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Cloning and characterization of *ifitm1* and *ifitm3* expression during early zebrafish development

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Summary

The family of interferon-inducible transmembrane proteins (IFITMs) plays a crucial role in inhibiting proliferation, promoting homotypic cell adhesion and mediating germ cell development. In the present study, the full-length cDNAs of zebrafish *ifitm1* (744 bp) and *ifitm3* (702 bp) were obtained by rapid amplification of cDNA ends (RACE). Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that *ifitm1* mRNA was expressed in the ovary, testis, brain, muscle, liver and kidney, while *ifitm3* mRNA was only detected in the ovary. Based on *in situ* hybridization, *ifitm1* mRNA was found to be strongly expressed in the ooplasm from stage I to stage II and *ifitm3* mRNA was also strongly expressed in the ooplasm from stage I to stage II, furthermore *ifitm3* expression ultimately localized to the cortex region beneath the plasma membrane of stage IV oocytes. During development, *ifitm1* expression was initially detected in the enveloping layer cells and deep layer cells of shield stage embryos. Then, throughout the segmentation phase (10.25–24 hours post-fertilization (hpf)), *ifitm1* expression was mainly detected in the head, trunk and tail regions. Unlike *ifitm1*, *ifitm3* expression was initially detected in sphere stage embryos and was then broadly expressed throughout the embryo from the 70% epiboly stage to 24 hpf. Interestingly, *ifitm3* was also expressed in primordial germ cells (PGCs) from the bud stage to 24 hpf. This expression analysis indicates that zebrafish *ifitm1* may play a critical role in early organogenesis and may perform immune or hematopoietic functions and *ifitm3* might be necessary for PGC migration and the formation of female germ cells.

Keywords: *ifitm1*, *ifitm3*, *In situ* hybridization, Primordial germ cell, Zebrafish

Introduction

Interferons (IFNs) are multifunctional cytokines that play important roles in the defense against viral or parasitic infections. They also exhibit anti-proliferative and pro-differentiation activities, which prompted an evaluation of their potential as antitumor agents (Samuel, 2001; Afonso *et al*., 1997). The pleiotropic effects of IFNs are mediated by various IFN-inducible transmembrane proteins (IFITMs) that are encoded by IFN-stimulated genes (ISGs) (Lewin *et al*., 1991).

Members of the IFITM family have been isolated in a number of mammalian species. In humans, IFITM genes were originally identified in neuroblastoma cells by their differential response to stimulation by interferon (Friedman *et al*., 1984). IFITMs belong to the CD225 superfamily, which is characterized by two highly conserved transmembrane regions, a highly conserved intracellular region, and termini with a high degrees of variance (Tanaka & Matsui, 2002). The family consists of five genes (*ifitm*1, *ifitm*2, *ifitm*3, *ifitm*5 and *ifitm*10), all of which are located on chromosome 11, and these genes encode proteins 125 to 133 amino acids in length that contain two transmembrane domains (Lewin *et al*., 1991). IFITM1, which is expressed by leukocytes and endothelial cells,

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has anti-proliferative effects and promotes homotypic cell adhesion. IFITM3 also inhibits cell proliferation, while the roles of *ifitm5* and *ifitm10* in humans are not well defined (Deblandre *et al*., 1995; Brem *et al*., 2003).

In mice, the *ifitm* gene family has also been characterized. The first murine *ifitm* gene (*ifitm3*) was identified in a screen for genes expressed specifically in early primordial germ cells (PGCs) (Saitou *et al*., 2002). *Ifitm3*, together with *ifitm1*, *ifitm2*, *ifitm5*, *ifitm6* and *ifitm10*, are on chromosome 7, whereas *ifitm7* is on chromosome 16. Murine *ifitm* genes encode proteins of 104–144 amino acids in length with two transmembrane domains (Tanaka *et al*., 2004). In mice, *ifitm1* is initially expressed in the extraembryonic tissue and the early mesoderm until differentiation occurs during murine embryonic development. This gene may be required for somite epithelialization and paraxial mesoderm formation. *ifitm3* is expressed in the proximal epiblast beginning around 5.5 days post coitum (dpc) embryos, and its expression is gradually restricted to PGCs as gastrulation proceeds. *Ifitm3* may play a role in germ cell development, whereas *ifitm2* is initially expressed at the late-streak stage (Lange *et al*., 2003). *Ifitm5* is not detectable in the early embryo from 5.5–9.5 dpc, but it is expressed in the developing bone beginning around 14.5 dpc. Recent studies have shown that members of the IFITM family may also be involved in the promotion and maintenance of the pluripotent state in many progenitor cells through their anti-proliferative effects (Johnson *et al*., 2006).

In teleost fish, only *ifitm1* from large yellow croaker and two rainbow trout *ifitms* (*ifitm1* and *ifitm2*) have been identified to date. RT-PCR analysis has shown that rainbow trout *ifitm1* mRNA is expressed in the head, kidney, gill and liver. This analysis also showed that the expression of *ifitm2* mRNA was highest in the gill, heart and liver, but absent from the head, kidney and blood (Johnson *et al*., 2006; Wan & Chen, 2008). However, little information is known about the expression profiles or functions of IFITMs in teleost fish. In the current study, we report the cloning and expression analysis of zebrafish *ifitm1* and *ifitm3*. The distribution of *ifitm1* and *ifitm3* mRNA was studied in adult zebrafish tissues and the developing embryo using RT-PCR analysis. These findings are used to discuss the potential functional roles these proteins play in developing and adult zebrafish.

Materials and methods

Experimental animals

Zebrafish (*Danio rerio*) were obtained from the Institute of Hydrobiology at the Chinese Academy of Science and were maintained at 28.5°C on a 14 h/10 h light/dark cycle. Embryos were collected from spontaneously spawning fish and cultured in egg water (Westerfield, 1993; Kimmel *et al*., 1995). Oocytes were staged based on physiological and biochemical characteristics, in addition to the morphological criteria described by Selman *et al*. (1993).

