1%) and 2 out of 7 fish (28.5%) (Figure 5B). X-ray images clearly showed that TPM3 mutants also affect the deformity of most fish in precaudal vertebrae, and TPM3(E151G) was more severe than TPM3(E151A).

3.4 TPM3 mutants' larva exhibited lower survival rate

We also noticed the survival rate of F1 larvae were affected by the TPM3 mutation. We found the larvae TPM3(E151A) and TPM3(E151G) exhibited significant lower survival rate than TPM3(WT). The survival rate for four independent lines of TPM3(E151G) were 41 of the 61 (67.2%), 18 of the 34 (52.9%), 1 of 15 (6.7%), and 8 of the 16 (50%) for TG1, TG2, TG3 and TG4 respectively. The survival rate for four independent lines of TPM3(E151A) were 38 of the 71 (53.5%), 4 of the 25 (16%), 12 of the 19 (63.2%), and 19 of the 26 (73.1%) for TG1, TG2, TG3 and TG4 respectively. In general, TPM3 mutants also decreased the survival rate, and TPM3(E151G) having lower survival rate than TPM3(E151A) (Figure 6).

3.5 TPM3 mutants swimming slower compared to TPM3(WT) transgenic fish

To examine whether TPM3 mutants affect muscles development and leading to swimming disability, T-maze was used to measure the swimming velocity and latency of adult TPM3 transgenic fish. We first examined the F0 adult fish of TPM3(WT), TPM3(E151A) and TPM3(E151G). There was no different between TPM3(WT) and TPM3(E151A) in velocity, however, TPM3(E151G) exhibited significant lower velocity from day 3 to day 5 compared to TPM3(WT) (Figure 7A). In latency, TPM3(E151G) and TPM3(E151A) significantly took longer time to find the target from day 3 to day 5 compared to TPM3(WT) (Figure 7B).

We also compared the F1 adult TPM3 transgenic fish, and found both TPM3(E151A) and TPM3(E151G) exhibited significant lower velocity compared to TPM3(WT) (Figure 8A). We also found the two groups of TPM3(E151G) with or without tail (abnormal) all swam slower than TPM3(WT). In latency, all of the TPM3(E151G) and TPM3(E151A) fish significantly took a longer time to find the target compared to TPM3(WT) (Figure 8B).

DanioVision was used to measure the swimming velocity of F1 larvae TPM3 transgenic fish at 7 dpf. We examined the swimming velocity for AB wild-type zebrafish, four independent lines for TPM3(WT), TPM3(E151A) and TPM3(E151G). There were no difference between AB(WT) and TPM3(WT) (Figure 9A), four independent lines of TPM3(E151G) all exhibited significant lower velocity compared to AB wild-type fish (Figure 9B). TPM3 (E151A) TG2 also had significant lower velocity compared to the wild-type fish (Figure 9B). In addition, we also measureed the swimming velocity of 3 dpf to 6 dpf, and

found that TPM3(E151G) larva exhibited lower velocity compared to AB(WT) (Supplementary figure 4).

3.6 TPM3 mutants exhibited weaker muscle endurance

Since T-maze swimming behavior test might mixed with learning and memory. To further examine the strength of the muscles, a total of thirteen groups of F1 adult zebrafish were tested for muscle endurance by swim tunnel. We examined AB wild-type zebrafish, four independent lines for TPM3(WT), TPM3(E151A) and TPM3(E151G). Critical swimming speed (*Ucrit*) for these thirteen groups were determined (Figure 10A). There was no different between AB wild-type and TPM(WT) adult fish in muscle endurance. Four independent lines of TPM3(E151G) exhibited significant poor muscle endurance compared with AB(WT). TG2 and TG4 of TPM3 (E151A) have significant weaker muscle endurance compared with AB(WT) (Figure 10A).

We wondered whether body weight or length of these thirteen groups making the difference in muscle endurance test. As shown in Figure 10B, only TPM3(WT) TG3, TPM3(E151G) TG1 and TG2 had significant smaller weight compared to AB(WT) (Figure 10B). After standardized the length, some of the fish including TPM3(WT) TG3 and TG4, TPM3(E151A) TG1, TPM3(E151G) TG1 and TG2 had significant shorter lengths, while TPM3(E151G) TG4 had significant longer lengths compared to AB(WT) (Figure 10C). Therefore, there was no correlation between the body weight or length and muscle endurance.

The four variation factors of critical swimming speed (*Ucrit*), gender, body weight and standard length were analyzed by principal component analysis. When two principle variation factors were selected, the total explanatory variation was 80%. The first principal component and the second principal

component accumulated 52.3% and 27.7%. When the two principal component factors represent all the factors, only 20% of the variation was ignored. We can observed that AB(WT) and TPM3(WT) were overlapping, which can be interpreted as the same group. TPM3(E151G) was divided into another group, which was obviously different from AB(WT) and TPM3(WT) group (Figure 11).

3.7 Evaluation of histopathology on adult TPM3 transgenic zebrafish

Muscle biopsy was an important diagnosis for confirming congenital myopathy. We used cryosection technique and histochemical staining to examine the muscle specimens of F1 adult TPM3 transgenic zebrafish. By using HE stain and Gomori trichrome stain, We observed that the muscle fibers of TPM3(WT) were arranged neatly and consistent. However, the muscle fibers of TPM3(E151G) displayed a disordered and disproportionate arrangement, which were similar to congenital fiber disproportion. Interestingly, TPM3(E151A) seemed resembled to Nemaline myopathy.

By using NADH-TR stain, We observed dark blues muscle fibers in the outer layer were slow muscle, white muscle fibers in the inner layer were fast muscle, and light blue in the middle layer were between fast muscle and slow muscle (Figure 12 and Figure 13). We also used ATPase staining for different pH to distinguish the type of muscle fibers, but all of the TPM3 transgenic zebrafish muscle could not be distinguished clearly (Supplementary Figure 5).

3.8 Screening of the therapeutic drugs using TPM3(E151G) larvae fish

Based on the results above, we found TPM3(E151G) mutant zebrafish exhibited the muscle weakness and slower swimming behavior resembled human patient. Since there is no cure for congenital myopathy, we would like

to use the TPM3(E151G) zebrafish model for screening therapeutic drugs. We treated F3 larvae TPM3 transgenic fish from 28 hpf to 7 dpf with five chemincals, and measured the swimming velocity at 7 hpf by DanioVision. We found that L-carnitine significantly improved the swimming velocity of larvae TPM3(E151G) fish. The other four chemicals including L-tyrosine, Taurine, Creatine and Terazosin could not improve the swimming velocity of larvae TPM3(E151G) fish (Figure 14A). L-carnitine treatment is specific to TPM3(E151G) since neither TPM3(E151A) fish (Figure 14B), nor AB(WT) (Figure 14C) can be improved by L-carnitine treatment. This results indicated that L-carnitine specifically improve the swimming velocity of TPM3(E151G) larvae, but not the swimming velocity of TPM3(E151A) and AB(WT) larvae.

3.9 Evaluation of the therapeutic using TPM3(E151G) adult fish

We wondered whether the chemical improved the swimming speed of TPM3(E151G) larvae can be applied to adult fish. We treated F2 adult TPM3(E151G) transgenic fish with L-carnitine and Terazosin which was reported to rescue the MN axonal outgrowth phenotypes in *smn* morphant zebrafish. Swim tunnel were used to measure the critical swimming speed (*Ucrit*) after one months of feeding drugs. We observed adult TPM3(E151G) fish exhibited significant poor muscle endurance compared to TPM(WT) with or without chemicals treatment. There was no significant difference in critical swimming speed, weight and length between treatment group and control group (Figure 15A and 15B).

The four variation factors of critical swimming speed (*Ucrit*), gender, body weight and standard length were analyzed by principal component analysis. When two principle variation factors were selected, I observed that treatment

group and control group were overlapping, which can be interpreted as the same group (Figure 16A and 16B). In adult TPM3 transgenic zebrafish model, L-carnitine and Terazosin cannot improve critical swimming speed (*Ucrit*).

Chapter 4 Discussion

4.1 Behavioral testing of swimming velocity and learning memory

The behavioral test of our TPM3 transgenic zebrafish model, we started with T-maze to measure the swimming velocity and memory test related to learning. In ethology, T-maze was a simple maze for animal cognitive experiments (Olton, 1979). The T-maze was widely used to measure the spatial memory and learning tasks in laboratory animals, such as rat or zebrafish. Zebrafish has quickly become an important model for studying the vertebrate neural development (Levin and Cerutti, 2009). Due to its easily to test the function of learning, T-maze has been widely used to study drugs and toxins that affect memory in zebrafish (Jatin Prakash, 2017). As with other study, our TPM3(WT) zebrafish can be trained, which means that TPM3(WT) zebrafish have good learning ability and memory during the T-maze behavior test. However, TPM3 mutant zebrafish differs greatly from TPM3(WT), TPM3 mutant zebrafish have deficits memory and unable to learn.

Previous study mentions that there were some muscle diseases were related to brain development. Muscleblind-like 2 (Mbnl2) knockout mice develop abnormal REM sleep propensity and deficits memory in myotonic dystrophy (Charizanis et al., 2012). In 67 cases of congenital fiber type disproportion, 2 cases reported with intellectual disability, 1 case reported with mild global development delay. Because some cases were young infants, the frequency of intellectual disability was minority (Clarke and North, 2003). Although TPM3 was expressed in the brain, whether the TPM3 mutation was directly related to the defects of brain development was still unknown. In our TPM3 zebrafish model, we have confirmed that TPM3 mutation may cause

defects in the brain development of zebrafish, which reduced learning ability and memory deficits. TPM3 mutation can cause myopathy and defects in the brain development. This hypothesis was only based on our TPM3 zebrafish model, we cannot confirm that clinical patients have learning and cognitive impairments. In order to eliminate the influence of learning in our experiments, we used swim tunnel for the behavior test without learning ability. The swim tunnel uses different water velocity to keep the fish in a state of swimming to test the endurance of the muscle. We used swim tunnel that was only related to muscle endurance and does not involve learning ability to conduct behavioral tests and drug screening tests in adult TPM3 zebrafish model.

4.2 How L-carnitine improve swimming velocity in larvae TPM3(E151G) zebrafish

Most of L-carnitine was intracellular, which stored in liver, skeletal muscle, heart and kidney (Jones et al., 2010). L-carnitine promoting long chain fatty acyl CoAs into the mitochondria for β-oxidation and nonoxidative glucose disposal (Alves et al., 2009; Cahova et al., 2015). The organic cation transporter-2 (OCTN2) enhance L-carnitine uptake inside cells. Carnitine acyltransferases were a family of enzymes that catalyze the reversible transfer of acyl groups between coenzyme A and L‑carnitine, and the conversion of acyl-CoA esters to acylcarnitine esters (Adeva-Andany et al., 2017). Neuroprotective in several animal models when administered with L-carnitine at super physiological concentrations. L-carnitine was used to reduce lactate levels and ATP elevation upon administration (Zanelli et al., 2005). A nemaline myopathy case with ACTA-1 gene mutation and carnitine deficiency, the first described in the study

with evidence of a disorder of mitochondrial fatty acid oxidation (Finsterer and Stollberger, 2015; Laing et al., 2009). The pathogenesis of congenital myopathy was up to now poorly understood. There were several gene mutation can cause congenital myopathy. The clinical phenotype caused by mutations in the same gene can display a broad spectrum even with the same amino acid substitutions (Nowak et al., 1999). We hypothesis that when we used high concentration of L-carnitine in our larvae TPM3(E151G) zebrafish model, L-carnitine will enhance long chain fatty acyl CoAs into the mitochondria for β-oxidation to generate ATP to rescue muscle weakness.

4.3 Comparison of larvae fish and adult fish in a drug screening platform for congenital myopathy

In our TPM3 transgenic zebrafish platform, we used larvae fish and adult fish for the drug screening. Not as expected, the results from larvae fish platform and adult fish platform were not consistent. We found that L-carnitine can improve the swimming velocity of larvae TPM3(E151G) zebrafish, but cannot rescue the muscle endurance of adult TPM3(E151G) zebrafish. This indicated that larvae fish and adult fish were different. At 24 hours post-fertilization (hpf), zebrafish muscle cells have finished segmentation (Ochi and Westerfield, 2007). Myogenic regulatory factors such as Myod, Myf5 and Myogenin were expression during the segmentation period (Coutelle et al., 2001; Weinberg et al., 1996). T-box transcription factors such as ntl and spt mutation can let embryos fail to form trunk somites and have a shortened in muscle development (Kimmel et al., 1989). Those gene performed high level in

the early embryonic stage, but not as same level in adult stage, it usually have lower expression level in adult stage.

