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Embryonic expression of zebrafish MiT family genes *tfe3b*, *tfeb*, and *tfec*

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Abstract

The MiT family comprises four genes in mammals: *Mitf*, *Tfe3*, *Tfeb*, and *Tfec*, which encode transcription factors of the basic-helix-loop-helix/leucine zipper class. Mitf is well-known for its essential role in the development of melanocytes, however the functions of the other members of this family, and of interactions between them, are less well understood. We have now characterized the complete set of *MiT* genes from zebrafish, which totals six instead of four. The zebrafish genome contain two *mitf* (*mitfa* and *mitfb*), two *tfe3* (*tfe3a* and *tfe3b*), and single *tfeb* and *tfec* genes; this distribution is shared with other teleosts. We present here the sequence and embryonic expression patterns for the zebrafish *tfe3b*, *tfeb* and *tfec* genes, and identify a new isoform of *tfe3a*. These findings will assist in elucidating the roles of the *MiT* gene family over the course of vertebrate evolution.

Keywords

zebrafish; Danio rerio; Mitf; transcription factor; gene duplication

INTRODUCTION

It has been suggested that over the course of metazoan evolution, organismal complexity has increased with the number of genes involved in transcriptional regulation rather than as a function of total gene number (Levine and Tjian, 2003). Sequence-specific DNA-binding proteins make up a subset of these regulators, and many families of such transcription factors have undergone expansion in multicellular organisms (Nowick and Stubbs, 2010). The potential for ever more complex regulation via networks of interacting transcription factors is especially evident for those proteins that function as dimers, such as those bearing helix-loop-helix (bHLH) domains, leucine zippers (bZIP), or both (bHLH-ZIP) (Amoutzias et al., 2008).

The MiT (microphthalmia/TFE) family comprises one small subfamily of basic helix-loop-helix/leucine zipper proteins that can form homodimers or heterodimers with each other but not with other bHLH or bHLH-ZIP proteins (Hemesath et al., 1994). *Mitf* was isolated as the gene responsible for the classic *microphthalmia* mouse mutant (Hodgkinson et al., 1993). Mitf is vital for the development of neural crest melanocytes as well as the pigmented retinal epithelium, and also plays an important role in osteoclasts and mast cells, at least in mammals (Steingrímsson et al., 2004). *Tfe3* and *Tfeb* were isolated as genes encoding E-box binding proteins in B cells (Beckmann et al., 1990; Carr and Sharp, 1990), while *Tfec* was originally identified based on similarity to *Tfe3* (Zhao et al., 1993). *Tfeb* is required for proper vascularization of the mouse placenta and inactivation of the gene results in

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embryonic lethality (Steingrímsson et al., 1998). *Tfe3* and *Tfec* are dispensable for early development (Steingrimsson et al., 2002), however *Tfe3* is involved in B cell activation (Merrell et al., 1997) and functions (with *Mitf*) in osteoclasts (Steingrimsson et al., 2002; Weilbaecher et al., 1998) and (with *Tfeb*) in T cells (Huan et al., 2006), while *Tfec* regulates gene expression in macrophages (Rehli et al., 2005; Zanocco-Marani et al., 2009). *Tfe3* and *Tfeb* have also been linked to the regulation of genes involved in metabolism (Nakagawa et al., 2006; Settembre et al., 2011). Chromosomal translocations involving *Tfe3* and *Tfeb* have been identified in a subset of renal carcinomas as well as other cancers (Davis and Fisher, 2007). Although expression of one isoform of *Mitf* is restricted to neural crest melanocytes , expression of other isoforms, and of other genes in the family, varies from tissue to tissue but is fairly broad (Kuiper et al., 2004; Steingrímsson et al., 2004).

The four-member *MiT* family of mammals likely arose via two rounds of whole genomic duplication (WGD) from a single ancestral gene (Simionato et al., 2007). *Drosophila* (Hallsson et al., 2004), *C. elegans* (Grove et al., 2009), and the basal chordate *Ciona intestinalis* (Satou et al., 2003) each contain single *MiT* genes. Zebrafish, as part of a vertebrate lineage, teleost fishes, thought to have undergone an additional WGD (Taylor et al., 2001), might be predicted to have more than four *MiT* genes reported in mammals. We previously identified duplicate *mitf* genes in the zebrafish (Lister et al., 2001). Here we describe the remainder of this family, which totals six members, and characterize the embryonic expression patterns of these genes.

RESULTS AND DISCUSSION

Isolation of additional members of the zebrafish MiT family

The identification of zebrafish orthologs of two of the four members of the MiT family has been previously reported; this included two *MITF* genes, *mitfa* and *mitfb*, as well as an ortholog of *TFE3*, *tfe3a* (Lister et al., 2001; Lister et al., 1999). Several molecular and bioinformatic approaches were taken to identify additional MiT family members encoded in the zebrafish genome, including degenerate PCR on cDNA and genomic DNA samples (data not shown), and mining of data from the zebrafish EST and genome sequencing projects. These efforts yielded an additional three genes, bringing the total to six. The same set of genes was identified in a bioinformatic survey of the basic helix-loop-helix transcription factor family of zebrafish (Wang et al., 2009), although this publication listed the size of the MiT subfamily as only five, treating the *mitf* duplicates as a single gene for reasons that are unclear.