Electronic cloning and sequence screen of ifitm-related ESTs in zebrafish

Electronic cloning was used to identify expressed sequence tags (ESTs) related to zebrafish *ifitm1* and *ifitm3*. The cDNA sequences of mouse *ifitm1* (GenBank accession no. NM_026820.3) and *ifitm3* (NM_025378.2) were used as probes for BLAST analysis of the zebrafish EST database at the National Center for Biotechnology Information (NCBI). The *ifitm1* (CN508493.1) and *ifitm3* (EH281751.1) related ESTs were identified using this approach.

RNA isolation, cDNA synthesis and RT-PCR

Total RNA from adult zebrafish tissue, zebrafish larvae and embryos at specific developmental stages were isolated using TRIzol reagent (Invitrogen Corp, Carlsbad, CA, USA). To remove any DNA contamination, DNase I (Promega, Madison, WI, USA) was used to treat the total RNA. Afterwards, 3 μg of total RNA was used for cDNA synthesis (37°C for 60 min) using 0.5 μg of oligo $d(T)_{18}$, 10 mM dNTP and 200 U of M-MLV reverse transcriptase (Promega).

RT-PCR was used to amplify zebrafish *ifitm1* and *ifitm3* cDNA from different adult zebrafish tissues and embryonic stages. First-strand cDNA was used as a template, and gene-specific primers were used to amplify the *ifitm1* and *ifitm3* transcripts (*ifitm1* Forward: 5′-ACTAACAGGCATCACTGCGTCA-3′ and Reverse: 5′-TTCTCTGTGTTTATTCATCCTCCA-3′; *ifitm3* Forward: 5′-GCACTCCGCTCACCAACTGT-3′ and Reverse: 5′-GGTGGTGGTAGGTTTGCTCG-3′). To verify RNA quality, zebrafish β-*actin* was used as an internal control (forward: 5′-CTGGGGCGCCCCAGG CACCA-3′ and reverse: 5′-CTCCTTAATGTCA CGCACGATTTC-3′). PCR was performed in a 25 μl reaction mix containing 10 mM Tris–HCl (pH 8.3), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each primer, and 1 U of *Taq* DNA polymerase. The amplification conditions included a 5 min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min.

Rapid amplification of cDNA ends (RACE)

All RACE assays were performed according to the BD SMART RACE cDNA amplification kit user manual.

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3′-UPM (5′-CTAATACGACTCACTATAGGGC-3′) was used as a common primer. To extend the 3′-end of the *ifitm1* cDNA, 3′-RACE was performed using *ifitm1* gene-specific primer1 3′if1GSP1 (5′-CCAGAGACCGCAGATTGCTTGGAGAC-3′) and 3′if1GSP2 (5′-CATCGCTTCTGTTATTCTTGGCGTC CTC-3′). First round touchdown PCR was performed with 3′if1GSP1 using the following conditions: 94°C for 30 s, followed by five cycles of 72°C for 2 min, 94°C for 30 s, and 70°C for 30 s, followed by five cycles of 72°C for 2 min, 94°C for 30 s, 68°C for 30 s, and finally 25 cycles of 72°C for 2 min; a final extension at 72°C was performed for 10 min. First round PCR product (diluted 1:50) was then used as a template for the second round of PCR amplification. Second round PCR was performed with 3′if1GSP2 under the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min and a final extension at 72°C for 10 min. The *ifitm3* gene-specific primer1 3′if3GSP1 (5′-CATTCTTGCTTCGGGCTGGAACCTC-3′) and 3′if3GSP2 (5′-ACCTACCACCACCTCCAAACCTTCT-3′) were used to extend the 3′-end of the *ifitm3* cDNA. The methods were the same as those used for *ifitm1*.

Conversely, 5′-RACE experiments were performed using a common primer 5′-NUP (5′-AAGCAGTGG TATCAACGCAGAGT-3′). To extend the 5′-end of the *ifitm1* cDNA, 5′if1GSP1 (5′-CAGACATTCTTTGA AGGGTTATTGGTGGC-3′) and 5′if1GSP2 (5′-GCA TAGTCTCCAAGCAATCTGCGGTCTC-3′) were used. To extend the 5′-end of the *ifitm3* cDNA, 5′if3GSP1 (5′-AGAAGCAGGACCAGCAGACCCAACA-3′) and 5′if3GSP2 (5′-AAGCCCAGGCAGCAGAAGTTGACG-3′) were used. The PCR conditions were similar to those used for 3′-RACE.

In situ hybridization and whole-mount *in situ* hybridization

Zebrafish *ifitm1* riboprobe was prepared from a 704 bp fragment (GU457425, nucleotides 19–722), and the *ifitm3* riboprobe was prepared from a 426 bp fragment (GU457426, nucleotides 70–495). Antisense riboprobes for each transcript were labelled with digoxigenin-UTP using SP6 RNA polymerase, while the control, sense probes were synthesized with T7. The hybridization signal was detected using NBT/BCIP according to the manufacturer's instructions (Roche).

In situ hybridization was performed at 65°C. The ovary samples were embedded in tissue freezing medium (Leica, Wetzlar, Germany) at −25°C and then cut into 8 μm sections. Frozen sections were placed onto gelatinized slides, and *in situ* hybridization analysis was performed as described (Dijkman *et al.*, 1995). For whole-mount *in situ* hybridization, the embryos were staged according to established morphological criteria using the protocol described by Westerfield (1993). Images of the embryos and all sections were captured using an Olympus BH-2 microscope (Olympus).

