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Otx but Not Mitf Transcription Factors Are Required for Zebrafish Retinal Pigment Epithelium Development

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Introduction

The retinal pigmented epithelium (RPE) is a monolayer of cells that lies between the retina and choroid and serves a variety of functions in the developing and mature eye [1]. These include providing nutritional support to the retina, regulating oxidative stress, and maintaining the outer segments of the photoreceptors. Defects in the RPE are implicated in a number of vision-related disorders such as age-related macular degeneration and retinitis pigmentosa [2,3]. The RPE also plays a vital role in early eye development. During the formation of the optic cup, the RPE encompasses the retina and completes the formation of the cup with the anterior and posterior sides of the presumptive optic cup joining together at the ventral optic fissure [4]. Failure of the RPE to develop properly at the optic fissure can lead to a gap in the ocular layers known as a coloboma, and in more severe instances can lead to retinal degeneration resulting in microphthalmia or anophthalmia [5]. Microphthalmia and the more severe anophthalmia are diagnosed in up to 25% of children with severe vision impairments and in approximately 1 of every 3,500 live births [6]. The zebrafish presents an excellent model to study these ocular disorders due to the similarities between human and zebrafish eye development, cell composition, and morphology [7].

Unlike the pigmented cells found in the skin and hair, which develop from migrating neural crest cells, the RPE develops from the neuroepithelium of the optic vesicle. All naïve cells of the developing optic vesicle originally express a similar set of transcription factors and can be induced to adopt either a RPE or retinal fate [8,9]. A combination of signals from surrounding tissues can induce the differentiation of these two cell types through the activation of retinal or RPE-specific transcription factors [10]. Two transcription factors that are up-regulated in the prospective RPE and are required for proper RPE differentiation in several species are Otx2 and Mitf [11,12].

Orthodenticle-related (Otx) proteins are paired type homeobox transcription factors that serve an essential role in vertebrate anterior head and brain development. In mice, Otx2 is initially expressed in the entire developing optic vesicle and is later restricted to the differentiating RPE [13]. Complete loss of Otx2 function in mice results in embryonic lethality, while heterozygous mutations have been found to produce RPE phenotypes, particularly when present in an Otx1 null background [12]. The ocular phenotype of Otx mutants is highly variable and is characterized by degradation of the retinal layers and a second retina-like unpigmented layer of cells replacing the RPE [12,14]. Heterozygous mutations in humans have also been identified with a similar ocular phenotype that varies from a small coloboma to a complete loss of ocular tissue [15].

Zebrafish, like other teleosts, underwent a genome duplication during evolution that resulted in many cases in two copies of genes present as single copies in mammalian genomes [16,17]. Zebrafish possess three orthologs, otx1a, otx1b, and otx2, to the two
mammalian Otx1 and Otx2 genes. Zebrafish Otx1a and Otx2 are evolutionarily well conserved compared to murine proteins with a 78% and 94% amino acid identity respectively [18,19]. The expression patterns and knockdown phenotypes of Otx1a and Otx2 suggest that these proteins serve conserved and partially redundant functions in zebrafish anterior brain and eye development [20]. A third zebrafish otx gene, otx1b, encodes a protein with almost equal similarities in amino acid composition to both murine Otx1 and Otx2, and a similar expression pattern to otx1a and otx2 [16].

Microphthalmia-associated transcription factor (Mitf) is a member of the basic helix-loop-helix/leucine zipper family of transcription factors. The mammalian Mitf gene produces numerous isoforms through the use of at least nine different alternative promoters and alternative splicing [21]. Mutations in mice that affect common exons, or RPE-specific isoforms of alternative promoters and alternative splicing [21]. Mutations in the mouse RPE [25]. These findings suggested that the RPE and MITFs are necessary for development of the RPE and proper eye morphogenesis in zebrafish.

