Cooperation of Mtmr8 with PI3K Regulates Actin Filament Modeling and Muscle Development in Zebrafish

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Abstract

Background: It has been shown that mutations in at least four myotubularin family genes (MTM1, MTMR1, 2 and 13) are causative for human neuromuscular disorders. However, the pathway and regulative mechanism remain unknown.

Methodology/Principal Findings: Here, we reported a new role for Mtmr8 in neuromuscular development of zebrafish. Firstly, we cloned and characterized zebrafish Mtmr8, and revealed the expression pattern predominantly in the eye field and somites during early somitogenesis. Using morpholino knockdown, then, we observed that loss-of-function of Mtmr8 led to defects in somitogenesis. Subsequently, the possible underlying mechanism and signal pathway were examined. We first checked the Akt phosphorylation, and observed an increase of Akt phosphorylation in the morphant embryos. Furthermore, we studied the PH/G domain function within Mtmr8. Although the PH/G domain deletion by itself did not result in embryonic defect, addition of PI3K inhibitor LY294002 did give a defective phenotype in the PH/G deletion morphants, indicating that the PH/G domain was essential for Mtmr8's function. Moreover, we investigated the cooperation of Mtmr8 with PI3K in actin filament modeling and muscle development, and found that both Mtmr8-MO1 and Mtmr8-MO2+LY294002 led to the disorganization of the actin cytoskeleton. In addition, we revealed a possible participation of Mtmr8 in the Hedgehog pathway, and cell transplantation experiments showed that Mtmr8 worked in a non-cell autonomous manner in actin modeling.

Conclusion/Significance: The above data indicate that a conserved functional cooperation of Mtmr8 with PI3K regulates actin filament modeling and muscle development in zebrafish, and reveal a possible participation of Mtmr8 in the Hedgehog pathway. Therefore, this work provides a new clue to study the physiological function of MTM family members.

Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome ten) and MTM (myotubularin myopathy) family factors are members of the growing class of dual-specificity phosphatases (DSPs), which can dephosphorylate the products of phosphoinositide 3-kinase (PI3K) and antagonize downstream effectors using 3-phosphoinositides as ligands [1,2]. They have been known to contribute to diverse processes that include cellular adhesion, signal transduction, and cell-cycle regulation[3]. Myotubularin-related (MTMR) phosphatases display a conserved active site motif CX5R, and an invariant sequence ‘CSDGWDR’ exists commonly in all the enzymatically active members[4–6]. Eight active members, including MTMR8, have been found in the MTM family. Several studies have further demonstrated that the PH/G domain functions to localize MTM to different subcellular compartments in the cell[7–9], and its deletion leads to activity loss of MTMR3 in vitro[10].

Mutations or altered expression of PTEN or MTM family members have been observed in human cancers[11–13] and in genetic developmental disorders[14,15]. Recent studies using RNA interference have revealed an unexpected role for several MTMs (including MTMR8) in promoting cell proliferation and survival [16,17]. MTM1 is mutated in individuals with X-linked recessive myotubular myopathy [14]. MTM1 is adjacent to MTMR1 on the X chromosome, which plays a role in muscle formation and represents abnormal mRNA splicing in myotonic dystrophy [18]. Function loss of MTM2 and mutation of MTMR15 causes Charcot–Marie–Tooth disease type 4B1 and 4B2[17,19], a severe demyelinating neuropathy characterized by muscle weakness and sensory loss in the lower extremities beginning in early childhood. In addition, MTMR6 and MTMR9 were identified genetically to be required in C. elegans for endocytosis[20,21]. Recently, the cell-therapy strategies, which use cultured myoblasts[22] or stem cells, have had notable successes in dystrophic mouse models[23] and DMD muscle[24–26], because transplant-
ing whole cells have the potential to correct the symptoms of disease although each has its inherent disadvantages[27].

PI3K is conserved across eukaryotic organisms and regulates many facets of pathways involving cellular growth, survival, metabolism, vehicle trafficking, and chemotaxis[20]. The pathways controlling random movement parallel those of the amplification step of chemotaxis that is controlled through a regulatory loop containing Ras, PI3K, PTEN, and F-actin[29]. The initial activation of Ras and PI3K is independent of F-actin polymerization. However, F-actin polymerization is essential for amplifying the signal and stabilizing the leading edge in neutrophils and Dictyostelium [30,31]. In Dictyostelium, F-actin recruits additional PI3K to the newly forming leading edge, enhancing the PI3P response and downstream effector function [29,30]. Cells with decreased PI3K activity exhibit a decrease in the second peak of RacB activation and F-actin polymerization, which has been linked to pseudopod extension.