Results

Cloning and sequence analysis of zebrafish ifitm1 and ifitm3

Two *ifitm*-related zebrafish ESTs (CN508493.1 and EH281751.1) were identified in the UniGene sequence database. Full-length cDNAs of *ifitm1* and *ifitm3* were obtained by performing RACE analysis and were submitted to the GenBank database (GU457425 and GU457426, respectively).

The 744 bp full-length cDNA of *ifitm1* contains a 345 bp open reading frame (ORF), which is preceded by a 63-bp 5′-untranslated region (UTR) and followed by a 336-bp 3′-UTR. A typical putative polyadenylation signal (AATAA) was found upstream from the polyA tail. The initiation codon was located at position 64–66 bp, whereas the stop codon was located at position 406–408 bp (Fig. 1*A*). The *ifitm1* gene is located on zebrafish chromosome 5 and contains two exons and one intron. Nucleotide sequence analysis indicated that the splice junctions between the introns and exons followed the 'AG-GT' rule. Zebrafish *ifitm1* encodes a 114 amino acid (aa) protein with a theoretical molecular weight of 12.558 kDa and an isoelectric point (pI) of 8.89.

The 702 bp full-length cDNA of *ifitm3* consisted of a 429-bp ORF, which extends from position 36 to 464. Alignment of the cDNA sequence with genomic DNA revealed that *ifitm3* is on zebrafish chromosome 25 and includes two exons and one intron, similar to *ifitm1*. The splice junctions between the introns and exons also adhered to the 'GT–AG' rule. The zebrafish *ifitm3* encodes a 142 aa protein with a theoretical molecular weight of 15.638 kDa and a pI of 8.10 (Fig. 1*B*)

Based on multiple alignments, zebrafish IFITM1 (ADK55689) shared 39, 41, 34, 33, 37, 41, 47, 46 and 50% aa sequence homology with zebrafish IFITM3 (ADD38964), Larimichthys *crocea* LcIFITM1 (ABY55168), rainbow trout OmIFITM1 (CAC83757), OmIFITM2 (CAC85160), mouse MmIFITM1 (NP_081096), MmIFITM3 (NP_079654), human HsIFITM1 (CAA59337), HsIFITM2 (CAG46672) and HsIFITM3 (NP_066362), respectively (Fig. 2).

According to protein–protein BLAST (BlastP) analysis at the NCBI database, a conserved domain (aa 19–99 and aa 26–108 in IFITM1 and IFITM3, respectively)

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(A)

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   1 gataaataagagctgaccactaacaggcatcactgcgtcacaacttggtgaattaagaca

  61 acaatgcagagctacccgcttagaggaaacccaatgcaggatgacaaaacctgcaacggt
        M  Q  S  Y  P  L  R  G  N  P  M  Q  D  D  K  T  C  N  G   19
 121 cagccagtggttgtgtccatgcctgcacaaaaattagatgatgatatcatttttccaca
     Q  P  V  V  V  S  M  P  A  Q  K  L  D  D  D  I  I  F  S  T   39
 181 ttcaattttcactactgcaacccttgttgtcttgggtttggagccttttacaactcagtg
     F  N  F  H  Y  C  N  P  C  C  L  G  F  G  A  F  Y  N  S  V   59
 241 aaggccagagaccgcagattgcttggagactatgcatcagcgagcacgtacggcactaga
     K  A  R  D  R  R  L  L  G  D  Y  A  S  A  S  T  Y  G  T  R   79
 301 gctcgacgtctgaacatcgcttctgttattcttggcgtcctctatggcattttactgatt
     A  R  R  L  N  I  A  S  V  I  L  G  V  L  Y  G  I  L  L  I   99
 361 gtcatccttgtctatgcttatcagcttagtcgttttagccaccaataaccccttcaaagaa
     V  I  L  V  Y  A  Y  Q  L  S  R  F  S  H  Q  *              114
 421 tgtctgtttgtatatcaaagcatttactctgtttccataaagcattttatatctgtttaa

 481 aatgtttgttaaggattatttaagtctttttttttattccatacaggcacattatttacc

 541 ttatttagagatgtgcttgcatttcctttatttgttttattgttttcttgtttatatcac

 601 actcatggtgtgatctcagcaattgtttatagaatgaatttttttttttatttatctat

 661 atactgtatatttttagtcacaccctatataaatatgtggaggatgaataaacacagag

 721 aactctaaaaaaaaaaaaaaaaaa
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(B)

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   1 gattcttccaggagtttggaccgctgatctccaggatggataacgccacgtacagctacg
                                        M  D  N  A  T  Y  S  Y    8
  61 tgaacgactgcactccgctcaccaactgtaaggccggccgtaaggctgggggctccacgg
     V  N  D  C  T  P  L  T  N  C  K  A  G  R  K  A  G  G  S  T   28
 121 tggtcaacatgagccatgcgggcaagaagccaccaaatgactacctggtctggtcactct
     V  V  N  M  S  H  A  G  K  K  P  P  N  D  Y  L  V  W  S  L   48
 181 gcaacactctttacgtcaacttctgctgcctgggcttcatggctctgatctactccatca
     C  N  T  L  Y  V  N  F  C  C  L  G  F  M  A  L  I  Y  S  I   68
 241 aggctcgagatcagaagaccctgggtgacatgcgtgcagcgcaggaatgctcagacaagg
     K  A  R  D  Q  K  T  L  G  D  M  R  A  A  Q  E  C  S  D  K   88
 301 ccaagtggtacaacattcttgcttcgggctggaacctcttgattccgcttctggtgttgg
     A  K  W  Y  N  I  L  A  S  G  W  N  L  L  I  P  L  L  V  L  108
 361 gtctgctggtcctgcttctggtgcatctgggaagctcagagggaacatttgatttcttcg
     G  L  L  V  L  L  L  V  H  L  G  S  S  E  G  T  F  D  F  F  128
 421 gtgaggatggattccagagcttcatgaagctcttcagcaggtagacagagcaactcgagc
     G  E  D  G  F  Q  S  F  M  K  L  F  S  R  *                 142
 481 aaacctaccaccacctccaaaccttatggactgttcatccctctcgactttatcagcatg

