Biochemical and Biophysical Research Communications 463 (2015) 912–916

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure and potential physiological role of zebra fish thioesterase superfamily member 2 (fTHEM2)

Shanshan Yu, Han Li, Feng Gao, Ying Zhou*

Non-coding RNA Laboratory, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

ARTICLE INFO

Article history:
Received 26 May 2015
Accepted 5 June 2015
Available online 9 June 2015

Keywords:
Zebra fish
THEM2
X-ray crystal structure
Thioesterase
Morpholino
Early development of embryo

ABSTRACT

Thioesterase superfamily member 2 (THEM2) is an essential protein for mammalian cell proliferation. It belongs to the hotdog-fold thioesterase superfamily and catalyzes hydrolysis of thioester bonds of acyl-CoA *in vitro*, while its *in vivo* function remains unrevealed. In this study, Zebra fish was selected as a model organism to facilitate the investigations on THEM2. First, we solved the crystal structure of recombinant fTHEM2 at the resolution of 1.80 Å, which displayed a similar scaffolding as hTHEM2. Second, functional studies demonstrated that fTHEM2 is capable of hydrolyzing palmitoyl-CoA *in vitro*. In addition, injection of morpholino against fTHEM2 at one-cell stage resulted in distorted early embryo development, including delayed cell division, retarded development and increased death rate. The above findings validated our hypothesis that fTHEM2 could serve as an ideal surrogate for studying the physiological functions of THEM2.

1. Introduction

Thioesterase superfamily member 2 (THEM2), also known as acyl-CoA thioesterase 13 (ACOT13), is considered essential for proliferation of mammalian cells [1]. THEM2, which consists of a single a hotdog domain, catalyzes hydrolysis of the thioester bond in a variety of acyl-CoA compounds *in vitro*. When catalyzing hydrolysis of fatty-acyl-CoA's, THEM2 displays relatively high catalytic efficiency towards medium-to-long-chain substrates, such as palmitoyl-CoA [2,3]. The thioesterase activity of THEM2 requires the formation of a homotetramer, in which a series of highly conserved residues from three monomers comprise the catalytic center. In both solution and crystals, THEM2 form a stable tetramer in a back-to-back mode [4].

In contrast to the elucidated atomic structures [3,4], only a few functional studies of THEM2 have been reported. THEM2 was implicated in hepatic metabolism *via* its interaction with phosphatidylcholine-transfer protein (PC-TP) [5,6]. Recent studies discovered both an increase of fatty-acyl-CoA concentration and a decrease of free fatty acid concentration in the liver of THEM2 knock-down mice, associated with reduced activation of peroxisome proliferator-activated receptor. The reduced level of THEM2 in mice also diminished hepatic glucose production when hepatocyte nuclear factor 4α(HNF4α) expression was reduced [7]. Besides liver, Cheng et al. reported the presence of THEM2 in other organs, which is highly expressed in kidney, and moderately expressed in brain, large intestine, and small intestine [1]. In addition, small interference RNA silencing in cell line HCT116 demonstrated the essential role of THEM2 for sustained cell proliferation [1].

Although the three-dimensional structure of human THEM2 (hTHEM2), in complex with its substrate analog, sheds considerable light on the catalytic mechanisms of THEM2 and its *in vitro* substrate preference [3], the detailed mechanisms underlying the physiological role of THEM2 in cell proliferation and embryonic development remains elusive. In addition, previous results showed that hTHEM2 is co-localized with microtubules [1], while the biological consequence of such co-localization has been little understood. Regarding the investigation on cellular and developmental functions, zebra fish is a well-established model organism, and contains a functional homolog of hTHEM2 in its genome. Therefore, the fish THEM2 (fTHEM2) may serve as an ideal surrogate for elucidating the physiological function of THEM2.