Materials and Methods

Ethics statement

All zebrafish procedures were performed in compliance with protocol AM10125, approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Zebrafish lines

Wildtype embryos were obtained through natural matings of adults of the AB strain or AB/WIK hybrids and were staged according to Kimmel et al. [28]. Genotyping of mitfα2 and mitfα602 fish lines was performed as described in [24]. mitfα mutant alleles ha3561 and ha5361 were obtained from the ZF-MODELS consortium. Genomic DNA from each allele was sequenced to confirm the mutations. Genotyping of each line was achieved through PCR with the primers listed in Table 1, followed by restriction enzyme digestion: the nucleotide change in ha3561 creates an AlwNI site while ha3567 destroys a MboI site.

The Et(1-5kb701;Gal4-VP16)s1003tTg(UAS:Elk-Kaede)s1999t double transgenic line was acquired from the Zebrafish International Resource Center (ZIRC). Adult transgenic fish were outcrossed to AB/WIK wild-type fish and embryos with no Kaede fluorescence were selected, raised, and genotyped at three months’ of age to determine s1003t (Gal4-VP16) carrier status. The UAS:mitfa construct was created using the Tol2 kit [29]. Briefly, p3E-2A-FLAG-mitfa-pA was combined with p5E-UAS-TP2, pME-GFP-nostop, and pDestTol2cryaa-DrEdpa to create pDestTol2cryaa-DrEdpa-UAS-TP2/EGFP-nostop/2A-FLAG-mitfa-pA. The UAS vector was combined in a 1:1 ratio with transposase mRNA for a final concentration of 25 ng/µl and injected into one to two cell wildtype AB embryos. Embryos with DrRed expression in the lens at 52 hpf were selected and raised to maturity and then mated pair-wise with wildtype AB/WIK fish. Putative germine founders (i.e., producing embryos with red lenses) were then mated directly to s1003t carriers.

Morpholino knockdown

Previously validated translation-blocking morpholino oligonucleotides against otx1a and otx2 [20], and against otx1b [30] were obtained from Gene Tools (Philomath, OR). A morpholino designed against the 5’ leader sequence found in the pCS2 expression vector, with sequence 5’ GAT CCT GCA AAA AGA ACA AGT AGC T 3’, was used as a control in all experiments. All morpholinos were injected at a concentration of 1.25 ng/embryo each unless otherwise noted. Larvae at five days post fertilization (dpf) were anesthetized in Tricaine and each eye was examined using a SZX12 dissecting stereomicroscope with DP70 camera (Olympus) and scored. To reduce the variability associated with morpholino injections when otx and mitf interactions were examined, larvae were only scored for phenotype when embryos of all four mitf genotypes (i.e. wild-type, both single mutants, and the double mutant) were successfully injected with the same needle. This experiment was repeated until at least three trials were obtained for each morpholino/morpholino combination. mitfα2 was used as the representative mitf single mutant in all experiments.

Histology

Specimens for histology were fixed in 4% PFA, equilibrated in 30% sucrose in PBS, and embedded in Tissue-Tek O.C.T (Sakura Finetek, Torrance, CA) prior to sectioning on a Shandon cryostat.
microtome (GMI, Ramsey, MN). 12 micron sections were stained in 1% Methylene blue, mounted in PBS and photographed with a Spot RT CCD camera (Diagnostic Instruments) on a Nikon ECLIPSE E800M microscope at 20× zoom. Measurements of the images were made using ImageJ software. Images were processed using Adobe Photoshop and Helicon Focus software.