 541 ttattttccttggctgtgtgaatttctcattatttagtttgttttgttccctaattcatg

 601 tccatgtactgtataactaatcctcaattttattttatgaagtaaattggatctgtagta

 661 tttaataaaatatttaatatcctcacaaaaaaaaaaaaaaaa
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Figure 1 (*A*) Full-length cDNA and the deduced amino acid sequence of zebrafish *ifitm1*. The initiation codon, stop codon and polyadenylation signal are all shown in bold. The amino acid sequence is represented using capitalized one-letter codes below the nucleotide sequence. (*B*) Full-length cDNA and the deduced amino acid sequence of zebrafish *ifitm3*. The initiation codon, stop codon and polyadenylation signal are all shown in bold. The amino acid sequence is represented using capitalized one-letter codes below the nucleotide sequence.

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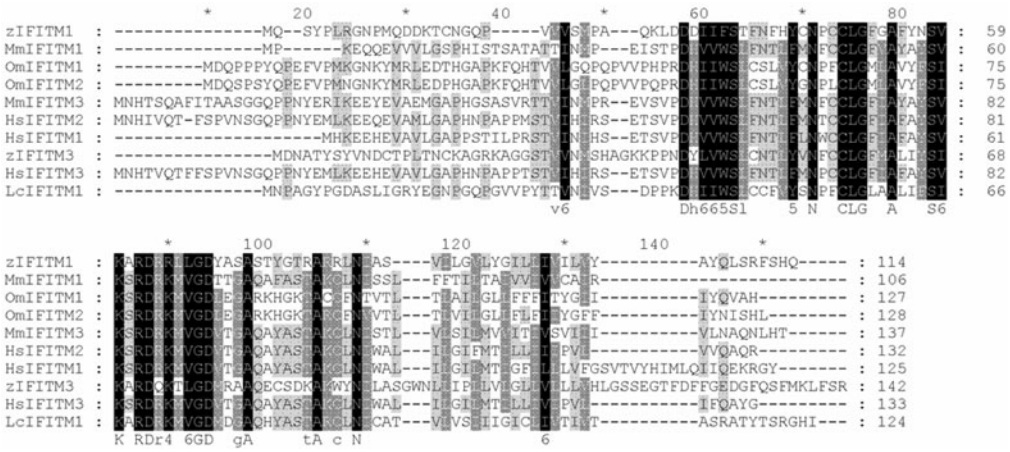

Figure 2 Multiple alignment of the deduced amino acid sequence of zebrafish zIFITM1 with zebrafish zIFITM3 (ADD38964), *Larimichthys crocea* LcIFITM1 (ABY55168), rainbow trout OmIFITM1 (CAC83757), OmIFITM2 (CAC85160), mouse MmIFITM1 (NP_081096), MmIFITM3 (NP_079654), human HsIFITM1 (CAA59337), HsIFITM2 (CAG46672) and HsIFITM3 (NP_066362) using ClustalX software. Grey shading indicates similar residues, whereas black shading indicates identical residues.

was identified in both proteins. This domain belongs to the CD225 family, which is characterized by an intracellular domain located between two conserved transmembrane domains. Structural analysis showed that zebrafish IFITM1 and IFITM3 consisted of five putative domains, including the N- and C-terminal extracellular domains, two putative transmembrane domains, and an intracellular domain between the two transmembrane domains. In regards to IFITM1, aa 1–36 of the N-terminal and aa 106–114 of the C-terminal domains are extracellular; aa 49–82 are intracellular, while aa 37–48 and aa 83–105 constitute the transmembrane regions. For IFITM3, aa 1–42 of the N-terminal and aa 118–142 of the C-terminal domains are extracellular; aa 65–97 are intracellular, while aa 43–64 and aa 98–117 constitute the transmembrane regions. According to the SignalP 3.0 server, neither protein is predicted to have a signal peptide or a signal anchor and are not likely to be secretory proteins.

To better understand the evolutionary relationship between zebrafish IFITM1 and IFITM3 and the IFITM family members in other species, a phylogenetic tree was constructed with the ClustalX and MEGA (Ver. 3.1) software using the neighbor-joining and maximum-likelihood methods. The results showed that zebrafish IFITM1 and IFITM3 lie within the IFITM group containing *Larimichthys crocea* IFITM1, rainbow trout IFITM1, IFITM2, mouse IFITM1, IFITM3, human IFITM1, IFITM2, and IFITM3. Zebrafish IFITM1 had a closer evolutionary relationship to *Larimichthys crocea* and rainbow trout IFITMs, while zebrafish IFITM3 was more closely related to mammalian IFITMs than zebrafish IFITM1 (Fig. 3).

Expression of zebrafish ifitm1 and ifitm3 mRNA in adult tissues

The adult tissue distribution of zebrafish *ifitm1* and *ifitm3* mRNA was determined using RT-PCR analysis. The results show that *ifitm1* is expressed in the ovary, testis, brain, muscle, liver and kidney, while *ifitm3* mRNA was only detected in the ovary (Fig. 4*A*). To examine the expression patterns of *ifitm1* and *ifitm3* mRNA during zebrafish oogenesis, we performed *in situ* hybridization analysis. *Ifitm1* mRNA was strongly expressed in the ooplasm from stage I to stage II, but became weaker in the stages III to IV oocyte. The decrease of the expression level of *ifitm1* mRNA may be due to the large accumulation of yolk within the cytoplasm during these stages (Fig. 4*Ba*, *Bb*). In addition, *ifitm3* mRNA was strongly expressed in the cytoplasm from stage I to stage II oocytes, and was decreased in stage III oocytes. However, it was ultimately localized in the cortical region beneath the plasma membrane of the lower hemisphere of the stage IV oocytes. In addition, the blue signal was not observed in the upper hemisphere of the stage IV oocytes (Fig. 4*C*). No signal was detected using control sense probes (Fig. 4*Bc*, *Cc*). Hematoxylin and eosin (HE) staining (Fig. 4*Bd*, *Cd*) was performed to characterize oocyte morphology.