To reveal the biological functions of MTM family members, we identified Mmr8 from the model animal zebrafish. Based on its expression pattern predominantly in the eye field and somites during early somitogenesis, we analyzed its physiological roles in embryo development by morpholino-mediated knockdown. We firstly showed that loss-of-function of Mmr8 led to defects in somitogenesis, and further examined the possible underlying mechanism and the PI3K domain function. Moreover, we investigated the cooperation of Mtmr8 with PI3K in actin filament modeling and muscle development, and revealed a possible participation of Mtmr8 in the Hedgehog pathway. The findings revealed a new role of Mmr8 and its functional mechanism in neuromuscular development.

Results

Molecular characterization and expression pattern of Mtmr8 in zebrafish embryos

The complete ORF for the Mtmr8 encodes polypeptides of 632 amino acids, which contains 14 exons and 13 introns (identical to human MTMR8). An amino acid sequence alignment of zebrafish, chicken and human Mtmr8 polypeptides is shown in Fig. 1A. Zebrafish Mtmr8 exhibit 63% and 64% identity, and 80% and 78% similarity, with human and chicken Mtmr8 respectively, and higher identities exist in the Myotub-related and PTPc_DSPc motifs (amino acids 155–263 and 264–432 of zebrafish Mtmr8). The high homology implies that the zebrafish Mtmr8 may have the same functions as in human.

Whole-mount in situ hybridization was used to analyze the expression pattern of Mtmr8 during zebrafish embryogenesis. The expression distribution of Mtmr8 was same to the result reported by Thise B and C [32]. As shown in Fig. 1B, Mtmr8 mRNA is expressed in prechordal plate and eye field at 50% epiboly (Fig. 1B-a). Between 1–13 somites, Mtmr8 transcript becomes restricted to eye field and somites (Fig 1B-b). At 19 somites, Mtmr8 expression is shown in the eye, telencephalon and ventral mesoderm (Fig 1B-c). At 24hpf, Mtmr8 is expressed predominantly in the eye and vasculature (Fig 1B-d). Later, Mtmr8 is expressed in the vasculature at 48hpf (Fig 1B-e).

Targeted knockdown of zebrafish Mtmr8 impaired embryo development

To determine the physiological effect of Mtmr8, we undertook loss-of-function experiments in zebrafish by using morpholinos. Firstly, we designed a splice junction morpholino targeted against the first coding exon-intron boundary to evaluate the knockdown effect and efficacy in vivo. As shown in Fig. 2A and Fig. 2B, the exon-intron morpholino (MO1) introduces intron 1 (about 5 kb) into the altered transcript, and includes a premature termination codon. Because the altered transcript is more than 5kb in its sequence, it could not be amplified from the morpholino-injected embryos in one minute by RT-PCR (Fig 2C). In the control embryos, the amplified PCR product is 310 bp by the same primers designed from the first and third exons (Fig 2C). And, the altered transcript sequence was further amplified and verified by bi-directional DNA sequencing. Microscope observation indicated that the morpholino-mediated Mtmr8 knockdown resulted in dramatic phenotypic abnormalities in somitogenesis (Fig 2D). At 24hpf, control experiments in which embryos were injected with Cont morpholino did not alter the wild-type phenotype throughout zebrafish development (Fig 2D-a), whereas the Mtmr8-MO1 morphants exhibited dose-dependent effect on embryos and resulted in mild and severe phenotype defects. In mild defects, morpholino-injected embryos showed delayed development, shorter and curled trunk and tail compared to wild-type or to control MO-injected embryos (Fig 2D-b). In severe defects, the morphants were marked by small heads, abnormal tail fins, and U-shaped somites (Fig. 2D-c). Fig. 2E shows the percentages of normal, mild and severe defect embryos under different injection doses of morpholino. Moreover, an increasing percentage of normal or mild defect embryos was observed when the embryos were co-injected with 100 pg capped Mtmr8 RNA and 6 ng Mtmr8-MO1 (Fig 2E). However, the defects could not be reduced when co-injected with 100 pg of capped GFP RNA and Mtmr8-MO1 (Data not shown). In addition, overexpression of zebrafish Mtmr8 by injection of capped RNA (100 pg) did not cause a visible phenotype. We used 100 pg doses in all the gain-of-function experiments described below if not indicated otherwise.