The primary structure of fTHEM2 (residues 1–141) resembles that of hTHEM2 (roughly 70% identical in sequence). However, the N-terminal region (residues 1–35) of fTHEM2 differs considerably from its counterpart in hTHEM2 (with a sequence identity of only 34%). It thus remains to be examined whether fTHEM2, with such

* Corresponding author.
E-mail address: zhouying@moon.ibp.ac.cn (Y. Zhou).

http://dx.doi.org/10.1016/j.bbrc.2015.06.034
0006-291X/

sequence difference from hTHEM2 in the N-terminal region, has comparable molecular and cellular functions. In light of this question, we seek to obtain structural information of fTHEM2 and to lay a solid foundation for the study of its biological function in embryogenesis. In this study, we report the expression, purification, crystallization and structural analysis of fTHEM2. Combined with the following cellular studies of fTHEM2 in embryonic development, our data demonstrated the important role of THEM2 in early development of embryo, and proved that fTHEM2 is ideal for future studies on physiological functions of THEM2.

2. Materials and methods

2.1. Ethics statement

All animal studies in this report were approved by the Institutional Review Board of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

2.2. fTHEM2 purification, crystallization and structure determination

fTHEM2 gene was amplified and cloned into pET22b vector between the restriction sites of NdeI and XhoI. The recombinant fTHEM2 protein was overexpressed in *Escherichia coli* BL21(DE3) and purified according to the purification procedure of hTHEM2 protein, which was described previously [4]. The dynamic light scattering analysis was performed using Dynapro Nano Star (Wyatt) in 20 mM Tris–HCl pH 7.5, 200 mM NaCl buffer at 25 °C. The purified fTHEM2 was crystallized by the sitting drop vapor diffusion method at 289 K. The drops were set up by mixing 1 μL of sample solution containing 10 mg/mL fTHEM2, in 50 mM Tris-Cl pH 7.5, 50 mM NaCl and 5 mM DTT and 1 μL of reservoir solution containing 0.1 M Ammonium Acetate, 0.1 M Bis-Tris pH 5.5 and 17% w/v PEG 10,000. One diffraction data set, at resolution of 1.8 Å, was collected at the Beamline 17U, Shanghai Synchrotron Radiation Facility (SSRF). The data set was processed with HKL2000 [6]. Molecular replacement was performed by MOLREP [8], using the monomeric hTHEM2 structure (chain A, PDB accession ID 2F0X) as the search model. After manual adjustment in Coot [9], the structure was further refined in the REFMAC5 [10]. The stereochemical quality of the final model was examined by PROCHECK [11]. The statistics of data collection and structure refinement were listed in Table 1. The structure of fTHEM2 has been deposited to the Protein Data Bank with an accession code 4ORD.

Table 1
Data collection and structure refinement statistics.

Data collection	
Space group	*C*2
Unit cell parameter	a = 77.1 Å, b = 74.4 Å, c = 96.6 Å β = 93.7°
Resolution range (Å)[a]	50–1.80 (1.86–1.80)
No. of total reflections	193,229 (18,737)
No. of unique reflections	50707 (5064)
Average redundancy	3.8 (3.7)
Completeness (%)	99.8 (100.0)
I/σ	15.5 (2.5)
R_{merge}[b]	0.087 (0.588)
Structure refinement	
R_{work}/R_{free} (%)[c]	19.3/22.5
RMSD bond length (Å)[d]	0.007
RMSD bond angle (°)	1.1

[a] Data for the highest resolution bin is in parentheses.
[b] $R_{merge} = \sum|Ii - Im|/\sum Ii$, where Ii is the intensity of the measured reflection and Im is the mean intensity of all symmetry-related reflections.
[c] $R_{work} = \sum||Fobs| - |Fcalc||/\sum|Fobs|$, where Fobs and Fcalc are observed and calculated structure factors, respectively. $R_{free} = \sum_T||Fobs| - |Fcalc||/\sum_T|Fobs|$, where T denotes a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.
[d] RMSD = root-mean-square deviation.