Expression of zebrafish *ifitm1* and *ifitm3* mRNA during early embryonic development

The mRNA expression patterns of zebrafish *ifitm1* and *ifitm3* mRNA were investigated in embryos from the 1-cell stage to 5 days post-fertilization (dpf) using

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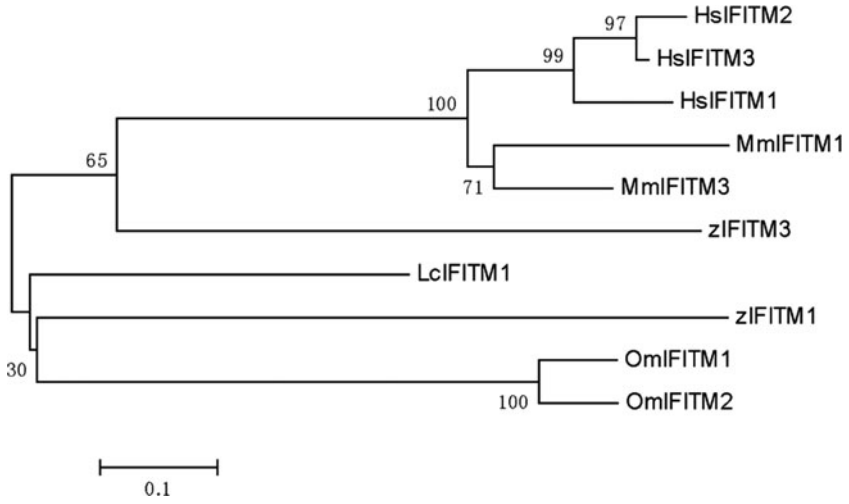

Figure 3 The phylogenetic tree of IFITM family proteins was constructed with the ClustalX and MEGA (Ver. 3.1) software using the neighbour-joining and maximum-likelihood methods. The numbers at the branches represent the bootstrap values (%) from 500 replicates.

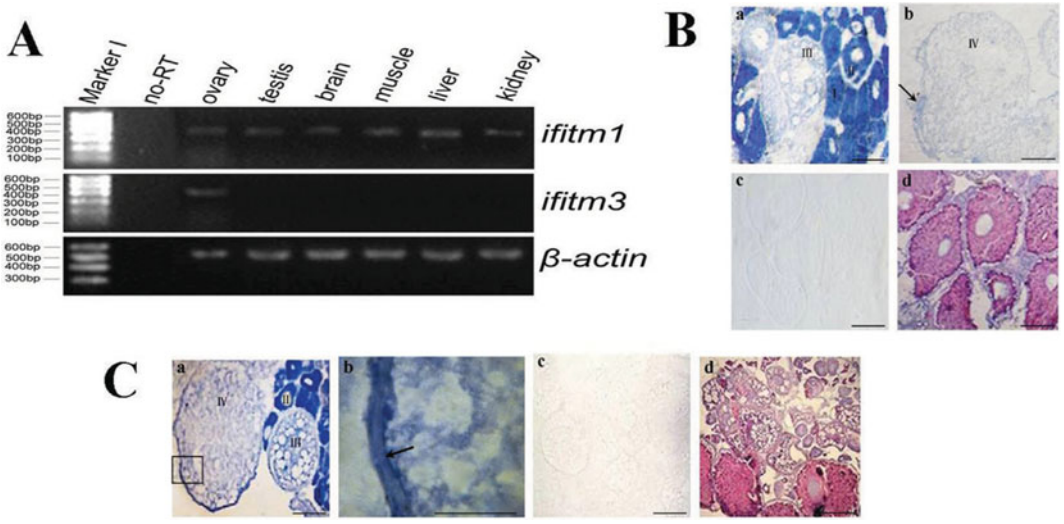

Figure 4 mRNA expression of zebrafish *ifitm1* and *ifitm3* in adult tissues. (*A*) Tissue analysis of zebrafish *ifitm1* and *ifitm3* mRNA expression using RT-PCR. RNA template and *ß-actin* were used as negative and internal controls, respectively. (*B*) Expression of zebrafish *ifitm1* mRNA in the ovary using *in situ* hybridization. (*Ba*, *Bb*) Based on antisense probe detection, intense positive signals were observed throughout the cytoplasm of stage I to stage II oocytes, and this signal decreased in the later stage oocytes. Arrow indicates the blue staining of the stage I oocyte; (*Bc*) negative control (sense probe); (*Bd*) HE staining. (*C*) Expression of zebrafish *ifitm3* mRNA in the ovary. (*Ca*) *ifitm3* mRNA was strongly expressed in the ooplasm from stage I to stage II and was mainly localized to the cortex region beneath the plasma membrane of stage IV oocytes; (*Cb*) partial enlargement of (*Ca*). The arrow points to the cortex region beneath the plasma membrane; (*Cc*) negative control (sense probe); (*Cd*) HE staining. I: stage I oocyte; II: stage II oocyte; III: stage III oocyte; IV: stage IV oocyte. Scale bars: 0.5 mm in all panels.