Role of Mtmr8 in regulating PI3K/Akt signaling pathway

Recent studies indicated that several MTMs might control PI3K/Akt activation by virtue of its ability to dephosphorylate PIP3[33]. However, there was no any direct report about the PI3P lipid phosphatase activity of Mtmr8. Because knockdown of Mtmr8 expression gave distinct developmental phenotypes, we asked whether the PI3P lipid phosphatase activity of Mtmr8 was conserved in the development of zebrafish. To confirm it, we compared the level of phosphorylated Akt (pAkt) to total Akt (Akt) in whole fish lysates prepared from wild type zebrafish and Mtmr8 morphant embryos. As shown in Fig 3, knockdown of Mtmr8 expression produces a significant increase in the relative amount of pAkt compared to Cont MO injected embryos, whereas the elevated level of p-Akt could be reduced by coinjection with Mtmr8 mRNA. These results indicate that zebrafish Mtmr8 exhibits PI3P lipid phosphatase activity and functions to negatively regulate the PI3-Kinase/AKT pathway.

Mtmr8 PH/G domain plays a critical role with PI3K in regulating zebrafish embryogenesis

PH/G domain plays an important role in the activity of MTMR family [10]. To delete the PH/G domain of zebrafish Mtmr8, we designed a splice junction morpholino targeted against the second coding exon-intron boundary. In the Mtmr8-MO2 morphants, the targeted exon was eliminated, which encodes partial of the PH/G domain, as shown by a smaller PCR transcript which was verified by bi-directional DNA sequencing (Fig 4A). However, unlike Mtmr8-MO1 morphants (Fig 4B-a), the Mtmr8-MO2 morphants display no obvious defects, except mild abnormal in the head (Fig 4B-b). Intriguingly, previous studies showed that the defects resulted by homozygous single or double mutants of pten+/− and pten−/−, were rescued by treatment with the phosphatidylinos-
Figure 1. Sequence comparison and expression pattern of the deduced zebrafish Mtmr8. (A) Amino acid alignment of Mtmr8 between zebrafish with human and chicken. Similar and identical amino acids are highlighted in grey and black boxes. And the percentages of identities and similarities in Mtmr8 were shown compared zebrafish with others. Arrowheads indicate the location of introns and are flanked by the corresponding exon numbers. The region encompassing the Myotub-related and PTPc_DSPc domain is underlined in grey and black lines. The rectangular box indicates the CX5R active site motif of enzymatically active members in the MTM family. (B) Expression pattern of zebrafish Mtmr8. Whole-mount RNA in situ hybridization were performed using a Mtmr8 specific antisense riboprobe on embryos at the indicated stages. The arrows indicate the signals in the anterior and head. Embryos in panels are lateral view with the animal pole toward the top, and the right picture of panel b is dorsal view. The embryos in other panels are lateral views, with dorsal toward the top and anterior to the left. All scale bars are 100 μm.

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Mtmr8 in Muscle Development

**A**

![Diagram showing gene splicing](image)

**B**

WT zebrafish *Mtmr8*

MEHIITPK VENVKLNRYTEKKSALGTLYLT
ATHLIVYEQTSNTRKEAW………QESS *

*Mtmr8*-MO1 mediated mis-splicing

MEHIITPK VGLSQQVFSCKVGRSLFIGEMSIN *

**C**

![Image of gene expression analysis](image)

**D**

- WT control, 24 hpf
- Mild morphant, 24 hpf
- Severe morphant, 24 hpf

**E**

![Bar chart showing percentage](image)
Mtmr8 in Muscle Development

Figure 2. Targeted knockdown of Mtmr8 using splicing morpholino in zebrafish embryos. (A) Diagram of splicing junction morpholino targeted against Mtmr8 exon-intron boundary. (B) Amino acid sequences of wild type Mtmr8 and Mtmr8-MO1 mediated mis-splicing Mtmr8. Sequencing of the RT-PCR products revealed the mis-splicing transcript leading to a premature stop (asterisk) and causing a truncation in the protein (exon 1 in bold, the other exons in plain, and intron in plain and underlined). (C) RT-PCR detection of Mtmr8 transcript at 24hpf in WT and Mtmr8-MO1 (6 ng) morpholino-injected embryos, comparing cryptic spliced transcript in the morpholino injected embryos to the cDNA PCR products. (D) Live morphology of WT control zebrafish embryo (a) and Mtmr8-MO1 knockdown embryo (b, c) at 24hpf. Injection volume was about 2 nL at 1-cell stage embryos. All scale bars are 100 μm. (E) Statistical data of three independent experiments on Mtmr8 knockdown as well as its overexpression and Mtmr8 mRNA rescue. Results are represented as mean±SD of three separate experiments (60 embryos in each experiment).