Figure 1 shows an alignment of all six zebrafish MiT proteins. The predicted sizes of the newly identified proteins are: Tfe3b, 445 amino acids; Tfeb, 493 amino acids; and Tfec, 396 amino acids. In the course of this work we uncovered an error in our originally published sequence for *mitfb* (Lister et al., 2001). The cDNA reported was actually chimeric; the 5′ end of *mitfb* was conjoined to a portion of an incompletely processed transcript of *slc30a10*, in the opposite orientation. (This also led to the determination of an incorrect map position for *mitfb*, see below.) The alignment shows the corrected sequence for Mitfb, with a predicted size of 500 amino acids. It should be noted that the expression pattern observed by RNA in situ hybridization with a probe corresponding to the corrected sequence of *mitfb* is identical to that originally described for this gene (Lister et al., 2001), which was obtained from a probe that contained a portion of *slc30a10* (data not shown). Outside of the highly-conserved basic region and helix-loop-helix/leucine zipper domains, the predicted MiT proteins show the strongest similarity in the N-terminus, including charged/helix-forming (CH) and glutamine-rich/basic (QB) regions previously noted (Rehli et al., 1999), MAP kinase consensus phosphorylation site, and transcriptional activation domain (AD). In the C-terminus, a serine-rich region is conserved, although Mitfa and Mitfb lack the consensus

cAMP-dependent protein kinase phosphorylation site (Ubersax and Ferrell, 2007). The zebrafish-specific exon 5a found in both *mitfa* and *mitfb* is not found in any of the other MiT genes. Interestingly, the four residues making up the leucine zipper are also not completely conserved: while the third and fourth positions are leucines in every zebrafish MiT protein, the first position is a leucine in only 3 of 6, and the second position in 5 of 6. The predicted leucine zipper of Tfeb contains leucine at only 2 of the 4 positions.

A phylogenetic tree of MiT proteins (using entire protein sequences) from human, mouse, chicken and zebrafish, confirms the assignments of the three newly identified genes as a second *tfe3* (*tfe3b*; ZDB-GENE-010919-3), *tfeb* (ZDB-GENE-090807-3) and *tfec* (ZDB-GENE-041210-21)(Figure 2). The Mitf and Tfe3 proteins share the most similarity, with Tfeb being the most divergent member of the family, in agreement with a previous analysis of mammalian MiT proteins which used only the bHLH-ZIP domains (Rehli et al., 1999). To assess BLAST searches were made against other teleost genomes that have been or are in the process of being sequenced (http://www.ensembl.org/index.html), including the pufferfishes *Takifugu rubripes* and *Tetraodon nigroviridis*, Japanese medaka, *Oryzias latipes*, and stickleback *Gasterosteus aculeatus*. These findings are summarized in Table 1. Except for medaka, for which only one *tfe3* ortholog can be identified, all five teleost species contain two *mitf* genes, two *tfe3* genes, and single *tfeb* and *tfec* genes, suggesting that *tfeb* and *tfec* duplicate genes may have been lost prior to the teleost radiation.

Chromosomal assignments for all six zebrafish *MiT* genes can be made in the most recent zebafish genome assembly, Zv9 (http://www.ensembl.org/Danio_rerio/Info/Index; Table 1). *mitfa* and *tfe3a* had previously been mapped to chromosomes 6 and 8 respectively (Lister et al., 2001; Lister et al., 1999). Zebrafish *tfe3b* and *tfeb* reside on chromosome 11 while *tfec* is found on chromosome 4. *mitfb* was previously localized to linkage group 13 by radiation hybrid mapping (Lister et al., 2001) with primers which were derived from the 3′, *slc30a10* portion of the original clone, but the latest genome assembly places it on chromosome 23 instead. (*slc30a10* indeed maps to chromosome 13; http://www.ensembl.org/Danio_rerio/Gene/Summary?g=ENSDARG00000034877;r=13:53636202-53640824;t=ENSDART00000052076).

Multiple MiT isoforms are expressed during zebrafish embryonic and larval stages

Because some of the sequences we derived originated from genomic DNA or were merely predicted in silico, we first used RT/PCR to determine if these genes were expressed during embryogenesis. RNA was isolated from newly-fertilized embryos as well as 72 hours post-fertilization (hpf) larvae, first-strand cDNA synthesized and PCR was performed with gene-specific primer pairs flanking at least one intron. Primers against *tfe3a*, which is already known to be expressed during embryogenesis (Lister et al., 2001) were used as a positive control. As seen in Figure 3A, expression of *tfe3b*, *tfeb*, and *tfec* could all be detected at 72 hpf. Two of the three new genes, *tfe3b* and *tfeb*, as well as *tfe3a*, could be detected as maternal transcripts.

In various assemblies of the zebrafish genome, alternate first exons have been predicted for *tfe3a*, *tfeb*, and *tfec*. For zebrafish *tfec*, an alternate first exon is located 5.7 kilobases upstream of the first coding exon; transcripts beginning with this exon would begin translation with the first ATG in exon 2 to yield a polypeptide of 325 amino acids (Fig. 4). In the case of *tfe3a* and *tfeb*, the putative alternate first exon occurs between exons 3 and 4, and includes an initiation codon in the correct reading frame (Figure 4). The first exon of transcript *tfe3a-002* would encode 18 novel amino acids, and give rise to an isoform with a total length of 292 amino acids, while the first exon of transcript *tfeb-002* encodes 28 amino acids, for a protein with a total length of 327 amino acids. Interestingly, an alternative promoter and first exon has been described in the analogous position in the human *TFEC*

gene (Kuiper et al., 2004); moreover, the polypeptides encoded by this exon in zebrafish *tfeb* and the human *TFEC* show significant similarity (Fig. 4). The shorter isoforms of Tfe3a and Tfeb would lack the conserved transcriptional activation domain encoded in exon 3 and might act to negatively regulate target gene expression (Roman et al., 1991; Zhao et al., 1993).