Skeletal muscle satellite (stem) cells were crucial for skeletal muscle growth and regeneration (Comai and Tajbakhsh, 2014). Energy ATP was essential for cells to conduct for the life. Cellular metabolism to regulate skeletal muscle stem cells was important (Ryall, 2013). Pax7 gene, which mainly express and play a critical role in satellite cell, and loss of Pax7 exhibit reduced postnatal muscle growth (Seale et al., 2000). They were quiescent under steady state conditions in adult stage, and will re-enter the cell cycle while the muscle damage (Sambasivan et al., 2011). However, satellite cells change during aging (Chakkalakal et al., 2012). Including genomic instability, DNA and oxidative damage, function of mitochondria changed and aging. Those reason were cause loss of satellite cells and decreased regenerative capacity (Garcia-Prat et al., 2016).

L-carnitine can improve swimming velocity of larvae TPM3(E151G) may because L-carnitine enhance long chain fatty acyl CoAs into the mitochondria for β-oxidation to generate energy for skeletal muscle satellite (stem) cells to regenerate, then recovered the swimming velocity of TPM3(E151G) zebrafish to near normal zebrafish in larvae. But in adult fish, skeletal muscle satellite (stem) cells due to its aging or loss function for a long time. Therefore, even have enough energy, it still cannot improve the muscle endurance in adult TPM3(E151G) zebrafish. Moreover, we found that Pax7 gene expression level in F1 adult TPM3(E151G) mutant zebrafish were decrease (Supplementary Figure 6).

Although we found L-carnitine can improve the swimming velocity of larvae TPM3(E151G) zebrafish, but clinical patients had completed muscle

development at the time of diagnosis, it similar to adult fish. There were no known cures of congenital myopathy, so it is necessary and urgent to develop drug screening for this rare human disease.

4.4 Future perspective

Next generation sequencing for the muscle specimens were performed to identify the dysregulated genes/pathways. We extracted the skeletal muscle RNA of F1 adult TPM3 transgenic zebrafish. Compared with TPM3(WT), TPM3(E151A) zebrafish had 913 genes down regulation and 673 genes up regulation. TPM3(E151G) zebrafish had 819 genes down regulation and 937 genes up regulation. We found that E151A and E151G zebrafish had 370 genes down regulation and 160 genes up regulation at a same time (Supplementary Figure 7). Down regulation genes were related to anatomy structure development, cellular protein modification process and cytoskeleton organization. And up regulation genes were related to response to stress (Supplementary Figure 8). In the future, we will further investigate the most important dysregulated genes that cause congenital myopathy and develop a personalized drug for this rare human genetic disease.

Figures and Tables

Figure 1. Confirmation the construct of TPM3 transgenic zebrafish by colony PCR

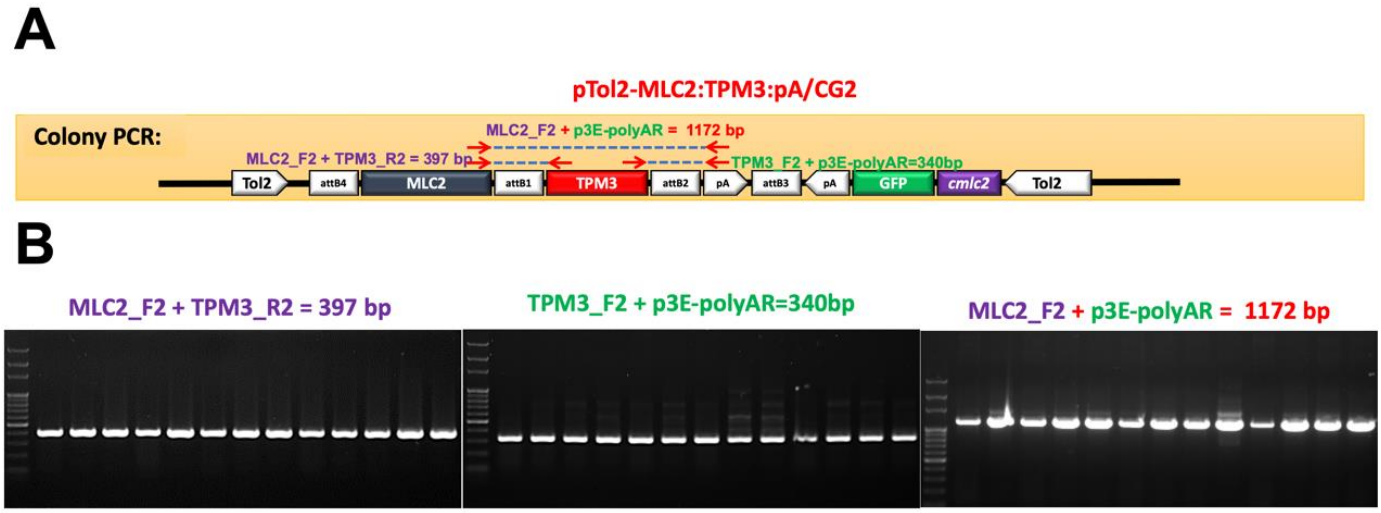


Figure 1. Confirmation the construct of TPM3 transgenic zebrafish by colony PCR

(A) Three pairs of primer used to confirm the construct of pTol2-MLC2:TPM3:pA/CG2. (B) After gel electrophoresis, the size of all PCR products were what we expected.

Figure 2. Confirmation the sequence of TPM3 transgenic zebrafish

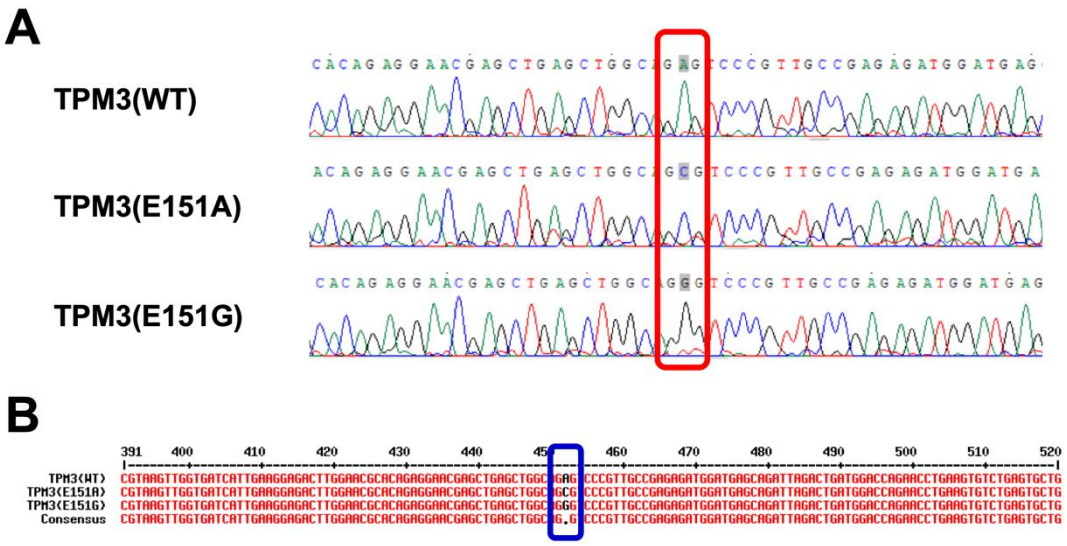


Figure 2. Confirmation the sequence of TPM3 transgenic zebrafish

(A) DNA sequencing to confirm TPM3 transgenic zebrafish. We can confirm that TPM3(WT) at nucleotide 452 was A, TPM3(E151A) at nucleotide 452 was C and TPM3(E151G) at nucleotide 452 was G. (B) Comparison the sequence result of TPM3 transgenic zebrafish.

Figure 3. Appearance of adult TPM3 transgenic zebrafish

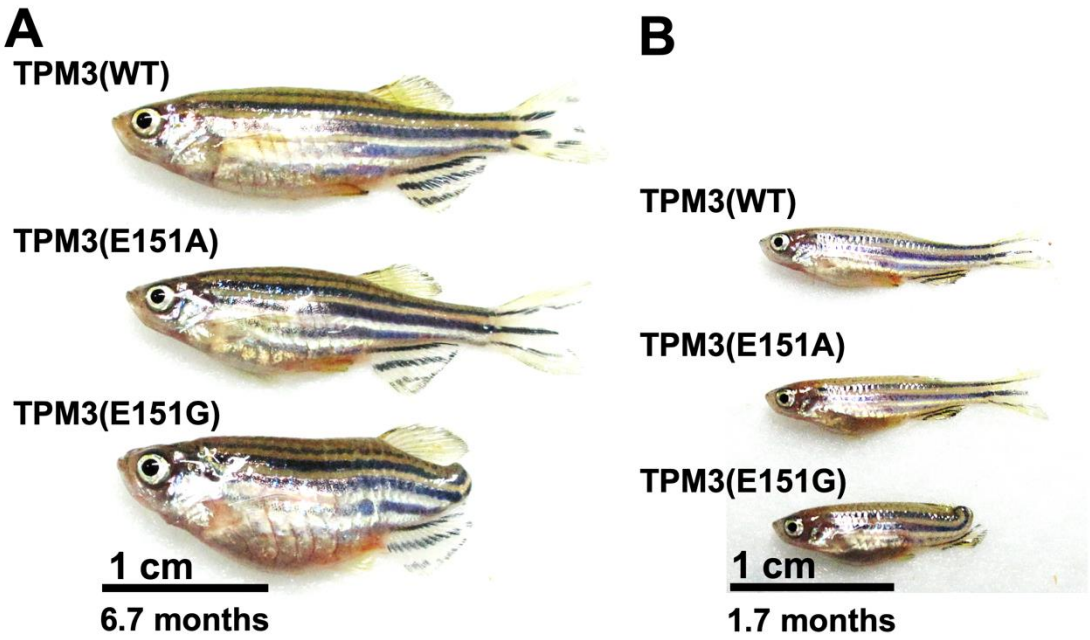


Figure 3. Appearance of adult TPM3 transgenic zebrafish

(A) Appearance of 6.7 months adult F0 TPM3 transgenic zebrafish. (B) Appearance of 1.7 months adult F1 TPM3 transgenic zebrafish.

Figure 4. Statistical the proportion of abnormal appearance of larvae TPM3 transgenic zebrafish

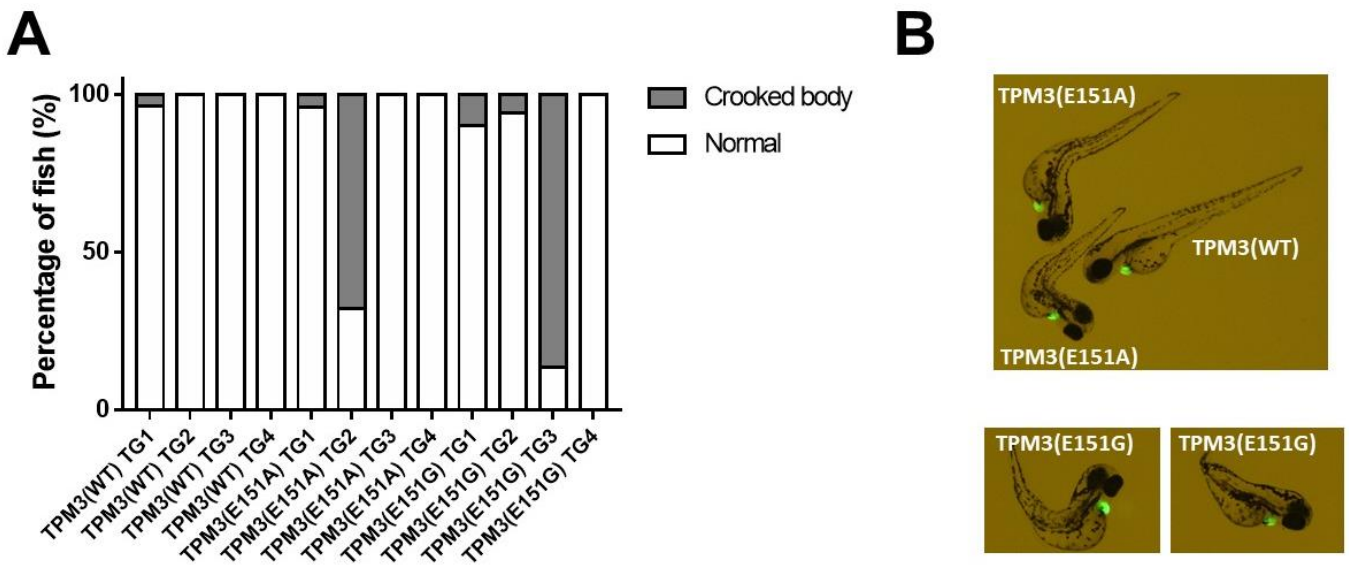


Figure 4. Statistical the proportion of abnormal appearance of larvae TPM3 transgenic zebrafish

(A) The proportion of abnormal appearance of F1 larvae TPM3 transgenic zebrafish, we observed four TG line of TPM3(WT), four TG lines of TPM3(E151A) and four TG lines of TPM3(E151G) at 2dpi. (B) F1 larvae TPM3 mutant transgenic zebrafish observed an abnormal appearance at 2 dpi.