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Mtmr8 cooperation with PI3K regulates actin filament modeling

Mtmr8 is a member of the growing class of dual-specificity phosphatases (DSPs) as PTEN, which controls cell random movement through the Ras, PI3K, PTEN, and F-actin regulatory loop [29]. To examine a possible requirement for Mtmr8 in integrity of the actin cytoskeleton, we used phalloidin-TRITC to stain filamentous actin in embryos at 24 hpf. In almost all the embryos treated with Mtmr8-MO1, we observed obvious defect of the actin cytoskeleton in a proportion of skeletal muscle fibres (Fig 5A-b), compared to the control (Fig 5A-a). However, when treated with Mtmr8-MO2, the actin cytoskeleton of Mtmr8-MO2 morphant was severely disturbed (Fig 5A-c).

Nexilin, a novel actin filament (F-actin)-binding protein, which was strongly expressed at somites at 24hpf [32]. We performed whole-mount in situ hybridization with zebrafish Nexilin riboprobes, and revealed that nexilin was significantly reduced and disorganized in the 24hpf Mtmr8-MO1 morphants (Fig 5B-b). However, the expression of nexilin was not obviously changed in Mtmr8-MO2 morphants (Fig 5B-c) and Cont MO morphants treated with LY294002 (Fig 5B-a). To further understand the function of Nexilin in integrity of the actin cytoskeleton, we performed morpholino to knockdown the zebrafish Nexilin. As shown by RT-PCR, in the control embryos, the amplified PCR product is 382 bp by primers designed from the first and second exons. However, in the Nexilin-MO morphants, because of inclusion of intron 1, the product is longer than 3 kb, which could not be amplified in one minute (Fig 5C). Embryos injected with the 3 ng Nexilin MO and stained with phalloidin, did show severe skeletal muscle detachments (Fig 5d-b,d), compared to the control (Fig 5d-a,c).

Mtmr8 is essential for the hedgehog pathway to regulate muscle development during embryogenesis

The Mtmr8 morphants have morphological traits in common with Hedgehog pathway mutants, such as U-shaped somite boundaries. To verify that the defects in Mtmr8 morphants are correlated with the changes in Hh signaling, we examined the expression of myod and patched1 (ptc1), a downstream target gene and receptor of Sonic hedgehog [35,36]. Compared with the control (Fig 6A-a,d), Myod and ptc1 expression is severely reduced and disorganized in Mtmr8 morphants (Fig 6A-b,c,e). Dominant negative PKA (dnPKA) mRNA, when injected into embryos, results in a broader expression of Hh target genes and a rescue of the Myod and ptc1 expression (Fig 6A-e,f). These results support the conclusion that Mtmr8 is required for Hedgehog signaling in zebrafish development.

To study the function of Mtmr8 in slow muscle development, the embryos were then stained with antibody F39, which detects mostly slow myofibrils, although it also reacts weakly with fast

Figure 3. Phosphatase activity of zebrafish Mtmr8. Different extracts from Cont MO embryos, Mtmr8 morphants and Mtmr8 morphants co-injected with Mtmr8 mRNA were prepared at 24 hpf (30 embryos/group) and subjected to Western blot detection with anti-Akt antibody or anti-phospho-Akt (pAkt) antibody. The corresponding histograms (right panels) plot the relative Ser(P)473-Akt to total Akt ratio (Relative p-Akt/Akt ratio), determined by band intensities that were analyzed by Scion software, with the ratio in experiment extracts normalized to the ratio determined in Cont MO injected embryo extracts. Results represent mean±SD of three separate experiments. *P<0.05.