2.3. Enzyme activity measurement

Hydrolysis of palmitoyl-CoA was measured in a 50 μL cuvette, containing 50 mM KCl, 10 mM Hepes (pH 7.5), 0.3 mM DTNB [5, 5′-dithiobis-(2-nitrobenzoic acid)] (Sigma), and 50 μM substrate. fTHEM2 was added to initiate the reaction. The cuvette was immediately loaded into a temperature-controlled spectrophotometer and absorbance at 412 nm was monitored at 1 min intervals for up to 30 min at 37 °C. For the measurement of fTHEM2 steady-state kinetic constants, the datasets of initial velocities, measured as a function of substrate concentrations, were analyzed using the Michaelis–Menten equation, $V = Vmax[S]/([S] + K_m)$; and $K_{cat} = Vmax/[E]$, where [E] is the total enzyme concentration and all measurements were repeated in triplicate.

2.4. them2 expression in zebra fish

To perform RT-PCR, mRNA was isolated from different stages of zebra fish embryos using TRIzol reagent (Invitrogen), and the total cDNA was synthesized using iScriptcDNA synthesis kit (Bio-Rad). The sequence of primers used to amplify *fthem2* cDNA were fTHEM2 up: 5′-ATGGCTTCATTAACGCTGAATAC-3′ and fTHEM2 down: 5′-TTAGCTGCCGAGGTGTTTTGTG-3′. For the control amplification of ornithine decarboxylase (ODC) cDNA, we used the following primers: ODC-BD up: 5′-CACTGGCGGTCAACATCATC-3′ ODC-BD down: 5′-GGTTCGGTCATTGGACACATC-3′.