### In-situ hybridization

Plasmid templates for synthesis of digoxigenin-labeled RNA probes were generated by PCR using the primers listed in Table 1; 

- *otx1a* was amplified from IMAGE clone 7138009 and cloned into pCR2.1-TOPO (Invitrogen), *otx2* (NM_1311251, nt 264–1133) was amplified from 24 hpf whole embryo first-strand cDNA and cloned into pCRRII-TOPO (Invitrogen). The *otx1a* template was linearized with Xhol and transcribed with SP6 RNA polymerase, while that for *otx2* was linearized with Xhol and transcribed with T3 RNA polymerase. Probes were generated by PCR using the primers listed in Table 1; probes were generated by PCR using the primers listed in Table 1; probes were generated by PCR using the primers listed in Table 1; probes were generated by PCR using the primers listed in Table 1;

- *otx1F* 5'-AGC ATA ATA GGT CCC CTG TAA C 3' 
- *otx1R* 5'-AAT ACA TGT AAA ACC TGA AAA GC 3' 
- *otx2F* 5'-ACC ATG ATG TCG TAT CTC AAG CAA C 3' 
- *otx2R* 5'-TCA CAA CAC TAT GGA TTT CCA GGA 3' 
- *silvBF* 5'-TGG ATA ACC GTA TTA CGG CC 3' 
- *silvBR* 5'-GCC GCA ATT AAC CCT CAC TAA AGG GAA CAC ACT TTG GTA AGC TCT GG 3'

**Table 1. Oligonucleotides used in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>hu3561F</td>
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</tr>
<tr>
<td>hu3561R</td>
<td>5’ AAT ACA TGT AAA ACC TGA AAA GC 3’</td>
</tr>
<tr>
<td>hu3857F</td>
<td>5’ AGC GCC CCC AAC AGT CCC AGG GCC T 3’</td>
</tr>
<tr>
<td>hu3857R</td>
<td>5’ CTG TGG CGA CCC CGG ATT AAT AAA GGG AC 3’</td>
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<tr>
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<td>5’ AAG GGC CTT AAA TAT CTC TGC 3’</td>
</tr>
<tr>
<td>otx1AR</td>
<td>5’ GGA TCC ATT AAC CCTCAC TAA AGG GAA CAC ACT TTG GTA AGC TCT GG 3’</td>
</tr>
<tr>
<td>otx2F</td>
<td>5’ ACC ATG ATG TCG TAT CTC AAG CAA C 3’</td>
</tr>
<tr>
<td>otx2R</td>
<td>5’ TCA CAA CAC TAT GGA TTT CCA GGA 3’</td>
</tr>
<tr>
<td>silvBF</td>
<td>5’ TGG ATA ACC GTA TTA CGG CC 3’</td>
</tr>
<tr>
<td>silvBR</td>
<td>5’ GCC GCA ATT AAC CCT CAC TAA AGG ACT AGT CAT ACC AGG ATC 3’</td>
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Results

Otx expression is required for proper zebrafish RPE development

Previous analysis of zebrafish Otx genes suggested that in addition to anterior brain defects, knockdown of Otx transcription factors can result in eye abnormalities [20]. We investigated the effect of the individual and combined loss of function for Otx transcription factors using previously characterized translation-blocking morpholinos for *otx1a* and *otx2* [20] and for *otx1b* at a concentration of 1.25 ng per embryo for each morpholino [30]. Knockdown of any of the Otx factors singly did not result in noticeable deficits in RPE pigmentation. The combination of *otx1a/otx1b* or *otx1b/otx2* morpholinos did produce the occasional absence of ventral RPE pigmentation but the combination of *otx1a* and *otx2* morpholinos consistently produced a significant delay in RPE pigmentation, leaving the neural crest derived melanocytes unaffected (Fig 1A,B). RPE pigmentation began to recover around 72 hpf, possibly due to the transient nature of the knockdown produced by the morpholinos.

At 5 dpf, the *otx* morphant phenotype was extremely variable both between larvae and between eyes of a single fish (Fig 1C,D).