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Figure 4. PH/G domain of Mtmr8 is required to cooperate with PI3K regulating embryo development and essential for the phosphatase activity of zebrafish Mtmr8 gene. (A) Splice junction morpholino targeted against Mtmr8 exon-intron boundary resulted to loss of PH/G domain. RT-PCR of Mtmr8 transcript at 24hpf in WT and Mtmr8-MO2 (6 ng) morpholino-injected embryos, comparing cryptic spliced transcript in the morpholino injected embryos to the cDNA PCR products. (B) Embryo phenotypes observed under the microscope. LY represents the PI3K.
Mtmr8 acts non-cell autonomously to F-actin modeling

Recent studies clearly showed that myotubularin specifically dephosphorylates phosphatidylinositol 3-monophosphate (PI3P). The action of myotubularin on PI3P levels may implicate two parallel pathways by acting both as a protein phosphatase decreasing PI3P level by down-regulating PI3K activity and, a lipid phosphatase directly degrading PI3P than PI4P in vivo[38]. Such a dual activity has also been suggested for PTEN[39]. Mutations in MTMR family proteins are associated with the human neuromuscular disorders X-linked myotubular myopathy (myotubularin and MTM1R) and type 4B Charcot-Marie-Tooth neuropathy (MTMR2 and MTMR13). These diseases arise from impaired development and/or maturation of skeletal muscle cells and myelinating Schwann cells, respectively. Although a significant body of evidence has linked MTMR to endocytic membrane trafficking events, their role in neuromuscular development is currently unclear. Our findings in zebrafish demonstrated that Mtmr8 is an essential component, together with PI3K, nexilin and F-actin, of the positive-feedback cycle that maintains the normal development of muscle. According to previous studies[40–42] and our current studies, a diagram is depicted to explain Mtmr8 function and the relationship between Mtmr8 and the hedgehog pathway (Fig 8). Until now, because of no available means to prevent myotubular myopathy, our findings for the first time provided a new clue to study MTM family related disease in vivo.

In this study, we presented evidence that the PH/G domain is indeed a function domain of Mtmr8 that contributes to the development of zebrafish. Deletion of the PH/G domain could result in increasing the relative amount of pAkt (Fig. 4C). Akt is activated by various growth factors and hormones such as IGF-1 and insulin. Activation occurs at the plasma membrane after PI3K-dependent generation of PI3P. Thereby, Akt is recruited to the plasma membrane via its PH (pleckstrin homology) domain and is activated by phosphorylation at Thr308 by PDK1 followed by phosphorylation at Ser473 by the mTOR complex[43]. Previous studies have demonstrated that the PH/G domain in a number of different MTMs bind phosphoinositides and also function to localize MTMs to different subcellular compartments in the cell[7–9]. All these indicated that Mtmr8 could inhibit PI3K/Akt activation through its functional PH/G domain, loss of which results in apoptotic signaling and cell death. Moreover, identifying the specific binding partners of the PH/G domains on the MTMs will provide important clues to the specific functions regulated by other MTMs as well as the mechanisms whereby loss of some MTMs lead to disease.

Recently studies demonstrated the existence of PI3K-dependent and -independent pathways for F-actin polymerization during chemoattractant-stimulated lamella extension in the human neutrophil. One pathway is dependent on PI3Kγ activation and downstream is dependent on PKCδ and Akt/PKB. This pathway controls the formation of 70% to 80% of the F-actin in the lamella region [44]. In vitro studies, activation of PI3K activity alone is sufficient to remodel actin filaments to increase cell migration through the activation of Akt and p70S6K1 in CEF cells [45]. However, in vivo, the F-actin polymerization and modeling is also Mtmr8-dependent. Although the PI3K/Akt was activated in Mtmr8 knocked embryos, it was not enough to model actin filaments alone. Nexilin is a F-actin binding protein and mediates cell motility, over-expression of which promoted cell migration and adhesion[46]. However, the mechanism of its function is not clear. In Mtmr8 deficient embryos, the expression of Nexilin was reduced, which induced the defect of F-actin modeling. Inhibition of PI3K with LY294002 did not alter the initial formation of these F-actin-rich cup structures at the plasma membrane but it did prevent Akt/PKB recruitment to these cups and their subsequent fusion into the large rings characteristic of normal[47]. However, the effects of inhibiting PI3K on the embryo cytoskeleton are not well characterized. Low dose of LY294002 didn’t obviously affect F-actin, whereas it severely disturbed the modeling of F-actin in Mtmr8 morphant and PH/G losing embryos. These indicated that the increasing of pAkt is a very important recovery mechanism for Mtmr8 deficient, although it could not completely rescue the defects.