RT-PCR. The results show that the expression of *ifitm1* was first detected in shield stage embryos, and *ifitm3* mRNA was first detected in sphere stage embryos. Transcripts of these two genes were present in embryos at all later stages but were not detectable in embryos from the 1-cell to the 1k-cell stage (Fig. 5).

We further investigated the spatial expression patterns of the identified zebrafish *ifitm1* and *ifitm3* mRNAs in embryos from the 1-cell stage to 24 hpf via whole-mount *in situ* hybridization. The *ifitm1* mRNA was not expressed from the cleavage stage to the sphere stage, and the *ifitm1* transcripts were localized in the blastoderm cells of the shield stage

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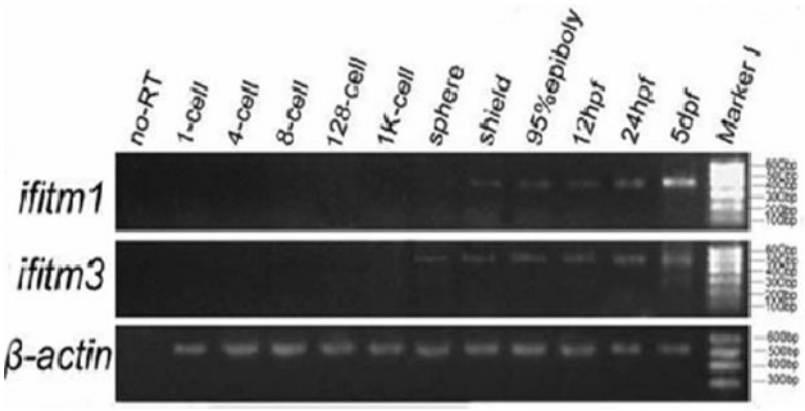

Figure 5 RT-PCR analysis of zebrafish *ifitm1* and *ifitm3* mRNA expression in embryos from the 1-cell stage to 5 dpf. *Ifitm1* was first expressed at the shield stage, and *ifitm3* mRNA was first expressed at the sphere stage.

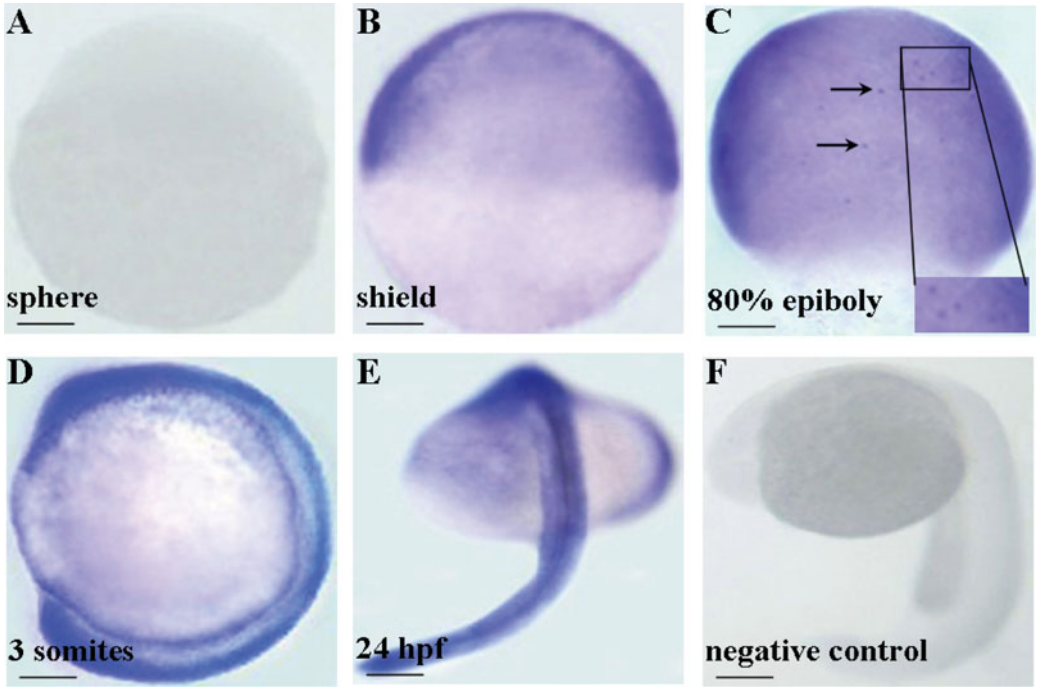

Figure 6 Expression of zebrafish *ifitm1* mRNA during early embryonic development. (*A*) No signal was detected at the sphere stage. (*B*) Zebrafish *ifitm1* expression began in the shield stage embryos. (*C*) *Ifitm1* transcripts appeared in PGCs at the 80% epiboly stage. The same regions are boxed and magnified. The arrows indicate the PGCs. (*D*, *E*) *Ifitm1* transcripts were detected in the head, trunk and tail. (*F*) No hybridization signals were detected in 24 hpf embryos using the sense probe (negative control). The signal was not detected from the 1-cell stage through the shield stage (data not shown). Scale bars: 0.2 mm in all panels.

embryos (Fig. 6*A*, *B*). *Ifitm1* mRNA was then detected in a ubiquitous expression, which, strikingly, included the PGCs at the 80% epiboly stage. Throughout the segmentation stage to embryos of 24 hpf, strong hybridization signals were mainly detected in the head, trunk and tail of the embryos, but could no longer be detected in the PGCs (Fig. 6*C*–*E*). In 24 hpf embryos, no signals were detected with the sense probes, which served as negative controls (Fig. 6*F*).