To quantify the effect, each eye from 5 dpf larvae was examined and scored as having either a normal RPE (no discernible phenotype), mild RPE deficits (incomplete closure of the optic fissure with a coloboma, but still an anterior and posterior connection ventrally), or major RPE deficits (complete lack of ventral RPE with no connection between the anterior and posterior RPE) (Fig 2A–C). Similar to what is observed in murine Otx mutants and in the zebrafish pigmentation phenotypes at 35 hpf, the knockdown of *otx1a*, *otx1b*, or *otx2* had little effect on eye phenotype (0%, 3% and 6% minor phenotype respectively) (Fig. 2D). The combination of *otx1a/otx1b* and *otx1b/otx2* morpholinos resulted in a higher percentage of minor phenotypes but failed to increase the severity of the eye phenotype. The combination of *otx1a* and *otx2* morpholinos however, resulted in defects in nearly all larvae (95%) with most (64%) having major disruptions of the developing RPE. These results suggest that Otx1a and Otx2 have the major, though partially redundant roles in zebrafish RPE development. The combination of *otx1b* with either *otx1a* and *otx2* morpholinos does not greatly increase the severity of the RPE phenotype, but the elimination of all three *otx*...
Dopachrome expression of melanin biosynthesis pathway gene morpholinos alone rarely produced small ventral deficits in the pigmentation analysis, the injection of any of the three morphants.

Double cone photoreceptors (Fig. 3B). No additional layer of cone monoclonal Zpr1 antibody [34] that is specific for red/green the mouse and instead is merely absent (Fig. 3A). In addition to the expression in the neural crest remained unaffected (Fig. 5A–D). Expression of mitf in the RPE but not in the epiphysis was also significantly decreased in otx1a, otx1b, and otx2 morphants (Fig. 5E–H). The reduction in mitfa and mitfb expression varied somewhat between embryos, but otx1a morphants consistently displayed a greater reduction in expression when compared to otx1b and otx2 morphants (data not shown).

Mitfa and Mitfb are not required for zebrafish RPE development

The loss of mitfa in zebrafish is not associated with eye defects, however mitfa is co-expressed with a second mitf gene, mitfb, at early stages [25]. To examine mitfb function in the developing RPE, two mutant alleles were obtained (Fig. 6A). The mitfbhu3561 allele has a nonsense mutation in exon 7 causing an amino acid change from arginine to a stop codon, which disrupts the putative binding domain of the protein. The mitfbhu3857 allele has a nonsense mutation in exon 2 causing a switch from leucine to a stop codon. Each of the two mitfb mutant alleles were intercrossed, however no obvious abnormalities in homozygous mitfb embryos or larvae were observed (Fig. 6C–E). 5 dpf homozgyous mutant larvae were cryosectioned, stained with methylene blue, and photographed. When the dimensions of the eye and the thickness of several ocular layers were examined, the analysis revealed no significant differences between the mitfb mutants and age-matched wildtype larvae in RPE thickness, outer nuclear layer thickness, or overall eye length (Fig. S3).

Because the possibility existed that mitfa was able to compensate for the loss of Mitf activity in mitfb mutants [25], mitfa null alleles mitfahu3857 and mitfa-2 were crossed to the mitfbhu3561 and mitfbhu3857 alleles respectively and then bred to obtain homozgyous mitfacmtf double mutant embryos. However, these mitfacmtf double mutants also did not display any RPE or ocular phenotype when compared to either age-matched wildtype or mitfa mutant embryos (Fig. 6F–H). There were no changes in photoreceptor layer for abnormalities at 5 dpf using the Zpr1 monoclonal antibody in either set of mitfacmtf double mutants when compared to wildtype or mitfa and mitfb single mutants (data not shown). In addition to not being necessary to early development, mitfa and mitfb do not appear to be required for the maintenance of the RPE layer during later life, as sectioned eyes of adult mitfa single and double mutants, which are viable, do not show any significant changes in the RPE or retinal layers (data not shown).

Otx expression positively regulates the expression of mitfa and mitfb

In murine experiments analyzing the regulatory relationship between Otx and Mitf transcription factors, the presence of either factor was found to be dependent on the other [10,12]. We therefore examined mitfa and mitfb expression through in situ hybridization at 21 hpf in otx1a, otx1b, and otx2 single morphants as well as in otx1a/otx2 morphants. The expression of mitfa in the RPE was reduced by all three otx single morpholino knockdowns while the expression in the neural crest remained unaffected (Fig. 4). Likewise, analysis of the RPE specific silb gene [35] at 24 hpf revealed a small area of absent expression in the ventral portion of the eye in a small percentage of single otx1a, otx1b, or otx2 morphants, and greater deficits resulting from the combination of otx1a/otx2 morpholinos and in otx triple morphants (Figure S2).