In the case of *tfeb*, support for the alternate isoform can be found in GenBank (Accession #CF265795) as an EST from a whole adult zebrafish library. To determine if this or other predicted isoforms are in fact expressed during embryogenesis we designed primers for their detection by PCR, along with primers specific to the isoforms for which there was already evidence from cDNA. All three alternate transcripts were detected in cDNA from 72 hpf larvae (Figure 3B). A *tfe3a* transcript starting from the alternate first exon but lacking exon 6 was detected at a low level (Fig. 3B, arrow); skipping this exon produces a frameshift that would lead to premature termination. Additionally, a *tfec* EST (I.M.A.G.E. clone #7226661) we obtained and sequenced was found to be full-length except for the absence of exon 7, which preserves the reading frame but encodes a protein missing the second helix and most of the adjacent loop and leucine zipper domains. To determine whether or not this was likely to represent an appreciable fraction of *tfec* transcripts, we conducted PCR with primers flanking the missing exon, however we were unable to detect a product representing the exon-skipping event at any stage tested (data not shown). We conclude that this clone represents a rare mis-splicing event rather than a transcript expressed during embryogenesis at significant levels.

Finally, cloning and sequencing of *tfe3b* RT/PCR products indicated use of multiple alternate splice donor sites in exon 4 (Figure 4). These alternate splice forms encode proteins with an additional two or six amino acids. Interestingly, this is the same position where the zebrafish *mitf*s, *mitfa* and *mitfb*, contain an additional exon (Fig. 1) and the melanocyte specific M-isoform of mammalian Mitfs contains an alternatively spliced six amino acid stretch which influences DNA binding affinity (Hemesath et al., 1994).

Embryonic expression of tfe3b, tfeb and tfec

Whole mount RNA in situ hybridization was used to determine the spatial expression of the newly isolated *MiT* genes during the first three days of embryogenesis. Each displayed a spatially-restricted pattern, as described in detail below.

tfe3b

Low level *tfe3b* expression, perhaps derived from maternal transcripts, is present throughout the embryo at the beginning of gastrulation and persists through early somitogenesis (Fig. 5A-C and data not shown). First likely zygotic expression of *tfe3b* is detected in the polster at the 3-somite stage (Fig. 5A). By six somites *tfe3b* begins to be expressed in scattered cells of the epidermis over the yolk (Fig. 5B,C) that are suggestive of ionocytes (Janicke et al., 2010). Beginning around the 16-somite stage, *tfe3b* transcripts can be detected in the lens placode (Fig.5D), and by 24 hours post-fertilization in the lens itself (Fig.5E,H). *tfe3b* expression can be seen in the intermediate cell mass (ICM) at 24 hpf (Fig.5F) but this has largely subsided by 36 hpf (Fig.5G). Expression can be observed at the mid/hindbrain boundary at 36 hpf (Fig.5H). *tfe3b* is expressed diffusely through much of the head by 48 hpf, and more intensely in the otic epithelium, gut, and ionocytes (Fig.5I) as well as olfactory pits and mouth (Fig.5J). Expression continues at 60 hours in ear structures, notably the dorsal otic vesicle protrusions that will begin to establish the semicircular canals (Haddon and Lewis, 1996) (Fig.5K,K'). While lens expression of *tfe3b* has declined by 72 hpf, expression can be detected at the ciliary margin (Fig.6L, arrow). *tfe3b* expression in the pharyngeal arches at this stage (Fig.5L,M) is consistent with the identity of these cells as

ionocytes associated with the developing gills (Varsamos et al., 2005). By 72 hpf *tfe3b* expression is also apparent in cartilage of the pectoral fins (Fig.5M).

tfeb

tfeb can be detected as early as the 8 somite stage, first in small groups of cells at the posterior edge of the optic primordia (Fig.6A) and a short time later in paraxial and lateral plate mesoderm (Fig.6B,C). By the 18-somite stage *tfeb* expression can be seen in the pronephros and the medial portion of the posterior somites (Fig.6D,F). *tfeb* expression is observed in the pancreas beginning at 24 hpf (Fig.6E) but has declined in this tissue by 48 hours (Fig.6K). Expression is likewise observed in the heart between 24 and 60 hpf (Fig. 5E,H,J,M) but declines thereafter; interestingly, sites of embryonic expression reported for a mouse *Tfeb* gene trap line included heart and kidney (McClive et al., 1998). Scattered *tfeb* expression along the dorsal midline is evident by 36hpf (Fig.6G,H) and can be seen in discrete cells, possibly iridophores (see below), at 60 hpf (Fig.6L). *tfeb* is expressed in the pectoral fin cartilage beginning around 40 hpf (Fig.6I). Small clusters of *tfeb*-expressing cells are apparent at the dorsal edge of the otic vesicle by 48 hpf (Fig.6J) and additional cells appear in the ear by 60 (Fig.6M,N) and 72 hpf (Fig.6O). Scattered *tfeb*-expressing cells are also observed deep in the brain along what may be commisural tracts (Fig.5K), and in the ventral hindbrain (Fig.5N,O); these cells may be macrophages or oligodendrocytes. Expression in the kidney continues to be robust at 60 hpf (L, inset; N), and by 72hpf the strongest expression of *tfeb* is observed in the pectoral fins and proximal convoluted tubules of the pronephros (Fig.5O,P,R). Expression can also be seen in the pharyngeal arches (Fig. 6P), optic fissure (Fig.6Q) and jaw musculature (Fig.6R).