Figure 5. The X-ray image of adult F1 TPM3 transgenic zebrafish

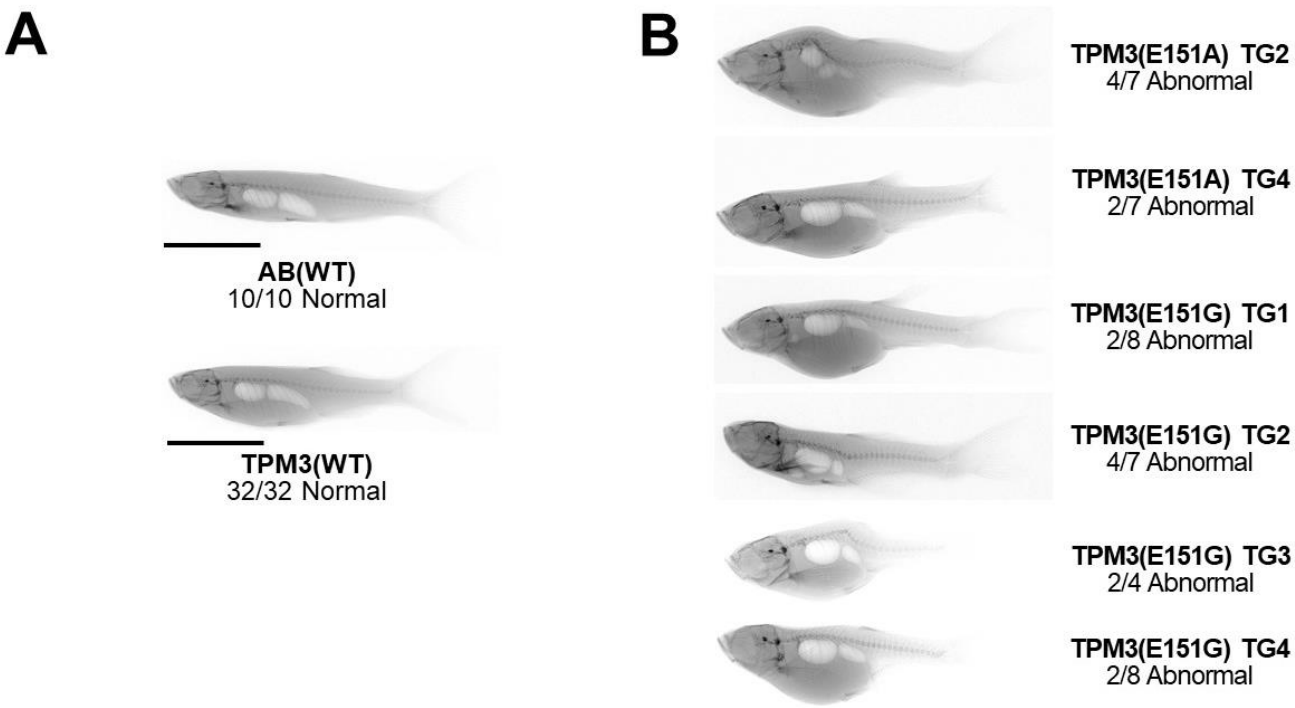


Figure 5. The X-ray image of adult F1 TPM3 transgenic zebrafish

(A) The skeleton of the zebrafish observed by X-ray. Zebrafish have 31 vertebrae in male and female fish. Adult AB(WT) display normal alignment during our examination. (B) Adult TPM3 mutant transgenic zebrafish display abnormal alignment in skeleton.

Figure 6. Statistical the survival rate of larvae TPM3 transgenic zebrafish

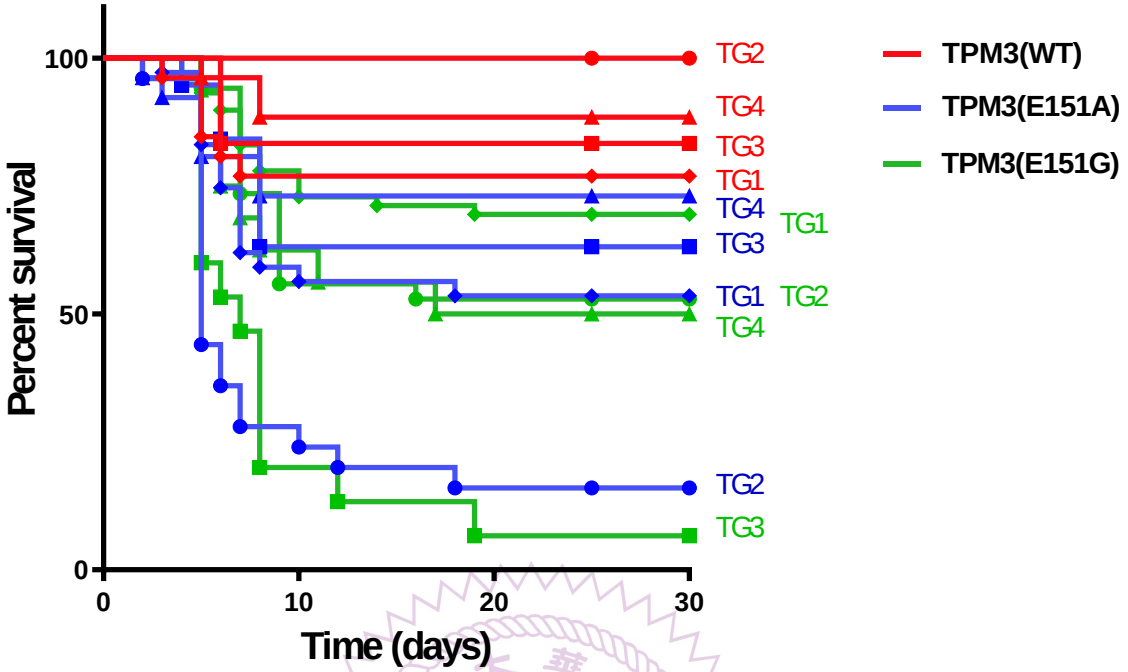


Figure 6. Statistical the survival rate of larvae TPM3 transgenic zebrafish

The survival rate of larvae TPM3 transgenic zebrafish were observed for 30 days. Red lines were four TG of TPM3(WT), blue lines were four lines of TPM3(E151A), green lines were four lines of TPM3(E151G).

Figure 7. T-maze behavior test for F0 adult TPM3 transgenic zebrafish

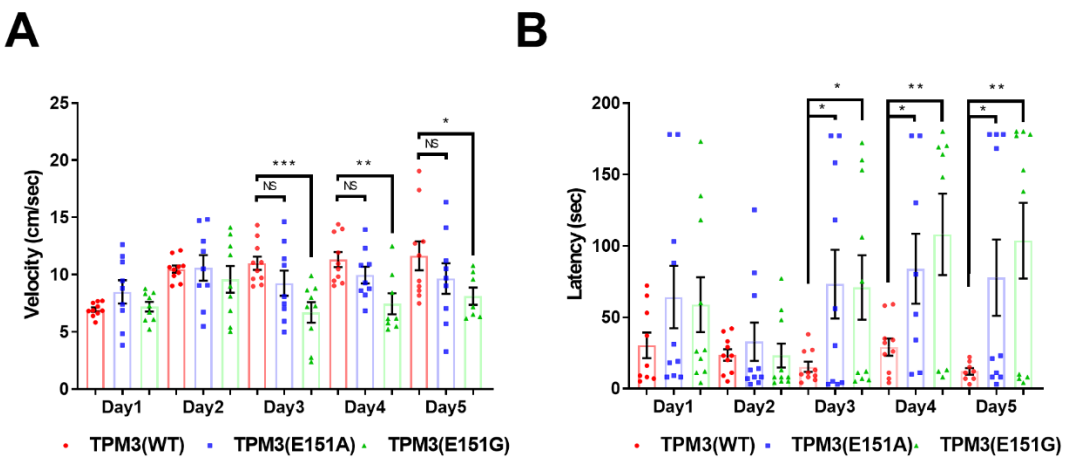


Figure 7. T-maze behavior test for F0 adult TPM3 transgenic zebrafish

(A) The swimming velocity of F0 adult TPM3 transgenic zebrafish during the five days of testing. Red plot was TPM3(WT) (n=10), blue plot was TPM3(E151A) (n=10), green plot was TPM3(E151G) (n=10). (B) Latency for memory test of F0 adult TPM3 transgenic zebrafish during the five days of testing. Red plot was TPM3(WT) (n=10), blue plot was TPM3(E151A) (n=10), green plot was TPM3(E151G) (n=10).

Figure 8. T-maze behavior test for F1 adult TPM3 transgenic zebrafish

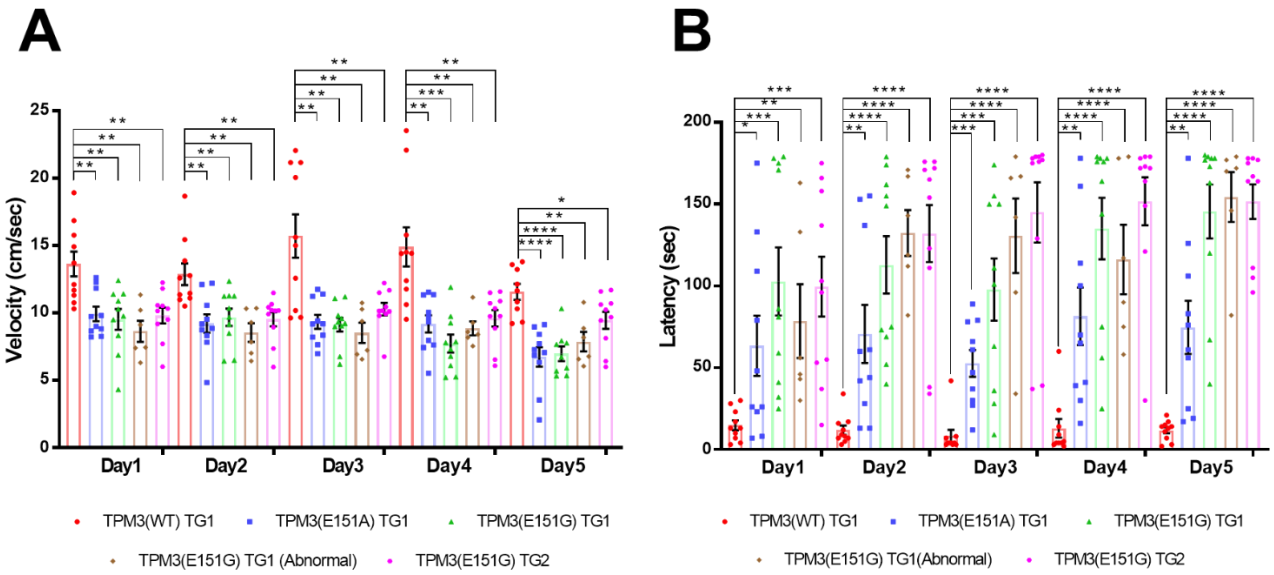


Figure 8. T-maze behavior test for F1 adult TPM3 transgenic zebrafish

(A) The swimming velocity of F1 adult TPM3 transgenic zebrafish during the five days of testing. Red plot was TPM3(WT) TG1 (n=10), blue plot was TPM3(E151A) TG1 (n=10), green plot was TPM3(E151G) TG1 (n=10), brown plot was TPM3(E151G) TG1 abnormal appearance (n=6), pink plot was TPM3(E151G) TG2 (n=10). (B) Latency for memory test of F1 adult TPM3 transgenic zebrafish during the five days of testing. Red plot was TPM3(WT) TG1 (n=10), blue plot was TPM3(E151A) TG1 (n=10), green plot was TPM3(E151G) TG1 (n=10), brown plot was TPM3(E151G) TG1 abnormal appearance (n=6), pink plot was TPM3(E151G) TG2 (n=10).