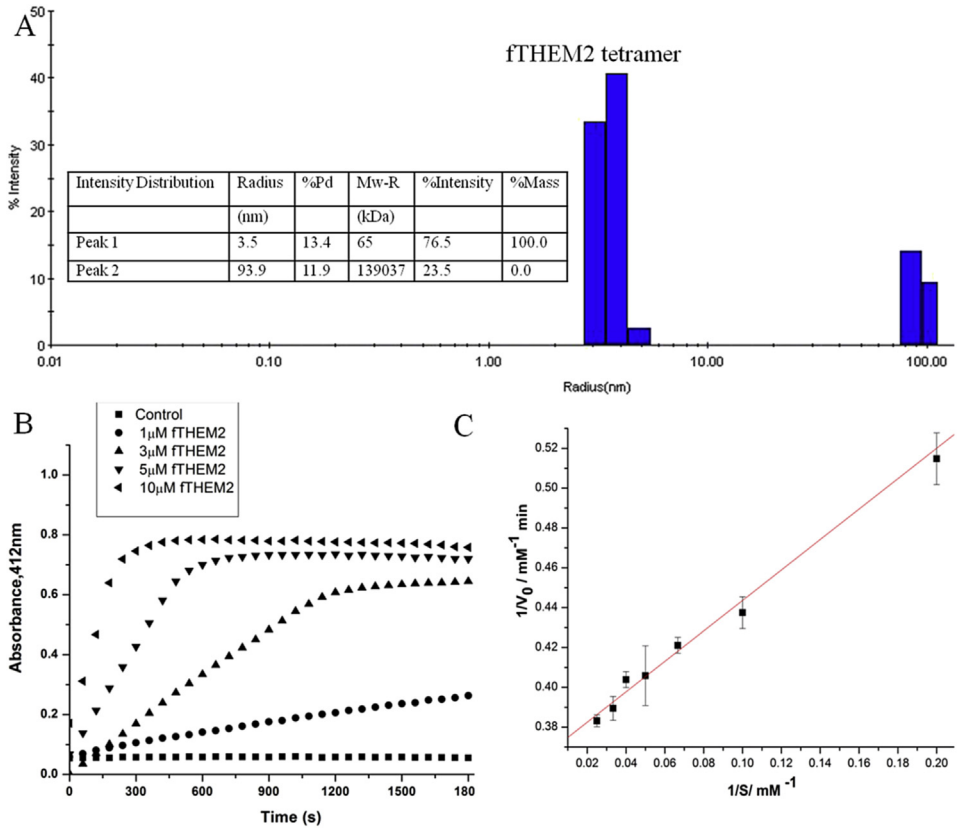
2.5. them2 knockdown in zebra fish

Morpholino (5′-CAAAACACAGAGAAGGACAAGTTCAC-3′) was designed to target the 5′ UTR of *fthem2* mRNA. The morpholino was injected into embryos at different doses and a standard control morpholino (Gene Tools) was used as the negative control. In the following development, live embryos were observed for phenotypes in a Leica microscope and the dead embryos were counted. For morpholino specificity assay, *fthem2* cDNA covering the UTR site were amplified with following primers up: 5′-CGGGATCCG-TATAACTTGGGCCAGCTTTC-3′ down: 5′-GCTCTA-GAGCTGCCGAGGTGTTTTGTGTG-3′ and cloned in frame with four flag tag into pCS2 vector. The efficacy of the morpholino was tested by western blot. First, the *fthem2* mRNA covering the UTR site was transcribed with RiboMAX Large Scale RNA Production Systems-SP6 and T7 (Promega) and injected into oocyte of *Xenopus* with or without fTHEM2 morpholino. After cultured for 24 h, oocytes were lysed and subjected to western blot probed with anti-flag antibody and anti-β-tubulin antibody.

3. Results and discussion

3.1. Protein purification and characterization

The *fthem2* gene was cloned and overexpressed in *E. coli*. The recombinant fTHEM2 protein was purified to homogeneity by affinity chromatography. The His-tagged fTHEM2 contained 149 residues with a theoretical molecular mass of 16.1 kDa. In order to examine the oligomeric state of fTHEM2 in solution, dynamic light scattering analysis was performed and two peaks were demonstrated (Fig. 1A). The major species was characterized with a molecular weight of 65 kDa, confirming that fTHEM2 exists as homotetramer in solution. The other species displayed measurable scattering intensity due to large physical size, but accounted only



Intensity Distribution	Radius	%Pd	Mw-R	%Intensity	%Mass
	(nm)		(kDa)		
Peak 1	3.5	13.4	65	76.5	100.0
Peak 2	93.9	11.9	139037	23.5	0.0

Fig. 1. Biochemical characterization of fTHEM2. (A) Dynamic light scattering analysis of fTHEM2. The properties of major scattering species are listed in the inserted table. (B) Palmitoyl-CoA can be hydrolyzed by varying concentrations of fTHEM2. (C) Calculation of enzymatic constants (K_m and k_{cat}) of fTHEM2. The double reciprocal plot (1/V vs. 1/[S]) is used to extrapolate the enzymatic constants, while the error bars represent standard deviations from three independent measurements.

trace amount in terms of mass percentage, which was most likely to be an experimental artifact or contaminant.

3.2. Three-dimensional structure

The structure of fTHEM2 was solved by molecular replacement, using a search model of the monomeric hTHEM2 structure (chain A, PDB accession ID 2F0X). Although four fTHEM2 molecules were identified per asymmetric unit, they do not belong to the same protein tetramer. Following the crystallographic symmetry, the fTHEM2 homotetramer could be successfully established and was used in the subsequent analysis.

The monomer of fTHEM2 clearly displays a hotdog fold (Fig. 2A), with six β-strands surrounding a long α-helix and an additional α-helix flanking the β1-strand. fTHEM2 dimerizes *via* a stable anti-parallel β-sheet formed at residues Ser84–Tyr91 on β3-strand from each monomer (Fig. 2B). The dimer is additionally stabilized by hydrogen bonds between the two central α-helices. Furthermore, two fTHEM2 dimers stack into a tetramer in a back-to-back mode (Fig. 2C), with two extended 12-strand anti-parallel β-sheets (Fig. 2B) from each dimer to form the tetrameric interface. The key residues involved in the tetramerization of fTHEM2 include Asn88, Asp86, Thr136, Met92, and Phe116.