tfec

tfec is first seen in the posterior optic primordia and a few cranial neural crest cells at the 8-somite stage (Fig.7A). This expression expands and begins to include trunk neural crest between 10 and 12 somites (Fig.7B-D). Between 16 somites and 24 hours, *tfec* becomes expressed throughout the retinal pigment epithelium (RPE) and peak neural crest expression shifts from rostral to caudal domains (Fig.7E-G). Although *Tfec* expression in mammalian neural crest has not been reported, *Tfec* is expressed in mouse RPE (Rowan et al., 2004). Beginning at around the 20-somite stage expression can be seen in the intermediate cell mass; this expression can still be observed at 36 hpf (Fig.7L) but is mostly gone by 60 hpf (Fig.7M). Around 24 hpf, *tfec*-expressing cells begin to form clusters over the yolk on either side of the midline (Fig.7H-J); at the same time, the posterior dorsal expression domain begins to thin out and to shift anteriorly. This pattern is strongly suggestive of neural crest-derived iridophore pigment cells, which is further supported by continued expression in the bilateral patches over the yolk from 48 to 72 hpf (Fig.7O-Q, arrowheads). Scattered expressing cells can be seen on the dorsal surface of the head at 36 hpf (Fig.7K), another region from which iridophores will later arise. Expression of *tfec* in the eye persists through 72 hpf (Fig.7N-Q). At 48 hpf a cluster of *tfec*-expressing cells can be seen just right of the midline over the yolk (Fig.7O). Expression increases in this domain at 60 hpf (Fig.7P) and by 72 hpf these cells appear to be forming into the swim bladder (Fig.7Q).

Summary and Conclusions

We have shown here that the zebrafish MiT family consists of six genes compared to the four of mammals; duplicate *mitf* and *tfe3* genes have been retained, but not *tfeb* or *tfec*. Surveys of avian genomes have suggested that they contain only two or three MiT genes (Liu et al., 2011). Single Mitf family members have been found in the demosponge *Amphimedon queenslandica*, the lancelet *Branchiostoma floridae*, and the tunicate *Ciona intestinalis* (Satou et al., 2003; Simionato et al., 2007). What function this single gene serves in any of these organisms is not known; what new functions the expanded gene family may

have acquired in more complex metazoans, and specifically how the six MiT genes may contribute to phenotypic diversity in teleost fishes, remain only partially answered questions.

The genes of the zebrafish *MiT* family display embryonic expression patterns that are surprisingly tissue-restricted and complex given the broad expression reported for their mammalian orthologs (Kuiper et al., 2004; Steingrímsson et al., 2004). While expression of some of the zebrafish *MiT* genes is clearly conserved with other organisms (e.g. *mitfa* in melanogenic cells, (Lister et al., 1999); *tfec* in RPE and *tfeb* in heart and kidney, this study), other aspects of expression do not appear to be conserved: for example, with the possible exception of *tfe3a* (Lister et al., 2001), expression of *MiT* genes does not appear to persist in hematopoietic tissues, although it may be that *MiT* gene expression is restricted to definitive hematopoiesis (de Jong and Zon, 2005). Likewise, the first osteoclasts do not appear in zebrafish until approximately 12 days post-fertilization (Hammond and Schulte-Merker, 2009), much later than our analysis here.

In several tissues, the expression of two or more genes appears to overlap and therefore the potential for regulation by heterodimeric complexes exist (Amoutzias et al., 2008). These include the intermediate cell mass (*tfe3a*, *tfe3b*, *tfeb*, *tfec*); retinal pigment epithelium (*mitfa*, *mitfb*, *tfec*), lens (*tfe3a*, *tfeb*), and ciliary margin (*mitfb*, *tfec*, *tfe3b*, *tfeb*) of the eye; ear, pectoral fins and pharyngeal arches (*tfe3b*, *tfeb*); and a subset of neural crest cells (*tfeb*, *tfec*). Much more extensive analysis with fluorescent in situ hybridization will be required to verify co-localization within any of these tissues. Such analysis, along with the generation of mutations and gene knockdown reagents in each member of the family, and overexpression of wild-type and dominant-interfering MiT proteins, will comprise the next steps in unraveling the functions of this set of genes.

EXPERIMENTAL PROCEDURES

Animal use

All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Embryos were obtained from natural matings of adults from the AB strain, or AB/WIK hybrids and staged according to Kimmel et al., 1995. Embryos were incubated in the presence of 0.003% N-phenylthiourea (PTU; Sigma P7629) to prevent melanin synthesis.

Cloning and phylogenetic analysis

A fragment of *tfe3b* was originally identified by degenerate PCR using primers and protocol previously described (Lister et al., 1999). A partial *tfeb* cDNA was first identified as an EST (fc83b12) and was subsequently extended by 5′SMART-RACE (Clontech) with the gene-specific nested primers B5RACE: 5′-TGG AGG TGG TTC TCC TCA GGG CTG C-3′, and B5NRACE: 5′-GGA GTT GCT GGG GAG GCC GTG G-3′. A fragment of *tfec* exon 8 was identified by performing PCR on genomic DNA with the degenerate primers F4: 5′- CCA GGA GCT GGA GAT NCA RGC NMG-3′ and R3: 5′-GGG GGA CAC GGA GGA CAR NAR NGG RTC -3′, designed using CODEHOP (Rose et al., 1998), and was extended by 5′SMART-RACE with the gene-specific nested primers C5RACE: 5′-GAG GTG AGA TGA CAG TTC GAC TGT GCC CA-3′, and C5NRACE: 5′-ATG CAG TCA TAC TGG GCA AAC CAT GAG CA-3′. PCR products were subcloned and sequenced, and used in BLAST searches against genome sequencing data as it became available. A full-length *tfe3b* clone to be used as a riboprobe template (see below) was obtained by RT/PCR of 72 hpf first-strand cDNA with the following primers: forward, 5′-GAA TTC CAC CAT GTC TTC GAG AGT GTT GCT C-3′; reverse, 5′-ACT AGT CTA ACA AGC ATG CTG GTT CTC CTC C-3′, followed by TOPO TA cloning (Invitrogen). The *tfec* cDNA missing exon 7

(I.M.A.G.E. clone #7226661, obtained from Open Biosystems) was excised from pME18S-FL3 with EcoRI and Spe I and cloned into pBluescript SK- (Stratagene).