Factors create a significantly more severe phenotype than what is observed in otx1a/otx2 morphants. This increase in severity was consistent even when the total concentration of the triple otx morpholino combination was reduced to that of the otx1a/otx2 morpholino combination (data not shown).

Any loss of RPE in zebrafish otx morphants occurs at the proximal ventral point of the optic cup and RPE deficits extend distally and dorsally with more severe phenotypes. Minor loss of RPE in these morphants does not have any noticeable effect on retinal lamination, although some layers may be thinner (Fig. 3A).

Major loss of RPE can disrupt the eye cup shape in the ventral portion of the eye with a variable loss of ventral ocular tissue. A rotation of the entire eye up to 90 degrees is also observed in many of the more severe RPE deficient zebrafish larvae (data not shown). Despite the rotation and missing tissue, the retina appears to be well organized in areas of the eye lacking RPE. Occasionally loss of ventral tissue. The dimensions of the ocular layers in the dorsal region of the eye remain comparable to wildtype eyes, even in the remaining RPE immediately adjacent to the affected ventral area (Figure S1).

The specific loss of ventral RPE in zebrafish otx morphants is similar to what has been described in murine Otx mutants. However in zebrafish otx morphants, the affected RPE does not appear to transdifferentiate into the retinal-like cells observed in the mouse and instead is merely absent (Fig. 3A). In addition to examining retinal lamination, 5 dpf larvae were assessed using the monoclonal Zp1 antibody [34] that is specific for red/green double cone photoreceptors (Fig. 3B). No additional layer of cone photoreceptors was observed in RPE deficient larvae and the photoreceptors appear largely unaffected by the loss of RPE in otx morphants.

Consistent with what is observed in the RPE phenotype and pigmentation analysis, the injection of any of the three morpholinos alone rarely produced small ventral deficits in the expression of melanin biosynthesis pathway gene dopachrome tautomerase (dct) when examined through in situ hybridization at 24 hpf, but the combination of otx1a and otx2 morpholinos created a significant loss of dct expression in the ventral RPE (Fig. 4).

Figure 1. Otx morpholino knockdown results in a variable degree of RPE deficits in zebrafish. (A–B) Control morpholino (A) and otx1a/otx2 morpholino (B) injected embryos photographed at 35 hpf. RPE pigmentation is reduced in otx1a/otx2 morphants (black arrows) while neural crest derived melanocytes are unaffected (white arrows). (C–D) Ventral view of 5 dpf control morpholino (C) and otx1a/otx2 morpholino (D) injected larvae. The phenotype of otx1a/otx2 morphants can vary in severity between eyes of the same embryo, with one eye displaying a minor coloboma phenotype (black arrow) and the other eye displaying a major coloboma phenotype (white arrow). doi:10.1371/journal.pone.0049357.g001

Otx and Mitf in Zebrafish Eye Development

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Mitf transcription factors may still have role in RPE development

The combined knockdown of Otx and Mitf transcription factors was explored to determine if mitfa and mitfb may still contribute to RPE development. To accomplish this, otx1a and otx2 morpholinos were injected into wildtype, mitfa mutant, mitfb mutant, and mitfa/mitfb double mutant embryos to determine if the loss of Mitf activity would increase the severity of the otx morphant phenotype.