Unlike *ifitm1*, *ifitm3* expression was initially detected in sphere stage embryos and was widely expressed throughout the embryo, from the 70% epiboly to 24 hpf (Fig. 7*B*–*F*). Interestingly, at the bud stage, *ifitm3* transcripts were detected in PGCs on the dorsal side of the embryo. The signal later accumulated in PGCs in the caudal portion of 24 hpf embryos (Fig. 7*D*–*F*). The *ifitm1* and *ifitm3* transcripts were not detected in embryos from the 1-cell to the 1k-cell stage (data not

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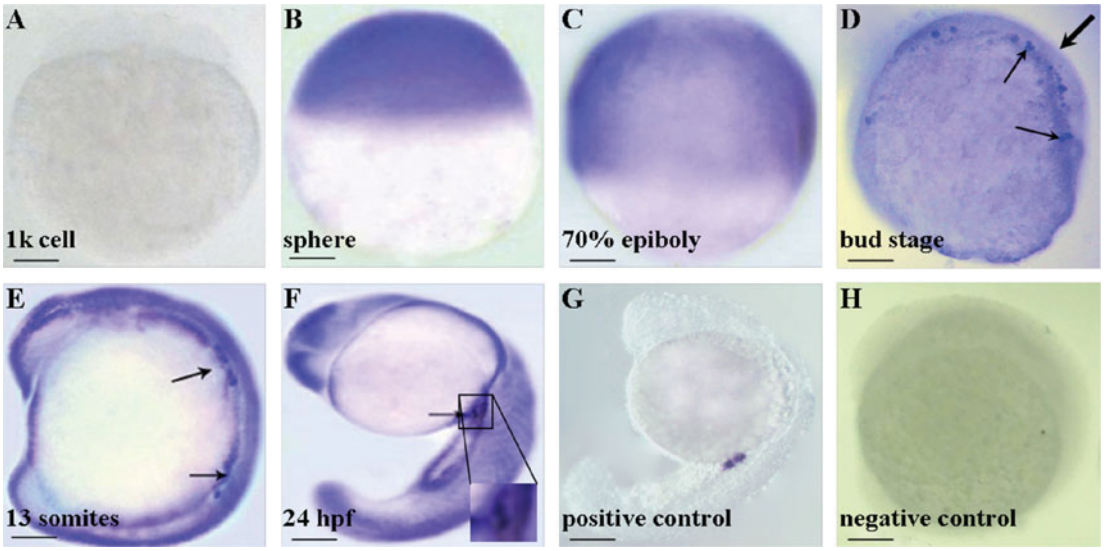

Figure 7 Expression of zebrafish *ifitm3 mRNA* during early embryonic development. (*A*) No signal was detected in the 1k-cell stage. (*B–F*) *Ifitm3* was first expressed in embryos at the sphere stage and became widely expressed in embryos from the 70% epiboly stage to 24 hpf. (*D–F*) The transcripts appeared in PGCs of embryos from the bud stage to 24 hpf. The same regions are boxed and magnified in (*F*). The signal was not detected from 1-cell stage until the sphere stage (data not shown). The thin arrows indicate the PGCs in (*D–F*). The thick arrow in (*D*) indicates the dorsal. (*G*) The transcripts appeared in PGCs of 24 hpf embryos using the *vasa* probe (positive control). (*H*) No hybridization signals were detected in somites stage embryos using the sense probe (negative control). Scale bars: 0.2 mm in all panels.

shown), which was consistent with the results of the RT-PCR analysis. Expression of the PGC marker gene *vasa* was used as a positive control to identify the PGCs in 24 hpf embryos (data not shown).

Discussion

Characterization of zebrafish ifitm1 and ifitm3

The *ifitm* gene family encodes proteins that contain two highly conserved putative transmembrane domains and an intracellular domain (Lewin *et al*., 1991; Tanaka & Matsui, 2002). In the present study, we cloned the 744 bp full-length cDNA of zebrafish *ifitm1*, which encodes a 114 aa protein, and the 702 bp full-length cDNA of *ifitm3*, which encodes a 142 aa protein. The deduced proteins of the two genes possess the stereotypical structural features of IFITM family members, including two conserved transmembrane domains with an intervening intracellular domain. Therefore, this analysis indicates that *ifitm1* and *ifitm3* belong to the IFITM family.

Phylogenetic analysis of the IFITM family proteins showed that zebrafish IFITM1 and IFITM3 lie within the IFITM group, consistent with their evolutionary conservation. Zebrafish IFITM1 has a closer evolutionary relationship to *Larimichthys crocea* and rainbow trout IFITMs, while zebrafish IFITM3 is more homologous to mammalian IFITMs than zebrafish IFITM1 (Fig. 3). We suggest that the functions of zebrafish IFITM3 may be distinct from those of IFITM1 but similar to those of mammalian IFITMs.

Expression of ifitm1 and ifitm3 mRNA in zebrafish adult tissues

Human IFITM1 is expressed in leucocytes and endothelial cells (Lewin *et al*., 1991). In teleost fish, the spleen, kidney and gills are major immune organs, which contain large numbers of immune cells including leucocytes and lymphocytes. In rainbow trout, *ifitm1* was highly expressed in the kidney, gills and liver (Johnson *et al*., 2006). In the present study, we demonstrated that zebrafish *ifitm1* mRNA was also expressed in the kidney and liver, organs associated with immunological functions, similar to rainbow trout *ifitm1* (Fig. 4A). The results of our study reveal that zebrafish *ifitm1* may play a role in tissues that perform immune or hematopoietic functions (Hoar *et al*., 1997).