Figure 9. The swimming velocity of larvae F1 TPM3 transgenic zebrafish

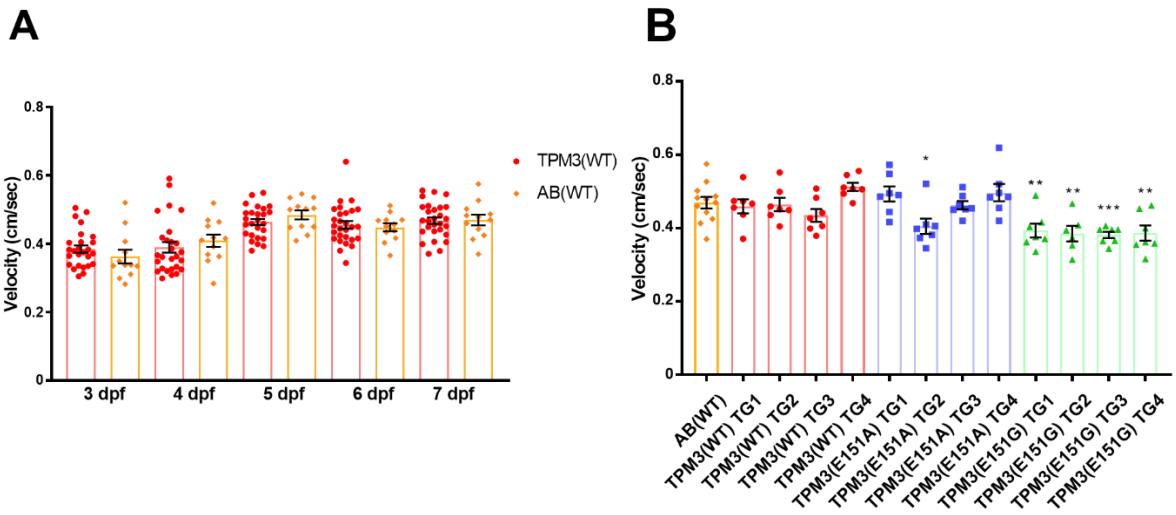


Figure 9. The swimming velocity of larvae F1 TPM3 transgenic zebrafish

Larvae fish swimming velocity was measured by DanioVision. (A) Red plot was TPM3(WT) fish (n=28), orange plot was AB(WT) fish (n=12). (B) All the statistics were compared with AB(WT). Orange plot was AB(WT) (n=12), red plot was four TG line of TPM3(WT) (n=8 for each line), blue plot was four TG line of TPM3(E151A) (n=8 for each line), green plot was four TG line of TPM3(E151G) (n=8 for each line).

Figure 10. Muscle endurance test for adult F1 TPM3 transgenic zebrafish

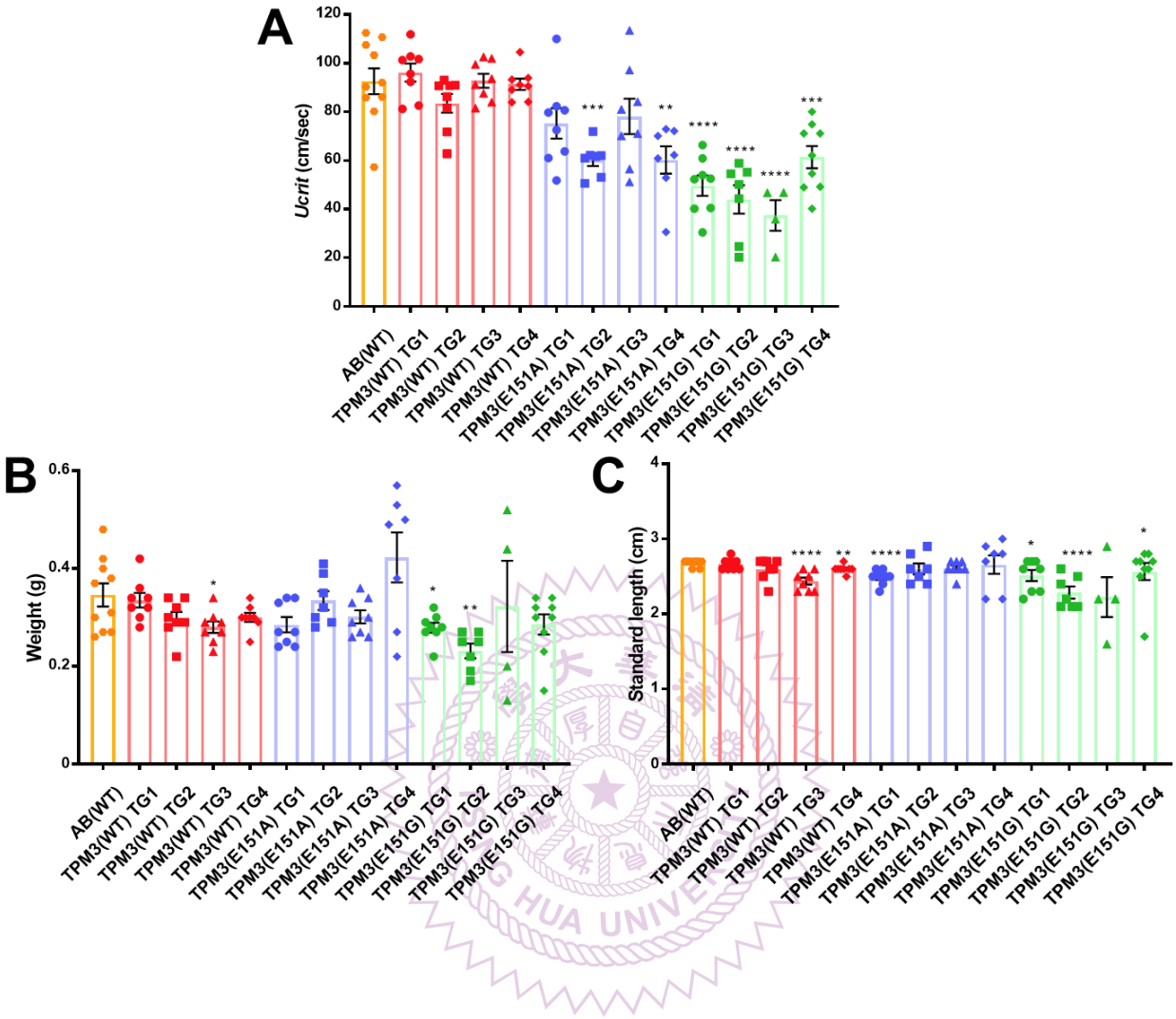


Figure 10. Muscle endurance test for adult F1 TPM3 transgenic zebrafish

Muscle endurance test used swim tunnel to measure the critical swimming speed (*Ucrit*). All the statistics were compared with AB(WT). Orange plot was AB(WT) (n=10), red plot was four TG line of TPM3(WT) (TG1 n=8, TG2 n=8, TG3 n=8, TG4 n=8), blue plot was four TG line of TPM3(E151A) (TG1 n=8, TG2 n=7, TG3 n=8, TG4 n=7), green plot was four TG line of TPM3(E151G) (TG1 n=8, TG2 n=7, TG3 n=4, TG4 n=9). (A) Critical swimming speed (Ucrit) of thirteen groups. (B) The body weight of thirteen groups. (B) The standard length of thirteen groups.

Figure 11. Principal component analysis of endurance test for adult F1 TPM3 transgenic zebrafish

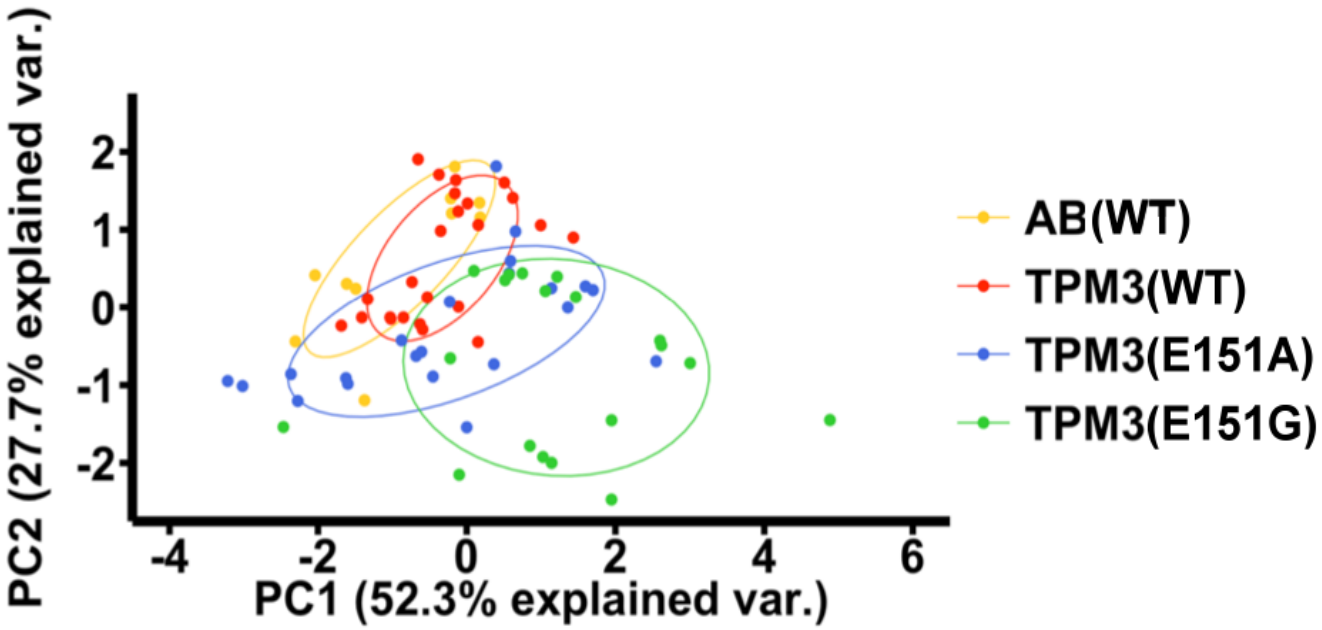


Figure 11. Principal component analysis of endurance test for adult F1 TPM3 transgenic zebrafish

The first principal component accumulated 52.3% and the second principal component accumulated 27.7%. Orange plot was AB(WT) (n=10), red plot was TPM3(WT) (n=24), blue plot was TPM3(E151A) (n=22), green plot was TPM3(E151G) (n=19).