Figure 2. Otx morphant phenotypes. (A–C) Eye phenotypes, assessed at 5 dpf, were scored as normal when the RPE displayed no visible defects (A), minor when coloboma was present but anterior and posterior ventral RPE were still contiguous (B), and major when the anterior and posterior ventral RPE were completely separated (C). (D) Histogram showing the eye defects induced by otx gene knockdown when injected at a morpholino concentration of 1.25 ng/embryo. Knockdown of individual otx genes caused only minor defects, while knockdown of otx1a and otx2 together, or all three otx genes together, produced a majority of embryos with major eye defects. otx1a, N = 108; otx1b, N = 230; otx2, N = 122; otx1a/otx1b, N = 222; otx1b/otx2, N = 256; otx1a/otx2, N = 214; otx1a/otx1b/otx2, N = 178.

doi:10.1371/journal.pone.0049357.g002

Figure 3. The loss of ventral RPE in otx morphants has little effect on retinal lamination. (A) Methylene blue stained sagittal section of an otx1a/otx2 morphant classified with a minor eye phenotype. The laminar structure of the retina is unaffected despite extensive absence of RPE. (B) Zpr1 staining reveals that red-green double cone photoreceptors differentiate normally despite the loss of RPE (white arrow). Scale bar is 25 micrometers.

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Figure 4. Otx knockdown causes a decrease in dct expression in the developing RPE. dopachrome tautomerase (dct) mRNA expression was analyzed through in situ hybridization at 24 hpf in otx morphants. dct expression was reduced only in the ventral most region of the optic cup in single morphants (B–D, white arrows) compared to controls (A). dct expression was significantly decreased in the ventral RPE of otx1a/otx2 morphants and to an even greater degree in otx1a/otx1b/otx2 morphants (E–F, white arrows). dct expression in neural crest melanocytes was unaffected (black arrows). Approximately 50 larvae were examined for each condition.

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Phenotypic variability of the combination of otx1a/otx2 morpholinos, even at reduced concentration, precluded the analysis of multiple trials as a single group, but each trial on its own had a significant increase in the percentage of mitfa;mitfb double mutant embryos with major RPE deficit phenotypes when compared to wildtype and single mitf mutant embryos (Fig. 7A and Fig. S4). In addition to the increased phenotypic severity, the combined knockdown of Otx1a and Otx2 also produced a significant decrease in dct expression in mitfa;mitfb double mutants when compared to wildtype otx morphants and uninjected mitf mutants (Fig. 7B,C; Figure 5. mitfa and mitfb expression is decreased in otx morphants. Expression of mitfa (A–D) and mitfb (E–H) was examined through in situ hybridization at the 21 somite stage in embryos injected with a control morpholino (A,E), or morpholinos against otx1a (B,F), otx1b (C,G), or otx2 (D,H). In situ hybridization analysis revealed a decrease in mitfa expression in the developing RPE cells (black arrows) but not in the neural crest cells (white arrows) of otx single morphants (D) and when compared to controls (A). mitfb expression was also reduced specifically in the RPE (black arrows) of otx morphants (F–H) compared to the controls, leaving epiphysis expression unaffected (white arrows). All morphants were processed simultaneously with approximately 50 larvae for each condition and the experiment was repeated twice.

Figure 6. Elimination of mitfa and mitfb activity has no detectable effect on RPE development in zebrafish embryos. (A) Diagram of mitfb cDNA showing exons, functional domains, and locations of mutations (arrows). The mitfbhu3857 allele contains a T>A nonsense mutation that causes a switch from leucine to a stop codon in exon 2. The mitfbhu3561 allele has a C>T nonsense mutation that results in a switch from Arginine to a stop codon in exon 7, in the basic-helix-loop-helix/leucine zipper binding domain (bHLH LZ). AD, transcriptional activation domain. (B) The mitfbhu3857 allele creates an AlwNI restriction site, allowing genotyping of animals by electrophoresis of digested PCR products. (C–H) Bright field images depicting the normal RPE pigmentation and eye development in 5 dpf larvae; wildtype (C), or mitfbhu3857 (D), mitfbhu3561 (E), mitfa^w2 (F), mitfbhu3857;mitfa^w2 (G), or mitfbhu3561;mitfa^d692 (H) homozygous mutants.