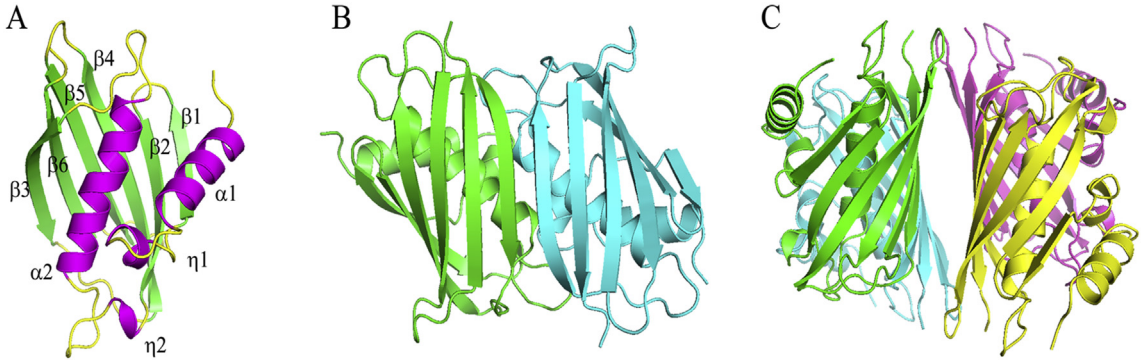


Fig. 2. The structures of fTHEM2. (A) The structure of fTHEM2 monomer. The secondary structural elements are denoted as labels, and colored as magenta (helix), green (stand) and yellow (loop), respectively. (B) The structure of fTHEM2 dimer. (C) The structure of fTHEM2 tetramer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The above mentioned monomeric, dimeric and tetrameric structures of fTHEM2 are highly similar to those of hTHEM2. In order to make better comparison, the structures of fTHEM2, hTHEM2, and hTHEM2 complexed with a substrate analog (undecan-2-one-CoA) were superimposed and carefully analyzed. First of all, almost identical subunit assembly and polypeptide folding was observed in these three structures (Fig. 3A). The quantitative analysis on the alpha carbons of fTHEM2 and hTHEM2 resulted a r.m.s.d value of 0.73 Å. In addition, the r.m.s.d values were also calculated for fTHEM2/hTHEM2-analog and hTHEM2/hTHEM2-analog, as 0.68 Å and 0.45 Å respectively. All the r.m.s.d values are lower than 1 Å, indicating very little positional shifts between the backbones of these three structures.

When tracing through the backbones of fTHEM2 and hTHEM2, some structural variation was detected. Besides direct observation on the 3D models (Fig. 3A), such variation could be easily identified at two regions on the plot of distances between superimposed backbone Cα's vs. residue positions (Fig. 3B). One is around the N-terminal α1 helix (residues 1–18) and the other is located at the C-terminus of the α2 helix (residues 73–77). Based on the multiple sequence alignment of THEM2 homologs from different vertebrate species, these two regions are also of high diversity. Although the overall structures of fTHEM2 and hTHEM2 are similar, these sequential and structural variations might enable some species-specific interaction networks.

3.3. Active site and thioesterase activity

The active site of hTHEM2 has been validated by both biochemical and structural characterization, and the key residues have been identified [3,4]. As above analyzed, fTHEM2 and hTHEM2 share similar architecture with many identical key residues. Such conservation in both sequence and structure allowed identification of the putative fTHEM2 active site, which is located at the subunit interface formed by three adjacent protomers (Fig. 3C). The binding pocket consists of many conserved residues, including Asp23, Asp66, Met67, Thr70, Met71, Pro81, Val83, Ser84, His135, and Lys137 from protomer I, Asn51, His57, Gly58, Tyr91, Met92, Asn93, and Ala94 from protomer II and Lys109, Gly111, Arg14, Thr113, and Leu114 from protomer III. Within these residues, Asn51, His57, Gly58, Asp66, Thr70, Ser84 are directly involved in catalysis [3] and mostly conserved (Fig. 3D).