Figure 12. 50X histopathological of F1 adult TPM3 transgenic zebrafish

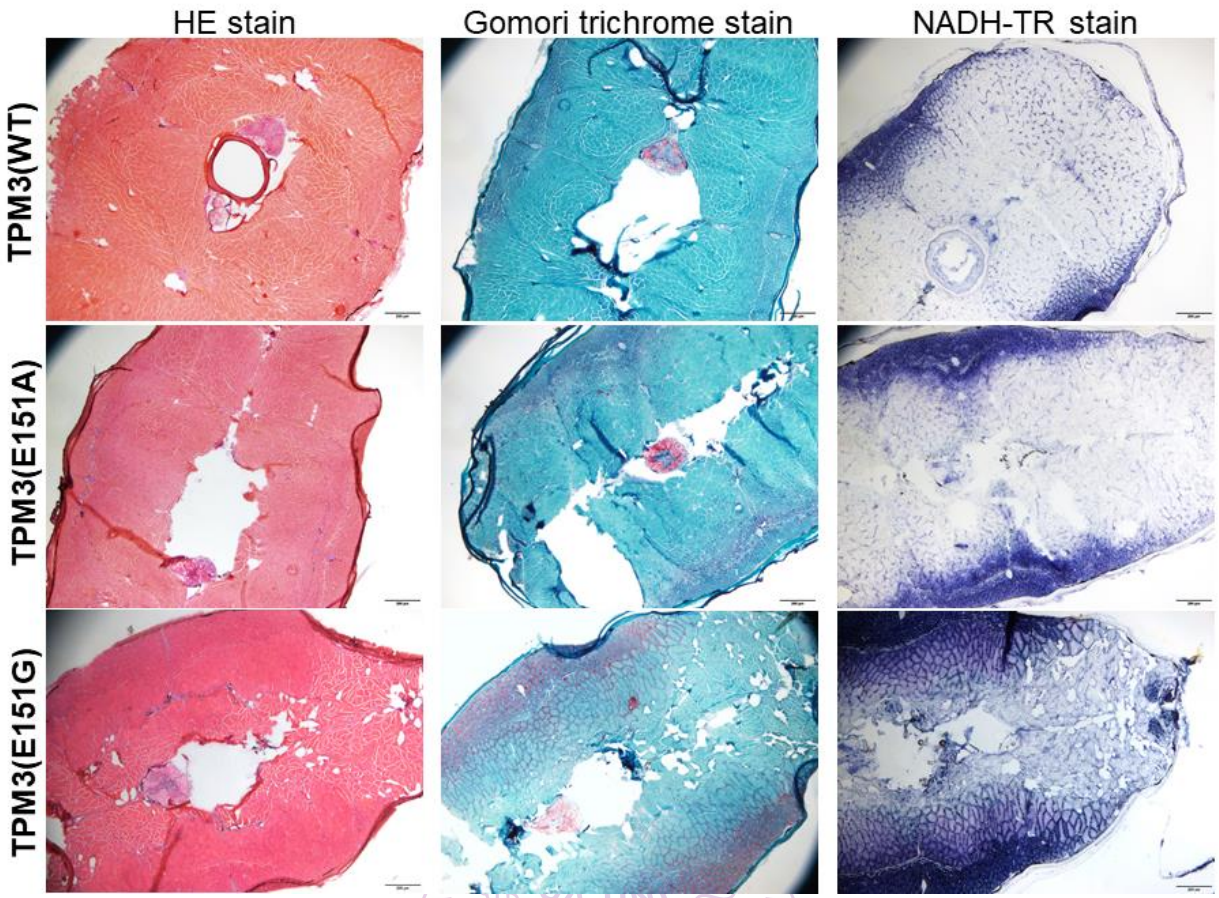


Figure 12. 50X histopathological of F1 adult TPM3 transgenic zebrafish

We used HE stain, Gomori trichrome stain and NADH-TR stain three different staining for diagnosis the histopathological of adult TPM3 transgenic zebrafish.

Figure 13. 400X histopathology of F1 adult TPM3 transgenic zebrafish

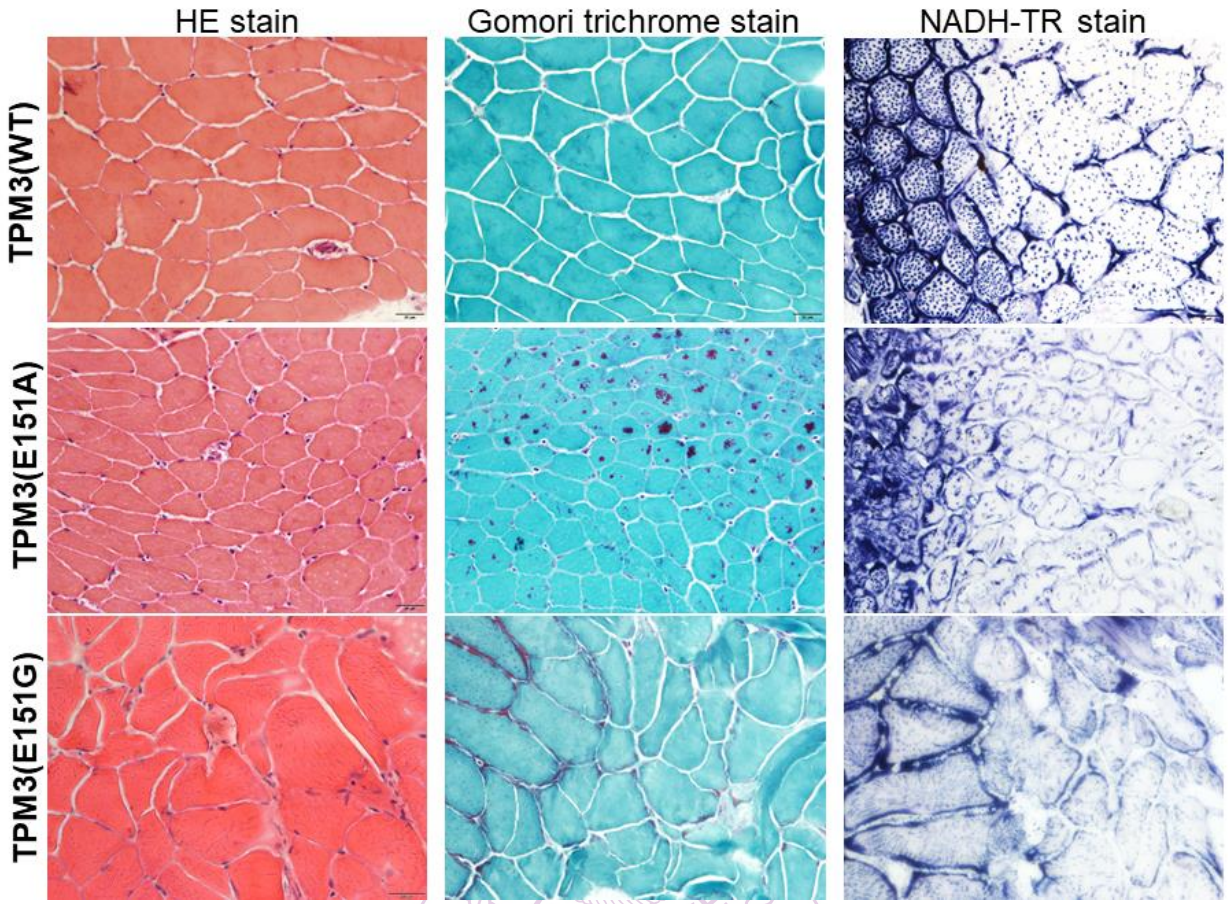


Figure 13. 400X histopathological of F1 adult TPM3 transgenic zebrafish

We used HE stain, Gomori trichrome stain and NADH-TR stain three different staining for diagnosis the histopathological of adult TPM3 transgenic zebrafish.

Figure 14. Chemicals treatment on F3 larvae TPM3 transgenic zebrafish

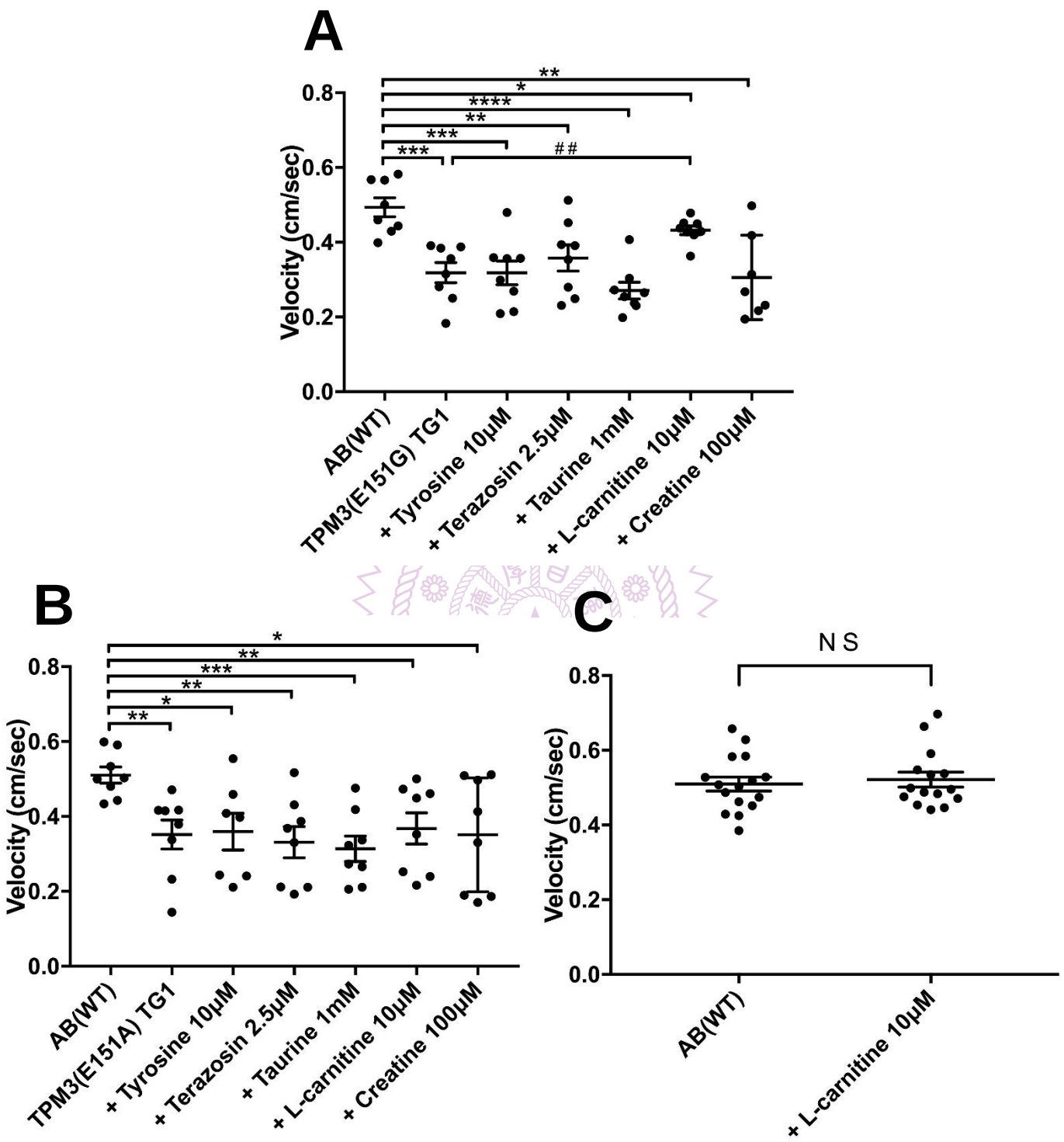


Figure 14. Chemicals treatment on F3 larvae TPM3 transgenic zebrafish

After chemicals treatment, larvae swimming behavior test at 7 dpf. (A) Five different chemicals treated F3 larvae TPM3(E151G) fish (n=8). (B) Five different chemicals treated F3 larvae TPM3(E151A) fish (n=8). (C) Larvae AB(WT) fish with and without L-carnitine treatment (n=16).

Figure 15. Chemicals treatment on F2 adult TPM3 transgenic zebrafish

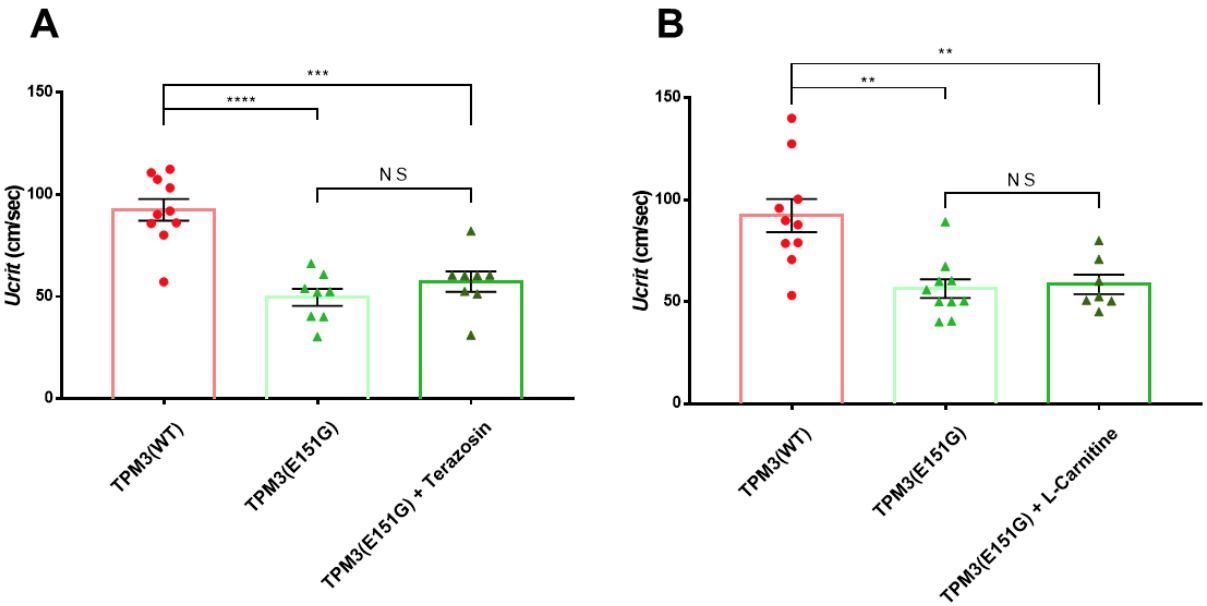


Figure 15. Chemicals treatment on F2 adult TPM3 transgenic zebrafish

Chemicals treatment on adult TPM3 fish measured the critical swimming speed (*Ucrit*) by swim tunnel. (A) Red plot was TPM3(WT) (n=10), light green plot was TPM(E151G) control group (n=8), dark green plot was TPM(E151G) treated with Terazosin (n=8). (B) Red plot was TPM3(WT) (n=10), light green plot was TPM(E151G) control group (n=8), dark green plot was TPM(E151G) treated with L-carnitine (n=8).