Nucleotide sequences have been submitted to GenBank with the following accession numbers: *tfe3a* isoform 2, JN105111; *tfe3b* isoform 1 JN105115; *tfe3b* isoform 2, JN105116; *tfe3b* isoform 3, JN105117; *tfeb* isoform 1, JN105112; *tfeb* isoform 2, JN105113; *tfec* isoform 2 JN105114; *mitfb* (corrected complete coding sequence), JN105118. The accession number for *tfec* isoform 1 is NM_001030105.

The zebrafish MiT family proteins were aligned using ClustalW in the VectorNTI AlignX package (Invitrogen). Zebrafish MiTs were also aligned with mouse, human and chicken MiT proteins using ClustalW. The accession number for the sequences used were: human MITF isoform 1, NP_937802; mouse Mitf isoform 2, NP_032627; chicken Mitf, BAA25648; human TFE3, NP_006512; mouse Tfe3, NP_766060; human TFEB, NP_001161299; mouse Tfeb isoform a, NP_035679; chicken Tfeb, NP_001026093; human TFEC, NP_036384; mouse Tfec, NP_112475; chicken Tfec, Q5XFQ6; zebrafish Mitfa, NP_570998; zebrafish Tfe3a, NP_571923; zebrafish Tfec, NP_001025276; yeast Rtg3p, NP_009447. The resulting msf file was used to build a phylogenetic tree with 1000 bootstraps using the protdist, neighbor, and consense programs of PHYLIP 3.69 (Felsenstein, 2005). The tree was drawn using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

BLAST searches were made against other teleost genomes available through Ensembl (http://www.ensembl.org/index.html): *Takifugu rubripes* (assembly 4.0, June 2005), *Tetraodon nigroviridis* (assembly 7, April 2003), *Oryzias latipes* (MEDAKA1, October 2005), *Gasterosteus aculeatus* (BROAD S1, February 2006).

RT/PCR

For expression analysis by RT/PCR, RNA was prepared from 25 embryos at each timepoint using Trizol (Invitrogen) and reverse-transcribed with oligo-dT (Bioline) and AccuScript High Fidelity RT (Stratagene). Primers were designed with the assistance of Primer3, v.0.4.0 (http://frodo.wi.mit.edu/primer3/) and are listed in Table 2. Agarose gel images were captured using an AlphaImager (Alpha Innotech).

In situ hybridization

Digoxigenin-labeled RNA probes were generated from the following plasmid templates: pCR4-tfe3b (nt 1-1356), linearized with Not I and transcribed with T3 RNA polymerase; pCR-tfeb 5′RACE (nt 63-1103), linearized with SpeI and transcribed with T7 RNA polymerase; pBS-tfec (nt 122-749, 898-1410), linearized with EcoRI and transcribed with T3 RNA polymerase. In situ hybridization was carried out as described previously (C. Thisse & B. Thisse, 2008). After clearing, samples were equilibrated in 50% glycerol/1x PBS for photography on a SZX12 dissecting stereomicroscope with DP70 camera (Olympus). For histology, already-stained specimens were equilibrated in 30% sucrose in 1x PBS, embedded in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA) and cryosectioned at 18 μM. Sections were mounted in 1x PBS and photographed with a Spot RT CCD camera (Diagnostic Instruments) on a Nikon ECLIPSE E800M microscope. All images were processed using Photoshop CS (Adobe).

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Figure 1. Multiple sequence alignment of the zebrafish MiT family

Alignment of amino acids sequences derived from ClustalW is shown. Red text on yellow background indicates highest similarity, followed by blue on cyan, black on green, and green on white. Vertical red lines indicate exon boundaries, and the basic region helix-loop-helix domain, and leucines comprising the leucine zipper are indicated. Abbreviations: CH, charged helical domain; QB, glutamine-rich, basic domain; MAPK, consensus MAP kinase phosphorylation site; AD, transcriptional activation domain; PKA, consensus cAMP-dependent protein kinase phosphorylation site.

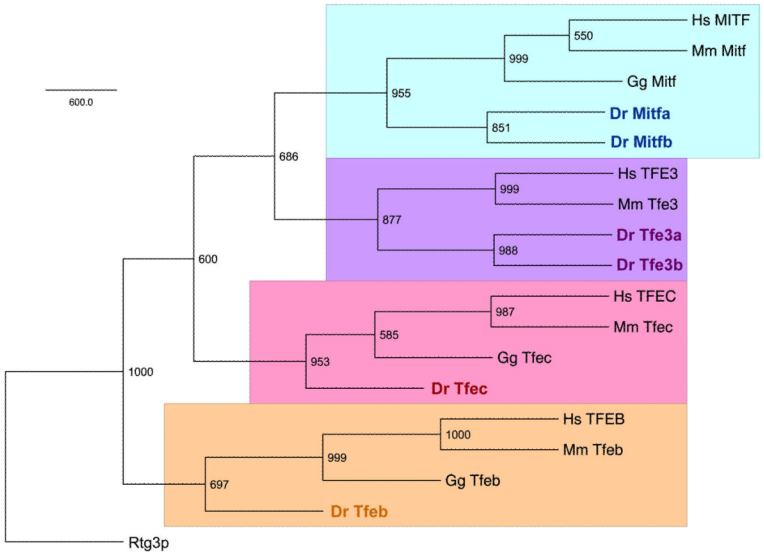

Figure 2. Phylogenetic tree of human, mouse, chicken, and zebrafish MiT proteins

A majority rule consensus tree is shown with bootstrap values indicated at nodes. The four MiT clades are each highlighted a different color. Abbreviations: Hs, *Homo sapiens*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Dr, *Danio rerio*.