The conserved binding pocket and catalytic residues in fTHEM2 indicated a conserved mechanism in substrate recognition and catalysis. In order to validate such hypothesis, *in vitro* enzymatic assays were conducted for fTHEM2 and the steady-state constants were calculated (Fig. 1C). Unsurprisingly, fTHEM2 exhibited similar enzymatic activities as hTHEM2. fTHEM2 could readily hydrolyze long-chain fatty-acyl-CoA compounds, such as palmitoyl-CoA (Fig. 1B) with K_m(fTHEM2) = 1.98 μM and k_{cat}(fTHEM2) = 2.26 × 10^{-2} s^{-1} (Fig. 1C), which are comparable to the Michaelis–Menten constants of hTHEM2 (K_m(hTHEM2) = 9 μM, k_{cat}(hTHEM2) = 1.70 × 10^{-2} s^{-1}) [3].

3.4. Disrupted embryo development by fTHEM2 knockdown

THEM2 is conserved in vertebrates (Fig. 3C) and has a single ortholog in zebra fish (fTHEM2). The embryonic expression pattern of *fthem2* was examined by means of RT-PCR with whole-mount mRNA. *fthem2* could be detected throughout the investigated stages (from 1-cell to 48 h), indicating that *them2* is a host gene and supplied both maternally and zygotically (Fig. 4A). To examine the consequence of *fthem2* disruption, the morpholino targeting the 5′ untranslated region of *fthem2* was prepared. The injection of