Figure 16. Principal component analysis of chemicals treatment on F2 adult TPM3 transgenic zebrafish

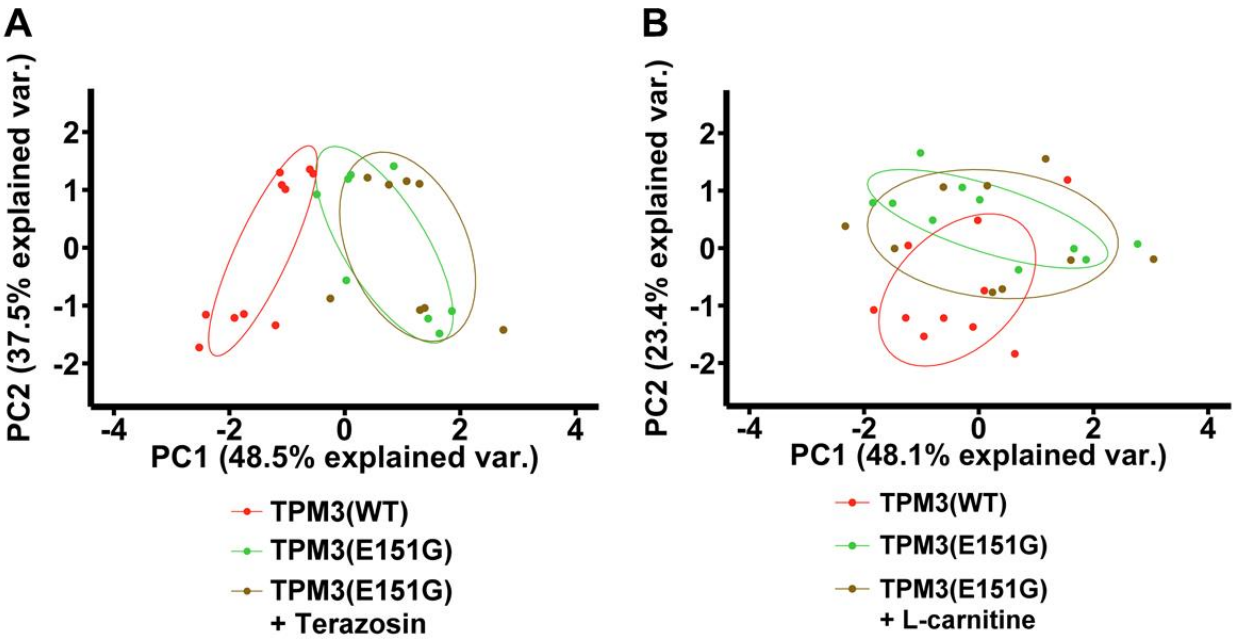


Figure 16. Principal component analysis of chemicals treatment on F2 adult TPM3 transgenic zebrafish

(A) The first principal component accumulated 48.5% and the second principal component accumulated 37.5%. Red plot was TPM3(WT) (n=10), green plot was TPM3(E151G) control group (n=8), brown plot was TPM3(E151G) treated with Terazosin (n=8). (B) The first principal component accumulated 48.1% and the second principal component accumulated 23.4%. Red plot was TPM3(WT) (n=10), green plot was TPM3(E151G) control group (n=8), brown plot was TPM3(E151G) treated with L-carnitine (n=8).

Table 1. The primer information for TPM3 gene amplification

Target Name	Primer Name	Sequence (5' to 3')	Location	Accession Number	Size (bp)
TPM3	TPM3-F	ATGGCTGGGATCACCACC	131-148	NM_153649.3	747
	TPM3-R	CTACATCTCATTCAGGTCAAGCAG	854-877		

Table 2. The primer information for generated pME-TPM3

Primer Name	Sequence (5’ to 3’)	Size (bp)
attB1-TPM3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGGGATCACCACC	805
attB2-TPM3-R	GGGGACAAGTTTGTACAAGAAAGCTGGGTCTACATCTCATTCAGGTCAAGCAG	

Table 3. The primer information for confirm LR recombination reaction

Primer Name	Sequence (5’ to 3’)	Size (bp)
MLC2-F2	CCATCACTTTCCCCCTACCT	805
TPM3-R2	CGGTCCAGCTCTTCTTCAAC	
TPM3-F2	AGCCAAGCTGGAAAAGACAA	340
p3E-polyAR	CCCCCTGAACCTGAAACATA	
MLC2-F2	CCATCACTTTCCCCCTACCT	1172
p3E-polyAR	CCCCCTGAACCTGAAACATA	

Table 4. The primer information for site-directed mutagenesis

Primer Name	Sequence (5’ to 3’)
TPM3 A452S-F	CTGAGCTGGCAGSGTCCCGTTGCCG
TPM3 A452S-R	CGGCAACGGGACSCTGCCAGCTCAG
TPM3 A452G-F	CTGAGCTGGCAGGGTCCCGTTGCCG
TPM3 A452G-R	CGGCAACGGGACCCTGCCAGCTCAG

Table 5. The primer information for confirm TPM3 transgenic zebrafish

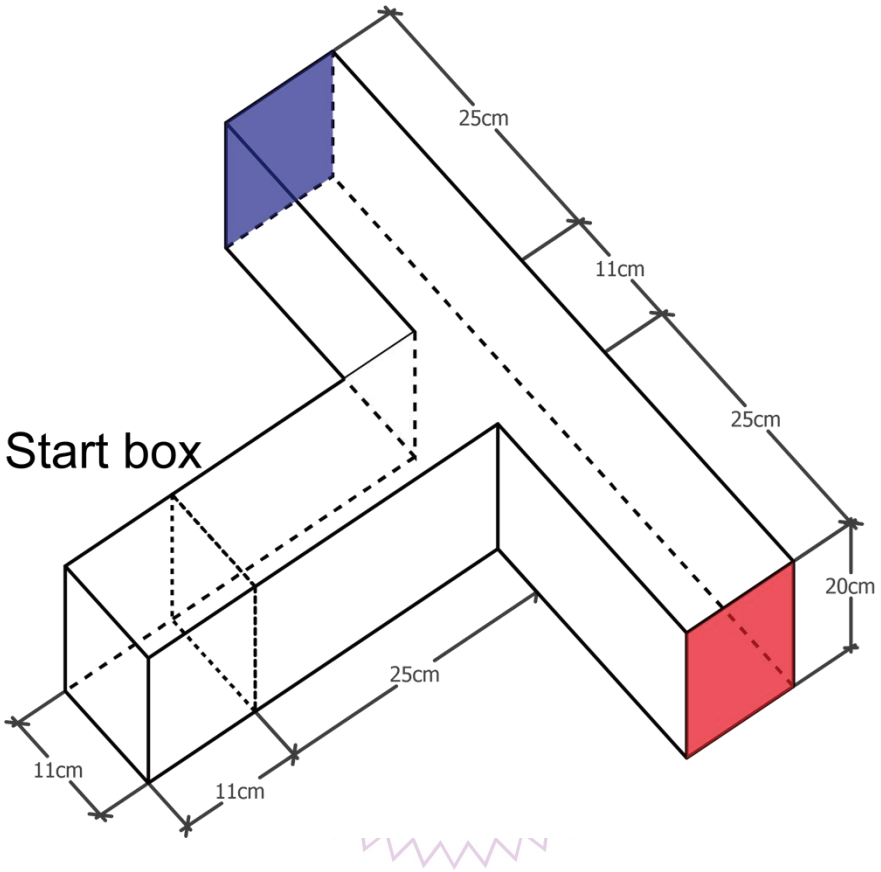
Primer Name	Sequence (5’ to 3’)	Size (bp)
TPM3-NF	GATGAAGAAAAGATGGAACTCCAGG	447
TPM3-R	CTACATCTCATTCAGGTCAAGCAG	

Table 6. The primer information for QPCR analysis in zebrafish

Target Name	Primer Name	Sequence (5' to 3')	Location	Accession Number	Size (bp)
TPM3	Q-TPM3-F	TAGACTGATGGACCAGAAC	613-631	NM_153649.3	199
	Q-TPM3-R	GGTGCATTTCAGTTTATCTTC	791-811		

Supplementary Information

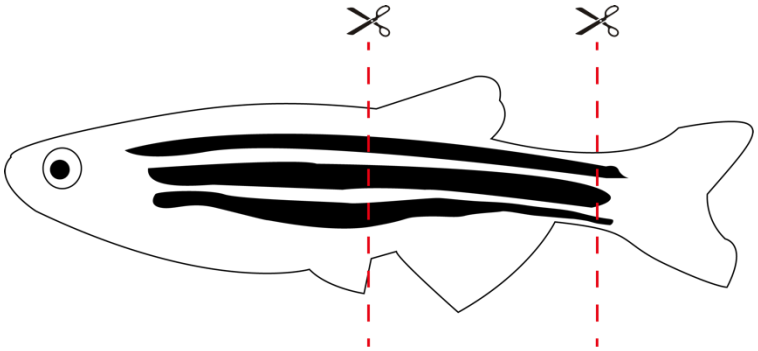
Supplementary Figure 1. A three-armed T-maze apparatus for adult fish behavior test



Supplementary Figure 1. A three-armed T-maze apparatus for adult fish behavior test

The fish was placed in the start box. We put blue cellophane in the left arm, red cellophane in the right arm.

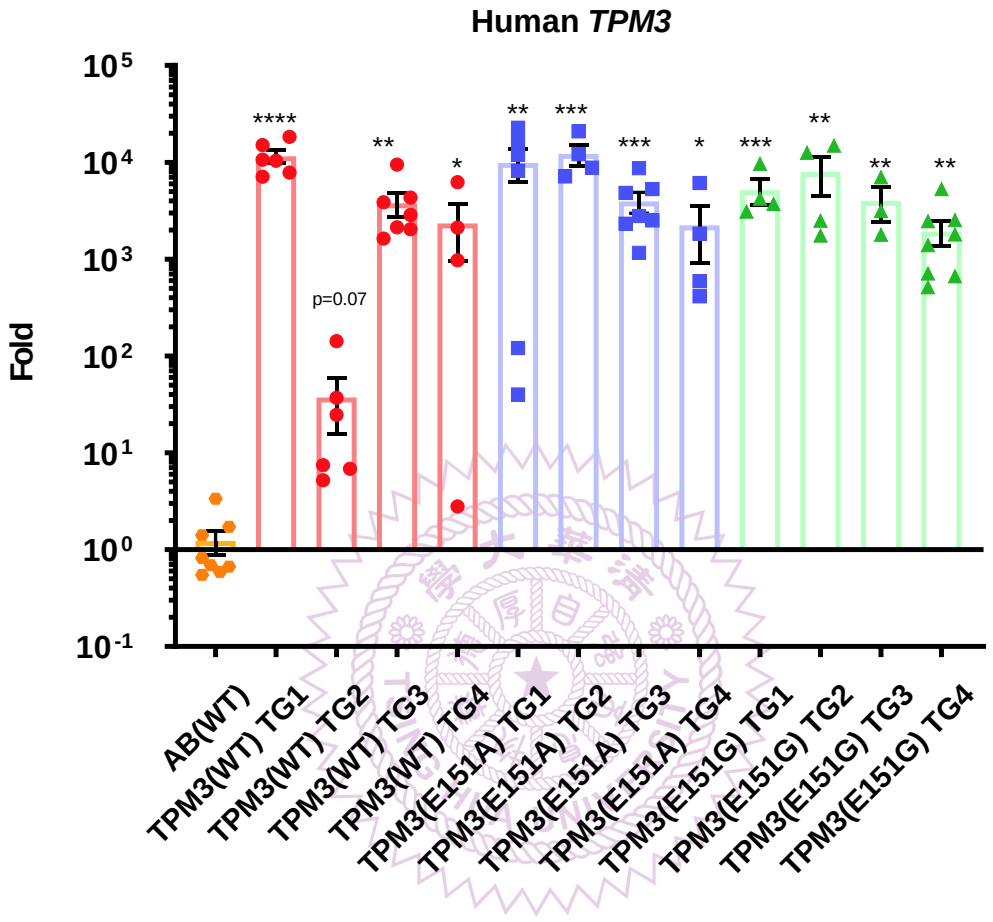
Supplementary Figure 2. Preparing the muscle tissue of zebrafish for frozen section



Supplementary Figure 2. Preparing the muscle tissue of zebrafish for frozen section

Once zebrafish was anesthetized, cut the muscle tissue between anal and caudal fins. This region contains mostly myotome and avoid the hollow regions that were difficult to section.