The zebrafish *Dazl* gene plays pivotal roles during zebrafish germ cell development. The *Dazl* mRNA was detected in the cytoplasm of stage I oocytes, and was expressed in the region near the cortex of stage II oocytes. Interestingly, *Dazl* mRNA was ultimately localized at the vegetal cortex region (lower hemisphere), but was not found at the other regions (upper hemisphere) of the stage III to stage IV oocytes (Howley & Ho, 2000). In this study, we also demonstrated that zebrafish *ifitm1* and *ifitm3* mRNA were expressed during oogenesis. Particularly, *ifitm3*

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was only transcribed in the ovary, but not in other tissues (Fig. 4A). By *in situ* hybridization analysis, we found that *ifitm1* and *ifitm3* mRNA were strongly expressed in the ooplasm of stage I to II oocytes. And *ifitm3* was ultimately localized in the cortex region of the lower hemisphere of the stage IV oocytes. The blue signal was not observed in the upper hemisphere (Fig. 4C). Our results demonstrate that the expression patterns of the *ifitm3* are similar to that of *Dazl* during the oocytes development. Thus, we hypothesize that zebrafish *ifitm3* is required during oogenesis. Future studies will be performed to investigate the functions of *ifitm3* in the female gonad.

Expression of zebrafish *ifitm1* and *ifitm3* during early embryonic development

Mouse *ifitm* genes exhibit a dynamic temporal and spatial expression pattern during early embryonic development. *Ifitm1* is initially expressed in the epiblast at the early gastrula stage and is expressed in the epiblast, mesoderm and the PGCs later in gastrulation. Subsequently, *ifitm1* is expressed in the paraxial mesoderm, but not in the PGCs, throughout the segmentation period. Mouse *ifitm1* is required for the transition of the PGCs from the mesoderm into the endoderm and plays an essential role during organogenesis (Lange *et al*., 2003; Tanaka *et al*., 2005). In this study, we show that zebrafish *ifitm1* transcripts were localized in the blastoderm cells of the shield stage embryos (Fig. 6*A, B*). Throughout the segmentation phase (10.25–24 hpf), strong *ifitm1* expression was primarily detected in the head, trunk and tail of the embryo, but could no longer be detected in the PGCs (Fig. 6*D, E*). The expression pattern of *ifitm1* is similar to that of mouse *ifitm1* during early embryonic development. These results suggest that *ifitm1* may play an important role during embryonic development (Hoar *et al*., 1997). A zebrafish *nanos* gene is essential for the development of PGCs. Nanos1–GFP (green fluorescent protein) 3′-UTR are the labels of the PGCs in zebrafish. Marion Ko *et al.* demostrated that differential GFP expression levels can distinguish between zebrafish germ cells and somatic cells by Nanos1–GFP 3′-UTR during the gastrulation stages (Köprunner *et al*., 2001). In our study, zebrafish *ifitm1* mRNA is expressed not only in the embryonic cells, but also in the PGCs in the 80% epiboly stage which is similar to those of *nanos* at the 50% epiboly stage (the gastrulation stages) (Fig. 6C). These data suggest that *ifitm1* mRNA may play roles in the PGCs. Further investigation is required to understand the functions of *ifitm1* in the PGCs at the gastrulation stages.

PGCs have been identified morphologically and can be used to confirm the migratory pathway of the PGCs from their location during the gastrula stages to the gonadal anlagen in zebrafish. In zebrafish, *vasa* mRNA is the component of the germplasm (molecular marker). And the *vasa* mRNA is uniquely localized to the cleavage planes at the 2- and 4-cell stages. By the 1k-cell stage, the PGCs assemble into four clusters and then begin to proliferate from the sphere stage to the 30% epiboly stage. During gastrulation, they transform from round, immotile cells into a polarized migratory population localizing at the anterior and lateral boundaries of the mesoderm. The PGCs continue to migrate toward the intermediate region bordering the mesoderm throughout the segmentation period and are bilaterally restricted to the anterior end of the yolk extension at 24 hpf stage (Mich *et al*., 2009). *Vasa* mRNA is a germ cell-specific marker that is used to identify zebrafish PGCs. In these studies, we found that zebrafish *ifitm3* mRNA was initially expressed in migrating PGCs at gastrula stages. During somitogenesis, its expression was restricted to the PGCs located at the anterior and lateral boundaries of the mesoderm. *Ifitm3*-expressing cells then migrated toward the region of the genital ridge and stayed in that position of embryos of 24 hpf. Our work demonstrates that zebrafish *ifitm3* mRNA is expressed in the migrating PGCs, which is similar to that of *vasa* in the developing zebrafish PGCs. Therefore, we infer that *ifitm3* is required for the migration and localization of PGCs in zebrafish. Further work should be focused on performing gene knock-down, such as antisense morpholinos, to elucidate its functions.

In *Drosophila*, maternal mRNAs are stable after fertilization and play roles in embryonic development (Akam, 1987). Oocyte-derived mRNAs are degraded shortly after fertilization and cannot direct more than the first few cell divisions in mammals (Thompson *et al*., 1998). In our study, we found that the *ifitm3* was expressed in developing oocytes, but not expressed in early stage embryos. We suggest that *ifitm3* is degraded after fertilization, which is similar to some maternal mRNAs in mammals. About the mechanism of maternal mRNA degradation after fertilization, recently, it has been reported that MiR-430 participates in the degradation progress in zebrafish (Giraldez *et al*., 2006). Further studies are needed to determine the mechanism of degradation of *ifitm3* mRNA in early stage embryos.

In conclusion, we identified zebrafish *ifitm1* and *ifitm3* and have determined that their corresponding proteins contain the typical structural features of IFITM family members. *ifitm1* mRNA is expressed in the ovary, testis, brain, muscle, liver and kidney, while *ifitm3* mRNA is only expressed in the ovary. During early embryo development, *ifitm1* mRNA is broadly expressed in the somatic cells, whereas *ifitm3* is specifically expressed in the PGCs. Expression analysis

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suggests that *ifitm1* may play an important role in early oogenesis, and *ifitm3* may be crucial for PGC migration. In future studies, antisense morpholinos should be used to knock-down expression of *ifitm1* and *ifitm3* to elucidate their functions.

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