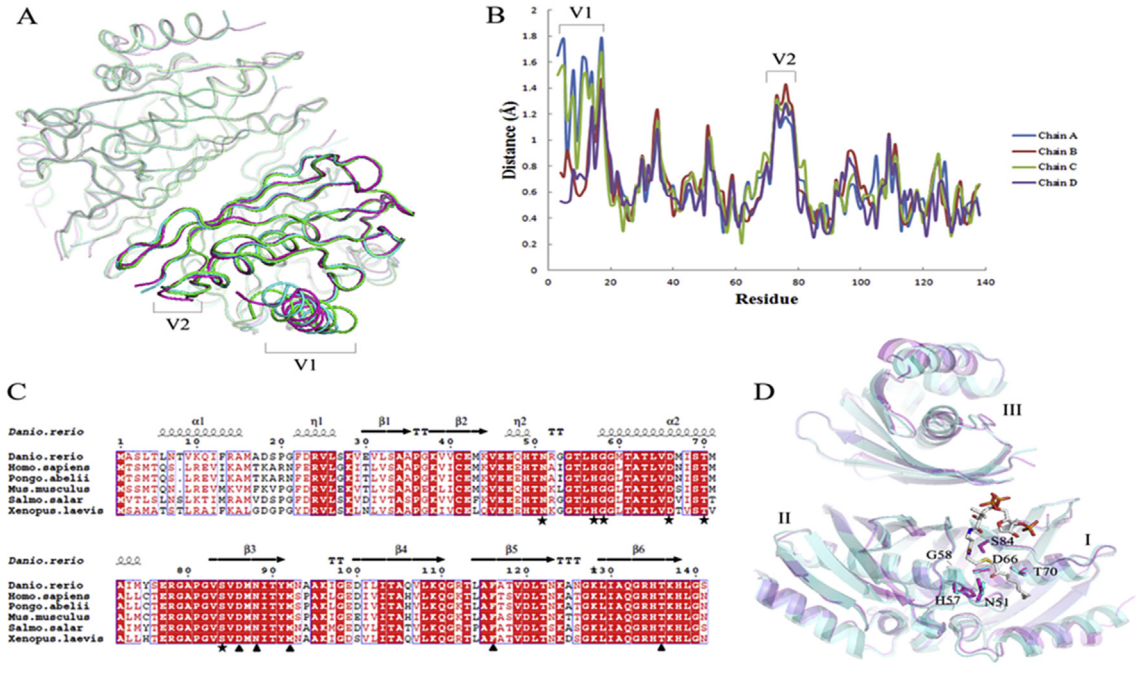


Fig. 3. Structural and sequence alignment of THEM2. (A) Structural alignment of fTHEM2 (magenta), hTHEM2 (green), and hTHEM2 complexed with undecan-2-one-CoA (blue). (B) Quantitation of backbone variation between fTHEM2 and hTHEM2 (apo) structures. Distances between Cα's in the two superimposed structures are plotted according to their positions in paired polypeptide chains. In panel A and B, V1 and V2 represent two regions with relatively high variation in backbones. (C) Multiple sequence alignment of THEM2 homologs from diverse vertebrate species. The conserved residues are labeled with red box, while the residues involved in tetramerization and active site are labeled as triangles and asterisks, respectively. (D) The putative active site of fTHEM2. The structures of fTHEM2 and hTHEM2/undecan-2-one-CoA complex are superimposed, with the polypeptide chains represented as ribbons (magenta for fTHEM2 and blue for hTHEM2) and the substrate analog represented as sticks. The active site is formed by three adjacent protomers (I, II and III), and the conserved residues involved in catalysis are labeled as in the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

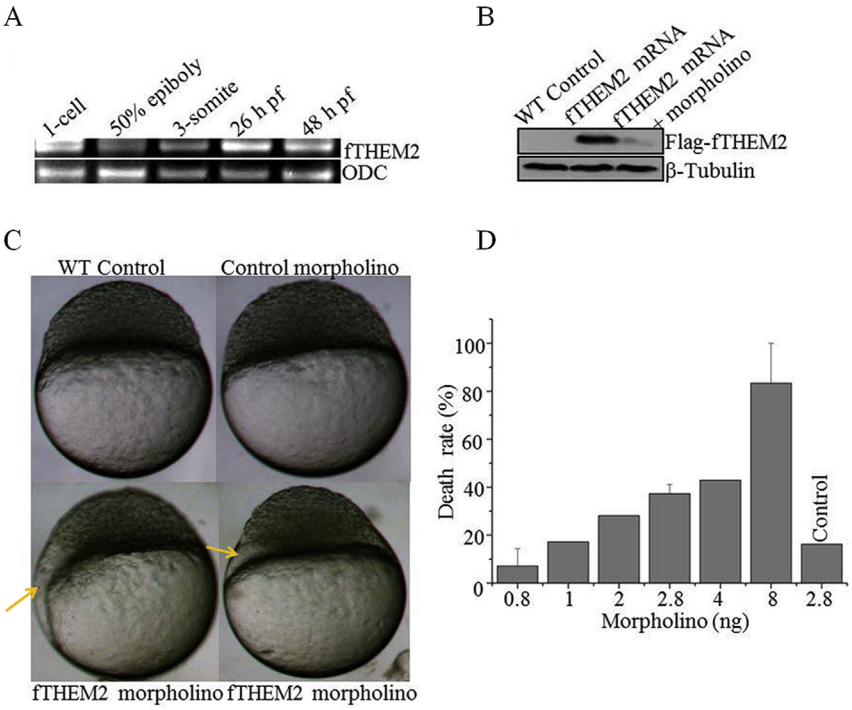


Fig. 4. hTHEM2 is required for normal embryonic development in zebra fish. (A) fTHEM2 expression patterns during embryonic development were analyzed by RT-PCR with whole-mount mRNA. (B) The efficacy of fTHEM2 morpholino. Xenopus cell lysates were analyzed by western blot probed with either flag antibody (top panel) or anti-β-tubulin antibody (bottom panel). (C) Control WT embryos and embryos injected with control morpholino or fTHEM2 morpholino were observed live. The regions with distorted cell division are labeled by arrows. (D) Histogram quantifying the death rates of injected embryo. The error bars represent standard deviations from three independent experiments.