The model about the mechanisms underlying the Hh signalling pathway is clearly shown by Masai et al.[41]. And Sonic hedgehog (Shh) has been reported to act as a mitogen and survival factor for muscle satellite cells. PI3K/Akt pathway is essential for Shh’s actions and directly involved in adult muscle cell proliferation and...
Figure 5. *Mtmr8* cooperates with PI3K regulating actin modeling. (A) Staining of embryos with phalloidin (F-actin) at 24hpf. (B) Nexilin mRNA expression at 24hpf. (C) RT-PCR of *Nexilin* transcript at 24hpf in WT and *Nexilin*-MO (3 ng) morpholino-injected embryos, comparing cryptic spliced transcript in the morpholino injected embryos to the cDNA PCR products. (D) The effects of *Nexilin* knockdown on embryo development (a, b) and F-actin (c, d). The pictures are representative of at least three experiments (30 embryos in each experiment). All scale bars are 100 μm.

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**Figure 6. The effect of Mtmr8 knockdown on hedgehog pathway and slow muscle development.** (A) The expression of myod and ptc1 at 24hpf in the Cont MO (a, d), Mtmr8-MO1 morphant (b, e) and it injected with dnPKA mRNA (c, f). (B) Whole-mount staining of 24 hpf embryo with F59 antibody (green) and Prox1 antibody (red) to reveal slow muscle cells. The pictures are representative of at least three experiments (30 embryos in each experiment). All scale bars are 100 μm.

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**Figure 7. Mtmr8 controls actin modeling non-cell-autonomously.** (A) Schematic depiction of cell transplant experiments. (B) Representative picture of a chimeric embryo at 24 hpf. The labeling cells were mostly located in somites of trunk and tail regions. (C) The effects of cell transplantation on F-actin.

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differentiation. Shh induces Akt phosphorylation in adult muscle cells and influences the PI3K/Akt pathways in a manner similar to IGF-I [48]. Although an increase in Akt phosphorylation could be detected in response to knockdown of Mtmr8, it was not enough to rescue the expression of Myod and Ptc1. Over-expression of dnPKA mRNA, a downstream of Shh, could reverse the defect induced by Mtmr8 deficient. Previous findings provide a basis for the synergistic role of PI3-kinase/Akt in Hh signaling in embryonic development and Hh-dependent tumors [49]. The PI3K inhibitor LY294002 inhibited migrating neo cells and was able to further inhibit residual dnPKA cell migration. PKA may play an important role in the signaling processes that lead to motility. Either inhibition or hyper-activation of PKA may inhibit cell migration, F-actin polymerization and synthesis [42,50–52].

Knockdown of Mtmr8 led to hyper-activation of PKA, which caused the reduction expression of Nexilin and F-actin. All these suggested that Mtmr8 and PI3K/Akt play a synergistic role in regulation of Hh signaling in embryonic muscle development.

Currently, many potential therapies are evaluated by introducing fluoscencently tagged cells into a diseased animal and then following their fate using fluorescence to determine if the introduced cells localize to muscle and participate in the repair process [27]. Mutation or knockdown of several genes in zebrafish have been shown to be the underlying basis of many muscular defects [53–56]. Cell transplantation experiments could confirm that the gene function is required cell-autonomously or non-cell-autonomously within the muscle development, which may be as a model to evaluate the possibility to use cell therapy for human genetic muscle disease during early fetus. Mtmr8 acts in non-cell-autonomous manner during embryos muscle development, which may suggest that cell therapy is not an efficient way to rescue the defect during early embryo development. The effectiveness of cell therapy could also be evaluated in human neuromuscular disorders caused by mutations in other myotubularin family genes.

From our studies, we have demonstrated that Mtmr8 have the same function as lipid phosphatas PTEN to dephosphorylate the PI3K products by down-regulating PI3K activity, and to regulate actin modeling and muscle development of zebrafish [57]. However, it is different from tumor suppressor gene, PTEN. Mtmr8 is not only an antagonist of PI3K, but also as a partner to balance the PI3K expression in embryo development regulation of zebrafish.