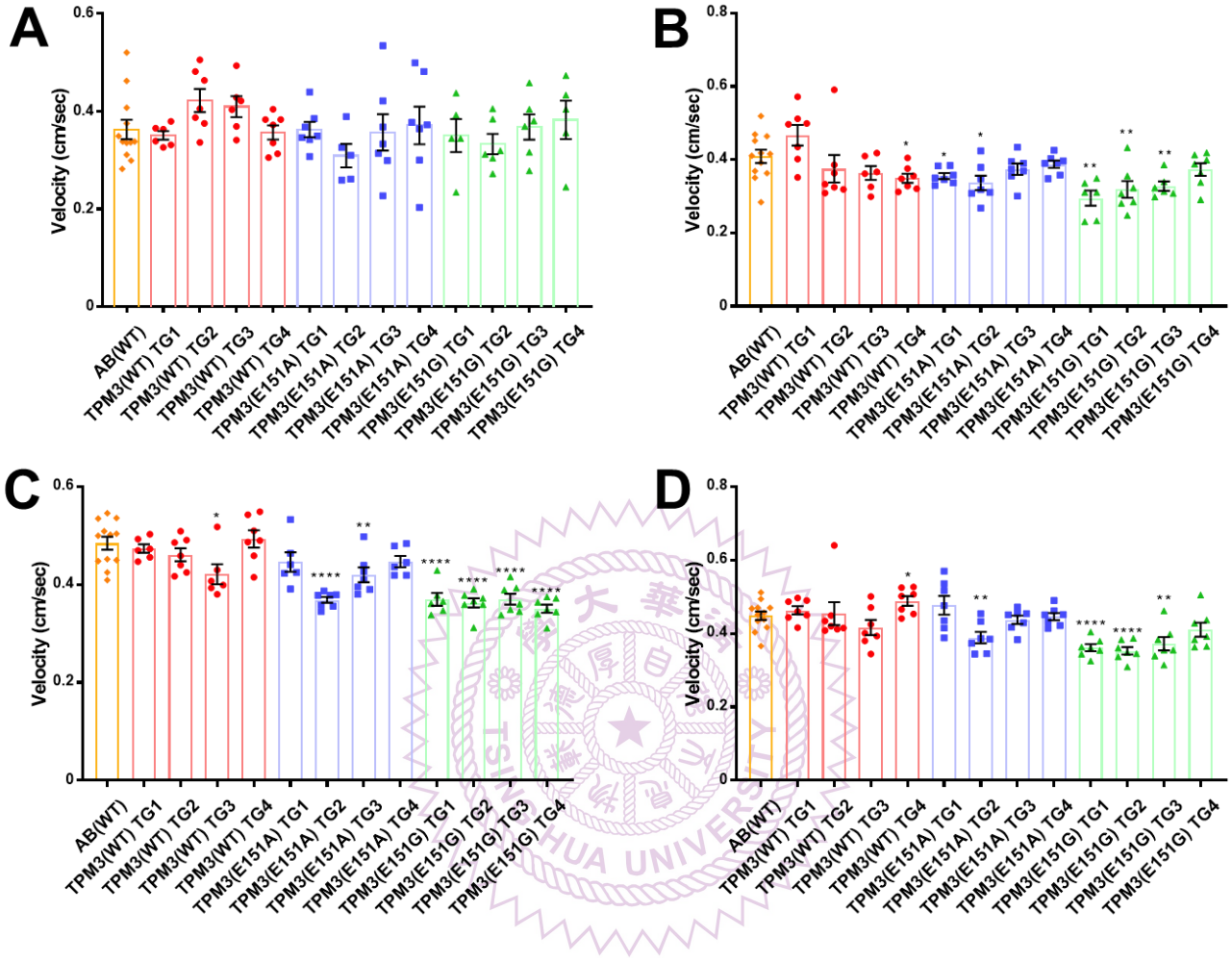
Supplementary Figure 3. Human TPM3 gene expression level of F1 adult TPM3 transgenic zebrafish



Supplementary Figure 3. Human TPM3 gene expression level of F1 adult TPM3 transgenic zebrafish

Orange plot indicated AB(WT) fish, red plot denoted TPM3(WT), blue plot represented TPM3(E151A), green plot indicated TPM3(E151G).

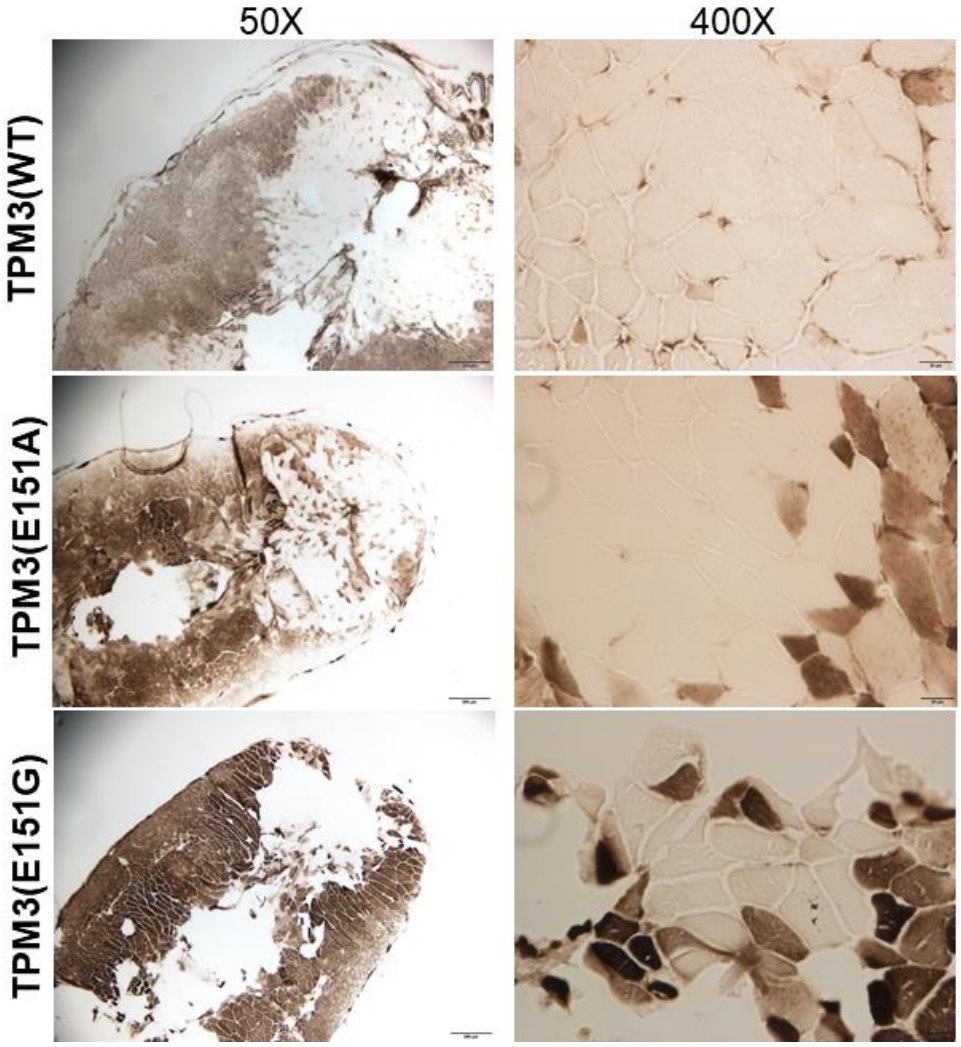
Supplementary Figure 4. The swimming velocity of larvae F1 TPM3 transgenic zebrafish at 3 dpf to 6 dpf



Supplementary Figure 4. The swimming velocity of larvae F1 TPM3 transgenic zebrafish at 3dpi to 6 dpi

Larvae fish swimming velocity was measured by DanioVision. Orange plot indicated AB(WT) (n=12), red plot denoted four independent lines of TPM3(WT) (n=8 for each line), blue plot represented four independent lines of TPM3(E151A) (n=8 for each line), green plot indicated four independent lines of TPM3(E151G) (n=8 for each line). (A) Swimming behavior test for 3 dpf larva. (B) Swimming behavior test for 4 dpf larva. (C) Swimming behavior test for 5 dpf larva (D) Swimming behavior test for 6 dpf larva.

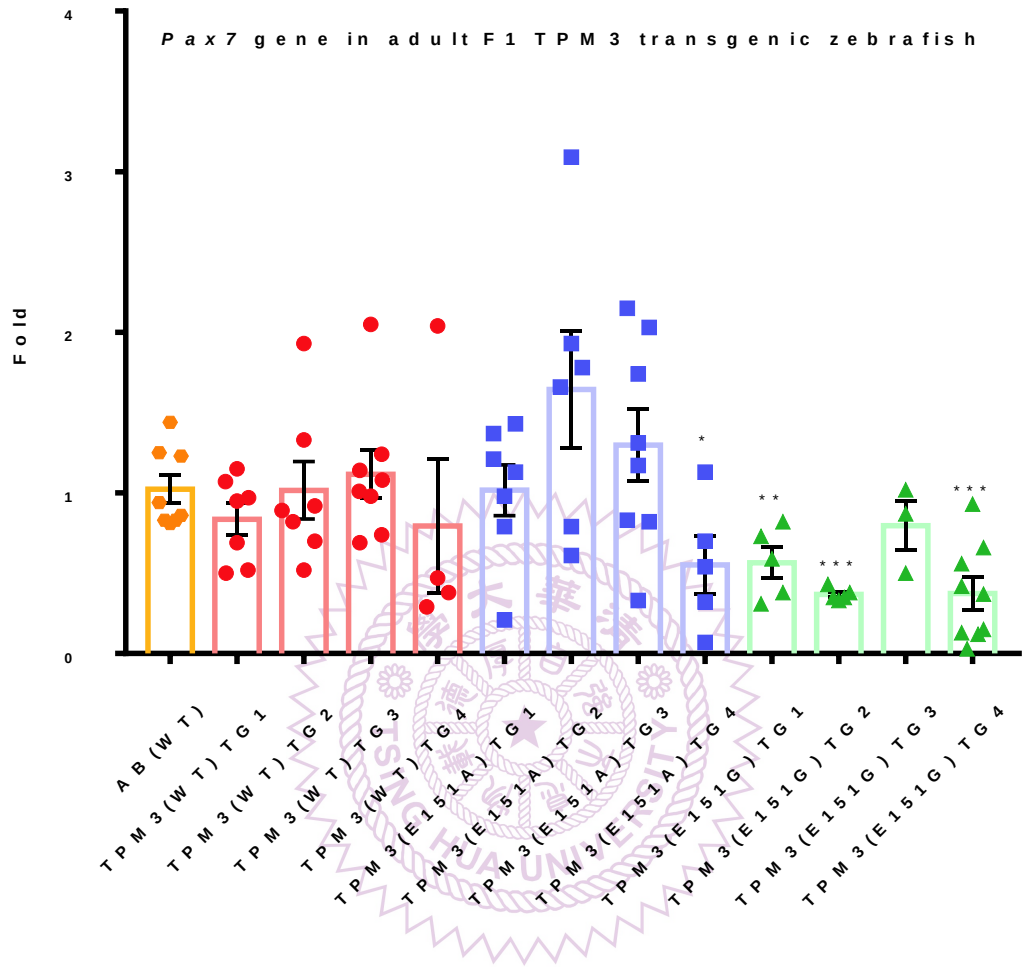
Supplementary Figure 5. ATPase 4.7 staining of F1 adult TPM3 transgenic zebrafish



Supplementary Figure 5. ATPase 4.7 staining of F1 adult TPM3 transgenic zebrafish

ATPase stain (pH=4.7) were used for diagnosis the histopathological of adult TPM3 transgenic zebrafish. Representative images of 50X and 400X of ATPase staining result were shown.