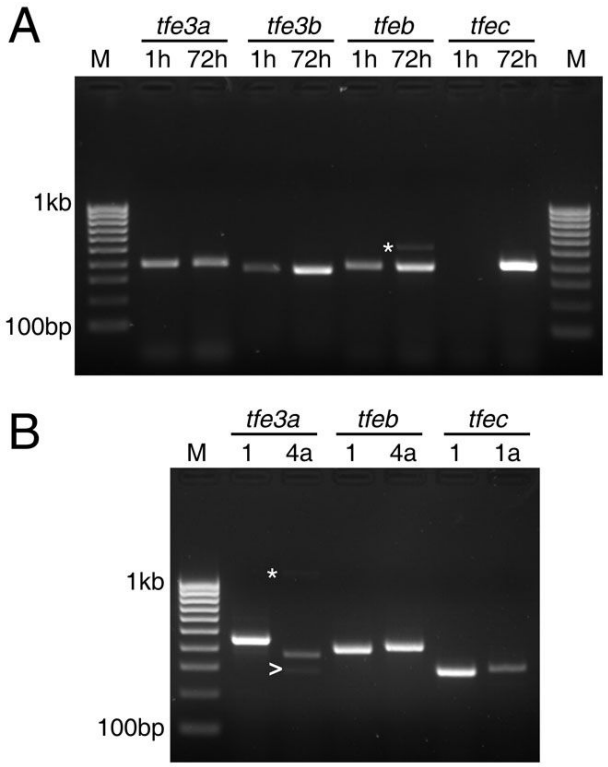

Figure 3. MiT isoforms expressed during embryogenesis

A. RT/PCR demonstrating expression of *MiT* genes at 1 and 72 hours post-fertilization. M, 100 basepair ladder markers. B. RT/PCR with isoform-specific primers at 72 hpf. Asterisks indicate non-specific products; arrow indicates *tfe3a* transcript lacking exon 6.

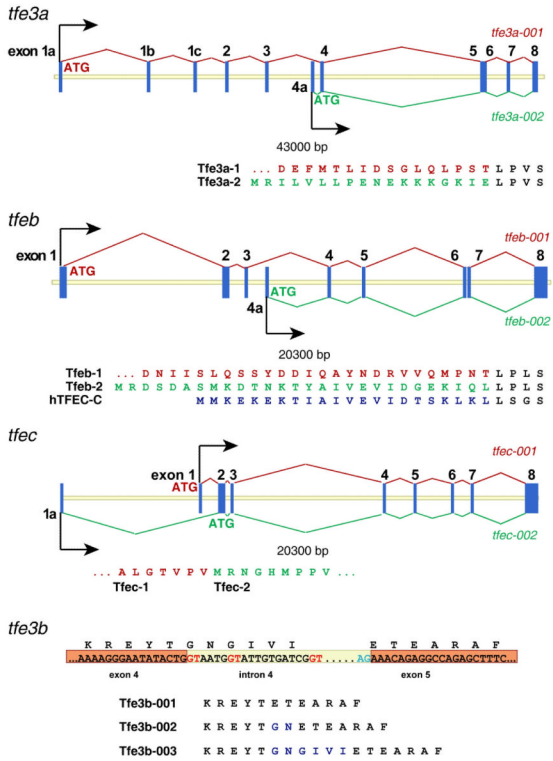

Figure 4. Structure of zebrafish MiT genes with alternative transcripts

tfe3a and *tfeb* each produce a transcript from an alternate promoter/first exon located between exon 3 and 4. Coding sequence of the alternate exon is shown beneath, along with the longer isoform and, for Tfeb, the human TFEC-C isoform. For *tfe3b*, alternate splice donor sites (shown in red) produce mature transcripts encoding an additional 2 (Tfe3b-002) or 6 (Tfe3b-003) amino acids.