fthem2 morpholino and *fthem2* mRNA covering 5′ untranslated region into oocytes of *Xenopus* resulted in a significant reduction in the expression level of fTHEM2 as determined by western blotting, which clearly demonstrated the efficacy of such morpholino to inhibit the *in vivo* expression of *fthem2* (Fig. 4B) Concurrent to the diminished fTHEM2 level, severe developmental defects emerged. First, the depletion of fTHEM2 significantly retarded embryonic development compared to the control embryos (wild type and/or those injected with standard morpholino controls). Failure of division was clearly observed in some cells at the edge of embryos (Fig. 4C). Second, the treated embryos exhibited a death rate of 40% at the 20-h stage, as opposed to the much lower death rate of 10% in the embryos injected with control morpholino (Fig. 4D).

The above mentioned observations obviously suggested a critical role that THEM2 played in early embryonic development, the disruption of which could make fatal consequences in zebra fishes. Interestingly, such increased death rate was apparently correlated with the retarded cell division. The importance of THEM2 in cell division and proliferation was previously demonstrated [1], based on the inhibited cell growth following *hthem2* silencing. Combined with our observation, these results appeared to indicate that THEM2 might be involved in some critical events during cell cycle.

Acknowledgments

This project was funded by the National Natural Science Foundation of China (31301107).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.034.

References

[1] Z. Cheng, S. Bao, X. Shan, H. Xu, W. Gong, Human thioesterase superfamily member 2 (hTHEM2) is co-localized with beta-tubulin onto the microtubule, Biochem. Biophys. Res. Commun. 350 (2006) 850–853.
[2] J. Wei, H.W. Kang, D.E. Cohen, Thioesterase superfamily member 2 (Them2)/acyl-CoA thioesterase 13 (Acot13): a homotetrameric hotdog fold thioesterase with selectivity for long-chain fatty acyl-CoAs, Biochem. J. 421 (2009) 311–322.
[3] J. Cao, H. Xu, H. Zhao, W. Gong, D. Dunaway-Mariano, The mechanisms of human hotdog-fold thioesterase 2 (hTHEM2) substrate recognition and catalysis illuminated by a structure and function based analysis, Biochemistry 48 (2009) 1293–1304.
[4] Z. Cheng, F. Song, X. Shan, Z. Wei, Y. Wang, D. Dunaway-Mariano, W. Gong, Crystal structure of human thioesterase superfamily member 2, Biochem. Biophys. Res. Commun. 349 (2006) 172–177.
[5] K. Kanno, M.K. Wu, D.S. Agate, B.J. Fanelli, N. Wagle, E.F. Scapa, C. Ukomadu, D.E. Cohen, Interacting proteins dictate function of the minimal START domain phosphatidylcholine transfer protein/StarD2, J. Biol. Chem. 282 (2007) 30728–30736.
[6] Z. Otwinowski, W. Minor, Processing of X-ray Diffraction Data Collected in Osillation Mode, Methods in Enzymology, Academic Press, New York, 1997, pp. 307–326.
[7] H.W. Kang, M.W. Niepel, S. Han, Y. Kawano, D.E. Cohen, Thioesterase superfamily member 2/acyl-CoA thioesterase 13 (Them2/Acot13) regulates hepatic lipid and glucose metabolism, FASEB J. 26 (2012) 2209–2221.
[8] A. Vagin, A. Teplyakov, MOLREP: an automated program for molecular replacement, J. Appl. Cyrst. 30 (1997) 1022–1025.
[9] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallogr. D. Biol. Crystallogr. 60 (2004) 2126–2132.
[10] G.N. Murshudov, A.A. Vagin, E.J. Dodson, Refinement of macromolecular structures by the maximum-likelihood method, Acta Crystallogr. D. Biol. Crystallogr. 53 (1997) 240–255.
[11] R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Cyrst. (1993) 283–291.