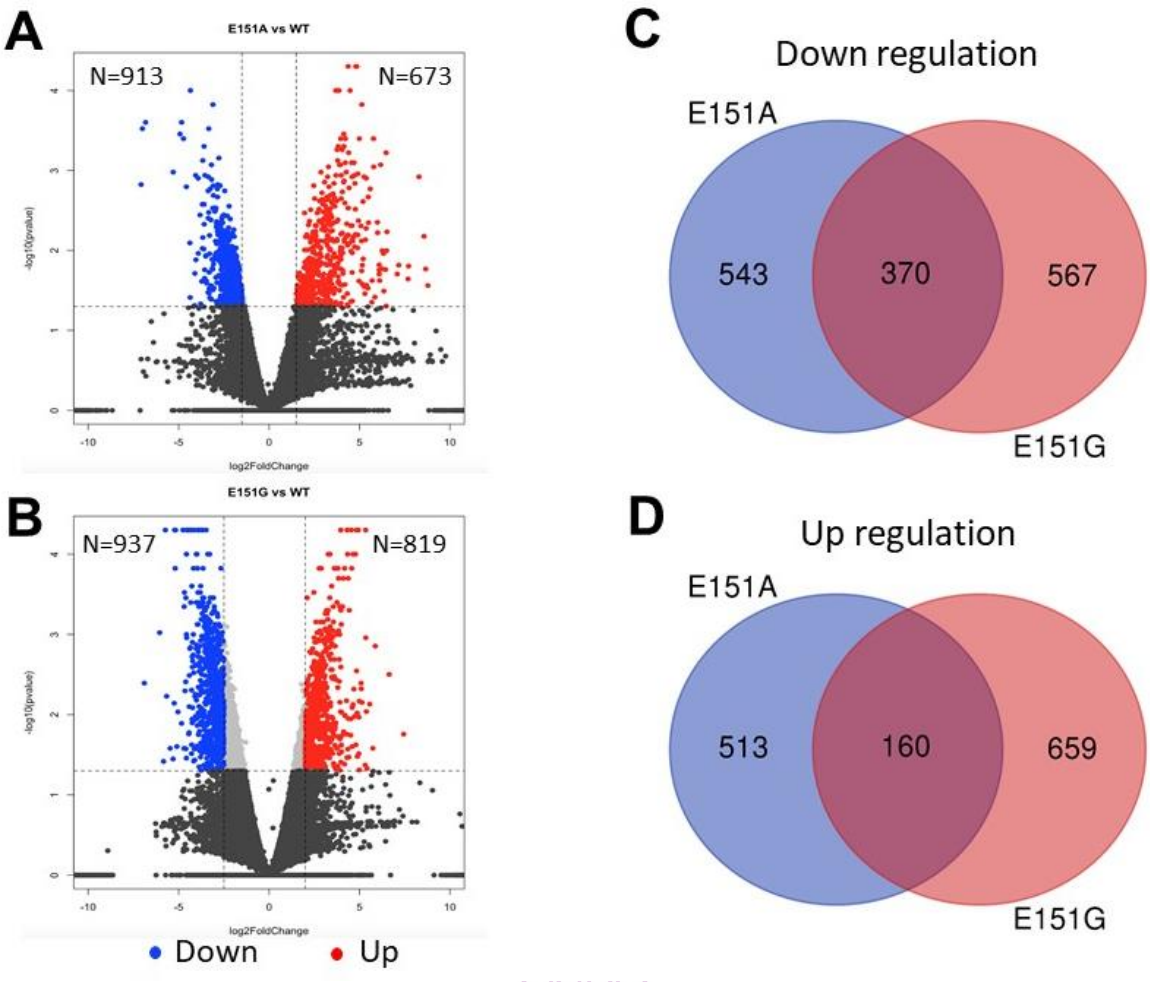
Supplementary Figure 6. Pax7 gene expression level of F1 adult TPM3 transgenic zebrafish



Supplementary Figure 6. Pax7 gene expression level of F1 adult TPM3 transgenic zebrafish

Orange plot indicated AB(WT) fish, red plot denoted TPM3(WT), blue plot represented TPM3(E151A), green plot indicated TPM3(E151G).

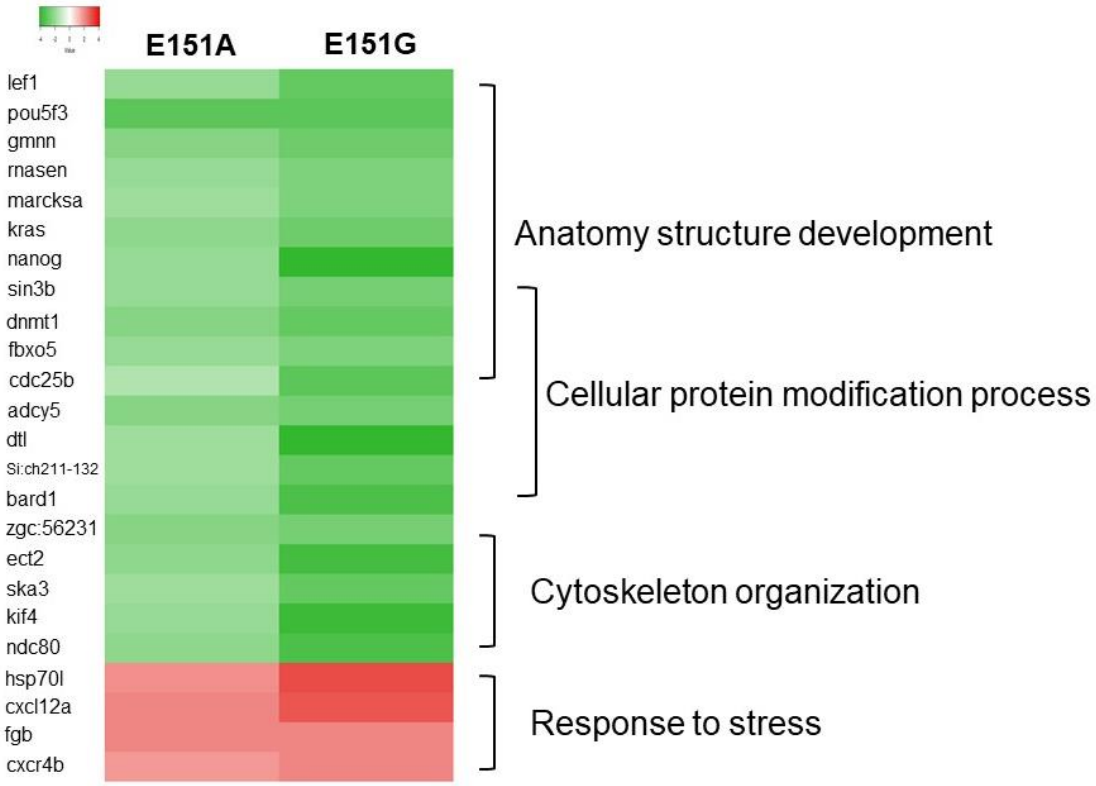
Supplementary Figure 7. Volcano plot and Venn diagrams of TPM3 transgenic zebrafish skeletal muscle by RNA-seq



Supplementary Figure 7. Volcano plot and Venn diagrams of TPM3 transgenic zebrafish skeletal muscle by RNA-seq

(A) Compared with TPM3(WT), TPM3(E151A) transgenic zebrafish had 913 genes down regulation and 673 genes up regulation. (B) Compared with TPM3(WT), TPM3(E151G) transgenic zebrafish had 937 genes down regulation and 819 genes up regulation. (C) TPM3(E151A) and TPM3(E151G) had 370 genes down regulation at a same time. (D) TPM3(E151A) and TPM3(E151G) had 160 genes up regulation at a same time.

Supplementary Figure 8. Heatmap of TPM3 transgenic zebrafish skeletal muscle by RNA-seq



Supplementary Figure 8. Heatmap of TPM3 transgenic zebrafish skeletal muscle by RNA-seq

In the heatmap, green color indicated the gene down regulation, red color indicated the gene up regulation.

Supplementary Data 1. Cloning of Tg(MLC2:TPM3(WT);myl7:EGFP) transgenic zebrafish

M13-R:AGGAAACAGCTATGACCATGA
BS1:AACAAAAGCTGGAGCTCCACCG
BamHI(GGATCC): single cut
attB1-TPM3-F:**GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGGGATCACCACC**
attB2-TPM3-R:**GGGGACCACTTTGTACAAGAAAGCTGGGTCTACATCTCATTCAGGTCAAGCAG**
TPM3-F:**ATGGCTGGGATCACCACC**
TPM3-R:**CTACATCTCATTCAGGTCAAGCAG**
MLC2_F2:CCATCACTTTCCCCCTACCT
TPM3_R2:CGGTCCAGCTCTTCTTCAAC
TPM3_F2:AGCCAAGCTGGAAAAGACAA
p3E-polyAR:CCCCCTGAACCTGAAACATA

>pTOl2-MLC2:TPM3(WT):pA/CG2
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Supplementary Data 2. Cloning of Tg(MLC2:TPM3(E151A);myl7:EGFP) transgenic zebrafish

M13-R:AGGAAACAGCTATGACCATGA
BS1:AACAAAAGCTGGAGCTCCACCG
BamHI(GGATCC): single cut
attB1-TPM3-F:**GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGGGATCACCACC**
attB2-TPM3-R:**GGGGACCACTTTGTACAAGAAAGCTGGGTCTACATCTCATTCAGGTCAAGCAG**
TPM3-F:**ATGGCTGGGATCACCACC**
TPM3-R:**CTACATCTCATTCAGGTCAAGCAG**
MLC2_F2:CCATCACTTTCCCCCTACCT
TPM3_R2:CGGTCCAGCTCTTCTTCAAC
TPM3_F2:AGCCAAGCTGGAAAAGACAA
p3E-polyAR:CCCCCTGAACCTGAAACATA

>pTOl2-MLC2:TPM3(WT):pA/CG2
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GAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGTGG

Supplementary Data 3. Cloning of Tg(MLC2:TPM3(E151G);myl7:EGFP) transgenic zebrafish

M13-R:AGGAAACAGCTATGACCATGA
BS1:AACAAAAGCTGGAGCTCCACCG
BamHI(GGATCC): single cut
attB1-TPM3-F:**GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGGGATCACCACC**
attB2-TPM3-R:**GGGGACCACTTTGTACAAGAAAGCTGGGTCTACATCTCATTCAGGTCAAGCAG**
TPM3-F:**ATGGCTGGGATCACCACC**
TPM3-R:**CTACATCTCATTCAGGTCAAGCAG**
MLC2_F2:CCATCACTTTCCCCCTACCT
TPM3_R2:CGGTCCAGCTCTTCTTCAAC
TPM3_F2:AGCCAAGCTGGAAAAGACAA
p3E-polyAR:CCCCCTGAACCTGAAACATA

>pTOl2-MLC2:TPM3(WT):pA/CG2
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Honors & Awards

- 2019 Honorable Mention Award of Poster Contest, College of Life Science in National Tsing Hua University

- 2019 First place of Poster Presentation, The 11th Asia-Pacific Forum of Medical Laboratory Sciences

- 2019 Outstanding Award of Poster Presentation, The 34th Joint Annual Conference of Biomedical Sciences

- 2018 Excellence of Flash Talk Presentation, Annual Taiwan Zebrafish Symposium

- 2018 第 20 屆罕見疾病博碩士論文獎助學金

- 2018 Outstanding Award of Poster Presentation, NHRI-NTHU Joint Research Conference