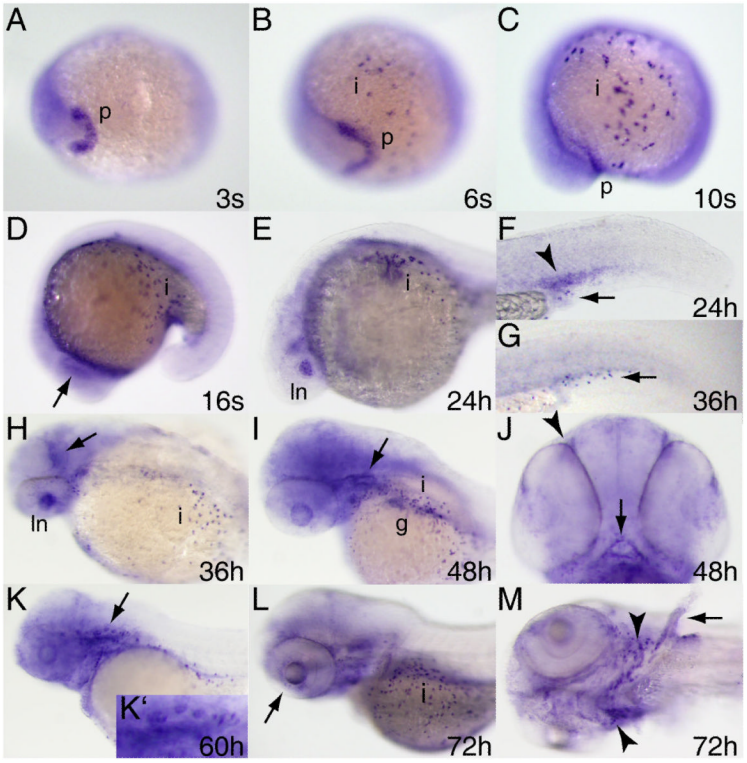

Figure 5. Expression pattern of *tfe3b* during embryogenesis

Whole mount RNA in situ hybridization was performed on embryos at the stages indicated. Expression begins in the polster (A,B) and is seen shortly thereafter in ionocytes (B,C). Expression is observed in the lens placode at the 16 somite stage (D, arrow), and thereafter (up to 36 hpf) in the lens itself (E,H). Expression in the intermediate cell mass (arrowhead, F) is seen at 24 hpf but has disappeared by 36 hpf (G; arrows indicate ionocytes). *tfe3b* is expressed at the mid-hindbrain boundary at 36 hpf (arrow, H) and in the otic epithelium (arrow, I) as well as gut tissue and ionocytes, and more diffusely through the head at 48 hpf. Expression is also seen in the olfactory pits (arrowhead, J) and mouth (arrow, J) at this stage. At 60 hpf, *tfe3b* is expressed in the epithelial protrusions of the dorsal otocyst (K, arrow; K', higher magnification). L) Ciliary margin expression (arrow). M) Expression in ionocytes associated with the branchial arches (arrowheads) and in pectoral fin cartilage (arrow). All views lateral except A,B,J, ventral view; H,I, dorsolateral view; M, ventrolateral view. Abbreviations: p, polster; i, ionocytes; ln, lens; g, gut.

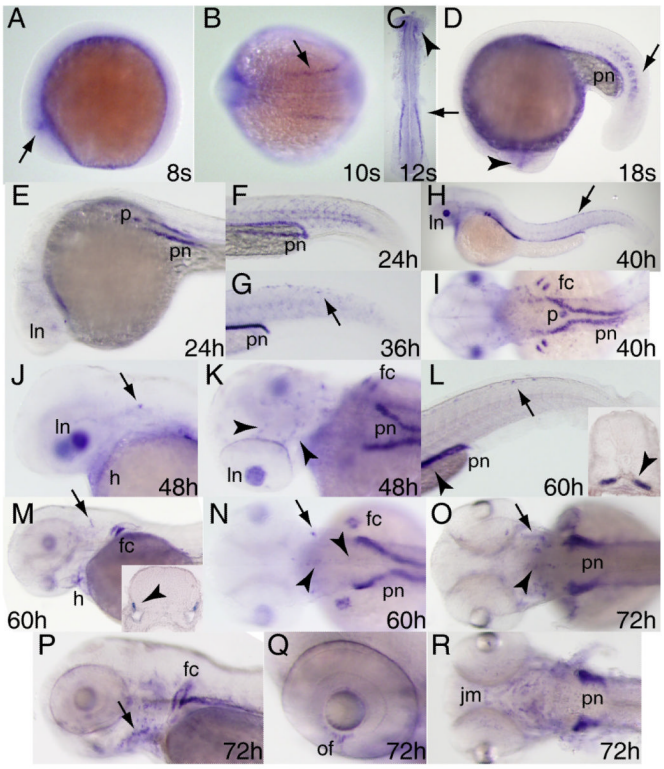

Figure 6. Expression pattern of *tfeb* during embryogenesis

Whole mount RNA in situ hybridization was performed on embryos at the stages indicated. Expression is first observed in the eye (arrowheads in A,C,D), and shortly thereafter in the paraxial and lateral plate mesoderm (arrows in B-D). Lens expression increases between 24 and 48 hpf (E,J) and diminishes thereafter. *tfeb* expression is observed in the pronephros along most of its length from 18 somites (D, shown in transverse section in inset L, arrowhead) up to 72 hpf when it is concentrated in the proximal convoluted tubules (O,R). Expression in the presumptive pancreas is seen at 24 hpf (E) and 40 hpf (I) but declines thereafter (K,N). *tfeb* is expressed in cells along the dorsal midline (arrows in H,L), scattered cells associated with the otic capsule (arrows in J,M-O; inset in M shows transverse section through otic capsule, arrowhead indicates expressing cells) cells in the brain (arrowheads in K,N,O) and associated with the pharyngeal arches (arrow, P). Expression is also seen at 72 hpf in the optic fissure (of, Q) and jaw musculature (jm, R). A,D-H,J,L,M,P,Q, lateral views; B,I,N,O, dorsal views; K, dorsolateral view; R, ventral view. Abbreviations: pn, pronephros; ln, lens; p, pancreas; fc, fin cartilage; h, heart.