**Materials and Methods**

**Maintenance of zebrafish**

A breeding colony of zebrafish (Danio rerio) were maintained at 28.5 °C on a 14 h (hour) light/10 h dark cycle [58]. All embryos were collected by natural spawning and staged according to Kimmel et al. [59]. Kinase inhibitors, LY294002 were dissolved in DMSO at stock concentration of 50 mM, and then diluted to final concentration of 10 μM in embryo media from 10 hpf to 24 hpf, which had no obvious effect on embryos survival, activity and health. Control embryos were treated with the equivalent amount of DMSO solution.

**RT-PCR and Western blotting**

Total RNAs were isolated by SV RNA Isolation Reagent (Promega) from different stages and tissues, and the concentration and quality were determined by agarose electrophoresis and spectrophotometer. After treating with DNase I (RNase-free, Promega), the RNAs (about 1 μg) were reverse-transcribed with MMLV (Gibco) at 37°C with oligo(dT)15 primer. β-actin was used as internal control genes. All samples were analyzed in triplicates. Phosphatase activity of zebrafish Mtmr8 and Western blot detection were performed according to previous reports [60,61]. Images of blots were captured with a scanner, and quantitative densitometric analysis was performed using Scion Image.

**Antisense morpholino and mRNA microinjection**

Morpholinos were synthesized by GeneTools LLC (Philomath, OR). Following are the sequences for various morpholinos: Mtmr8-MO1 (Splicing antisense), 5’-CACCTCCTGCTGACAGACCGTACCTTC-3’; Mtmr8-MO2 (Splicing antisense), 5’-GGCCAA-CATTACCCAGTGTTCCTTG-3’; Nexilin-MO (Splicing antisense), 5’-ATAGCGCTTACATCTTACGCATTT-3’; Standard control MO (Cont MO), 5’-CCTCTACCTGTTAGATTTGTTTATA-3’. For injection, MOs were injected into fertilized zebrafish

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**Table 1. Mtmr8 is non-cell autonomous for muscle F-actin modeling.**

| Donor genotype | Host genotype | Result |
|----------------|---------------|--------|
| Mtmr8 morphant | Wild-type | 15/15 Wild-type (100%) |
|                | 10/10 Wild-type (100%) |
|                | 13/13 Wild-type (100%) |
| Wild-type      | Mtmr8 morphant | 12/12 Severe defect (100%) |
|                | 13/13 Severe defect (100%) |
|                | 12/12 Severe defect (100%) |

Donor embryos were labeled with fluorescein dextran. At 24 hpf, donor and host embryos were observed under the microscope, and then fixed for phalloidin labeling to determine genotype (as shown in Fig 7C). The phenotype of the transplanted embryos (number and percentage) is reported.

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**Figure 8. A hypothesized signal pathway that Mtmr8 regulates zebrafish muscle development.** Knockdown of Mtmr8 promotes PI3K/Akt pathway and prevents Hh signaling pathway. The two pathways may interact with each other in regulating F-actin modeling and muscle development.

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eggs at the one-cell stage at a concentration of about 6 ng each embryo. After injection, embryos were incubated at 28.5 °C in Embryo Medium [50].

Plasmids pc82-Mtmr8 and pc82-shPKA were linearized for in vitro transcription. Capped sense RNAs were synthesized using SP6 RNA polymerase and the SP6 Capi-Scribe (Roche), following the manufacturer's instructions, re-suspended in water and injected at a concentration of 100 ng/μL.

Whole-mount in situ hybridization and immunohistochemistry

Embryos at different stages were collected and pre-treated and fixed as described [57]. Purified plasmids was linearized by selected restriction enzymes and used as templates for in vitro transcription using T7 or SP6 RNA polymerase to generate DIG-labeled (Roche) sense and anti-sense probes. In situ hybridization was performed as described [62].

An antibody was purchased from Developmental Studies Hybridoma Bank (DSHB, University of Iowa, USA), and Proxl antibody from Abcam (Cambridge, United Kingdom). Zebrafish embryos were fixed overnight in 4% paraformaldehyde at 4 °C, and then washed in 0.1% Triton X-100 in PBS (PTX) and dechorionated. They were then incubated for 1 h in 0.5% Triton X-100 in PBS, followed by 5-h incubation in block solution (10% normal goat serum, 1% DMSO, 0.1% Triton X-100 in PBS). Embryos were then incubated overnight at 4 °C in block solution containing Phalloidin and/or primary antibodies. They were then washed in PTX, and incubated for 5 h at room temperature with secondary antibodies. Antibody and Phalloidin staining of zebrafish embryos were performed as previously described [63].

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