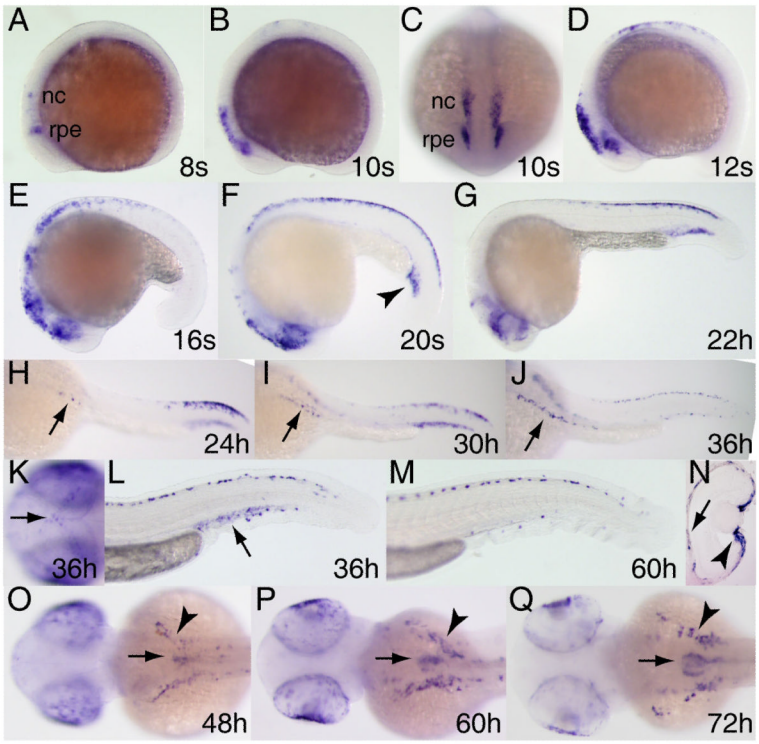

Figure 7. Expression pattern of *tfec* during embryogenesis

Whole mount RNA in situ hybridization was performed on embryos at the stages indicated. Expression begins in the posterior eye region/presumptive retinal pigment epithelium and a few neural crest cells at the 8-somite stage (A) and expands during somitogenesis (B-F). *tfec*-expressing cells populate bilateral patches over the yolk beginning at 24 hpf (arrows, H-J), increasing in number and extent through 72 hpf (arrowheads, O-Q), and are also found on top of the head (arrow, K) and dorso- and ventromedially in the trunk and tail (L,M), all positions characteristic of iridophores. Expression in the intermediate cell mass is seen by the 20 somite stage (arrowhead, F), has weakened by 36 hpf (arrowhead, L) and is gone by 60 hpf (M). Expression in the retinal pigment epithelium (arrow) and ciliary margin (arrowhead) is observed in transverse section of the eye at 60 hpf (N, dorsal is to the top). Beginning at 48 hpf, expression is observed in the developing swim bladder (arrows, O-Q). Lateral views A,B,D-G,L,M; frontal view, C; dorsolateral views, H-J; dorsal views K,O-Q. Abbreviations: nc, neural crest; rpe, (presumptive) retinal pigment epithelium.

Table 1

Teleost MiT genes

Species	Ensembl gene ID	map location	ortholog
Danio rerio	ENSDARG00000003732	chr 6	*mitfa*
	ENSDARG00000076049	chr 23	*mitfb*
	ENSDARG00000004729	chr 8	*tfe3a*
	ENSDARG00000019457	chr 11	*tfe3b*
	ENSDARG00000010794	chr 11	*tfeb*
	ENSDARG00000008423	chr 4	*tfec*
Oryzias latipes	ENSORLG00000003123	chr 5	*mitfa*
	ENSORLG00000013461	chr 7	*mitfb*
	ENSORLG00000014134	chr 5	*tfe3*
	ENSORLG00000015866	chr 5	*tfeb*
	ENSORLG00000010461	chr 23	*tfec*
Takifugu rubripes	ENSTRUG00000001600	scaffold 150	*mitfa*
	ENSTRUG00000012080	scaffold 60	*mitfb*
	ENSTRUG00000013198	scaffold 79	*tfe3a*
	ENSTRUG00000005584	scaffold 215	*tfe3b*
	ENSTRUG00000000653	scaffold 79	*tfeb*
	ENSTRUG00000016827	scaffold 8	*tfec* *
	ENSTRUG00000017315	scaffold 2576	*tfec* *
Tetraodon nigroviridis	ENSTNIG00000007360	chr 11	*mitfa*
	ENSTNIG00000012564	chr 9	*mitfb*
	ENSTNIG00000007324	chr 11	*tfe3a*
	ENSTNIG00000019469	unassigned	*tfe3b*
	ENSTNIG00000007281	chr 11	*tfeb*
	ENSTNIG00000018518	chr 19	*tfec*
Gasterosteus aculeatus	ENSGACG00000011875	group XVII	*mitfa*
	ENSGACG00000003425	group XII	*mitfb*
	ENSGACG00000010355	group XVII	*tfe3a*
	ENSGACG00000000794	scaffold 141	*tfe3b*
	ENSGACG00000000364	scaffold 27	*tfeb*
	ENSGACG00000018910	group IV	*tfec*

* Coding regions of ENSTRUG00000016827 and ENSTRUG00000017315 are nearly identical at the nucleotide level and appear to represent the same gene.

Table 2
Additional oligonucleotides used in this study

tfe3a 3′ forward	5′-CGC ATT CAG GAA CTG GAA AT-3′
tfe3a 3′ reverse	5′-TTT TGA GGC TCC TGG AGA AA-3′
tfe3b 3′ forward	5′-AAA TAC AAG CAC GCC ACC AT-3′
tfe3b 3′ reverse	5′-GAG AAG AGC ACA TCC GAA CC-3′
tfeb 3′ forward	5′-TAA ACG CAT GCA GAA GGA TG-3′
tfeb 3′ reverse	5′-TCG CAC AAG TCC AGA GAC TG-3′
tfec 3′ forward	5′-GAG CAG CAA CAC GCT CGT G-3′
tfec 3′ reverse	5′-CCG CTG GTG CTC CTC CTT-3′
tfe3a1a 5′ forward	5′-AGC CCG CTT GCT GTG CTC-3′
tfe3a4a 5′ forward	5′-GAG GAT TCT GGT TTT ACT GC-3′
tfe3a 5′ reverse	5′-TGC AGC TTG CGG ATG TAG TCG-3′
tfeb1 5′ forward	5′-CAC GAG AAC GAG ATG GAT GA-3′
tfeb4a 5′ forward	5′-GGA GAG GTG GAG GGA GAA CG-3′
tfeb 5′ reverse	5′-CCA CAG AGG CCC TCA GAA TA-3′
tfec1 5′ forward	5′-CCG ACT GTA GTT TCT ACA AGA TGA GA-3′
tfec1a 5′ forward	5′-ATG AGC CGC TGC TGT GTG GT-3′
tfec 5′ reverse	5′-CTC ACT GTC TTG ATT GTT GGC-3′