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compare to Fig. 4E). Injections of either otx1a or otx2 morpholinos singly into mitfbhu3857 mutants produced a significant increase in both the percentage of embryos with a phenotype, as well as the severity of those phenotypes in mitfa;mitfb double mutants when compared to the other genotypes (Fig S5). Taken together, this evidence suggests that mitfa and mitfb interact with otx to contribute to zebrafish RPE development although they are not necessary on their own.

The expression of otx1a and otx2 was also examined through in situ hybridization at 21hpf in mitfa2 and mitfbhu3857 single and double mutants. There was no observable change in otx1a or otx2 expression in any of the mitfa2 and mitfbhu3857 single or double homozygous mutants when compared to wildtype embryos (Fig. 8). This unidirectional regulation of Otx transcription factors differs from what is observed in mice and may help to explain why loss of Mitf transcription factors does not have an apparent effect on zebrafish RPE development.

Mitfa has the ability to promote pigmented cell fate in the developing retina

Due to the similar developmental origin, RPE and retinal cells have the ability to transdifferentiate between each cell type for an extended period of time in many organisms [36–38]. Loss of Mitf activity in murine and quail mutants causes hyperproliferation and a conversion of the prospective RPE layer into a second inverted retinal layer [10,39,40]. Conversely, the retinal misexpression of Mitf is able to direct the transdifferentiation of retinal cells into a second pigmented layer [22,41,42]. To examine the ability of Mitfa to promote an RPE cell fate in developing retinal cells, a transgenic line was created with a 5' UAS sequence driving expression of mitfa in tandem with an EGFP reporter, along with an alpha crystallin promoter driving expression of DsRed in the developing lens to allow for identification of germline transormants (Fig. 9A).

UASmitfa founders were bred to the enhancer trap line E(l5 kep701:Gal4-VP16)s1003t, expressing Gal4-VP16 in the developing retina [43]. A portion of embryos from such matings were observed to have GFP expression in the developing retina starting around 24 hpf, with robust expression by 28 hpf (Fig 9B). GFP expression was not observed in embryos that did not also later express DsRed in the developing lens. A small percentage of embryos also displayed scattered GFP expression and pigmentation in other tissues including the heart, where this expression was often accompanied by circulation problems (data not shown). When sorted for GFP expression and processed for in situ hybridization with a probe for mitfa at 28 hpf, only GFP expressing embryos displayed mitfa expression in the developing retina, suggesting that the Gal4-UAS elements were functioning correctly in the transgenic larvae (Fig. 9C,D). Cryosections of 5 dpf GFP

Figure 7. The combined knockdown of Otx and Mitf transcription factors creates a more severe phenotype. (A) The effect of otx1a/otx2 knockdown was compared between wildtype, mitfa2 and mitfbhu3857 single mutants, and mitfbhu3857;mitfa2 double mutants. Morpholinos were injected at a total concentration of 2 ng/embryo using the same needle for all genotypes. Results from one representative experiment are shown. The percentage of severe RPE defects was significantly increased (P<0.0001) in the mitfbhu3857;mitfa2 double mutant background compared to wildtype and single mitfa and mitfb mutants. Wildtype, N = 252; mitfa, N = 196; mitfb, N = 158; mitfa;mitfb, N = 170. (B,C) At 24 hpf dct expression is nearly eliminated in mitfbhu3857;mitfa2 embryos injected with the otx1a/otx2 morpholino combination (C) compared to sibling embryos injected with control morpholino (B).

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**Figure 8.** *otx1a* and *otx2* expression is not altered in *mitfa* and *mitfb* mutants. (A–D) No difference in *otx1a* expression was revealed through in situ hybridization in wildtype (A), *mitfa*<sup>−/−</sup> (B), *mitfb<sup>hu3857</sup> (C), and *mitfb<sup>hu3857</sup>;mitfa<sup>−/−</sup> (D) embryos at 21 hpf. (E–H) Likewise, *otx2* expression was unchanged in wildtype (E), *mitfa<sup>−/−</sup> (F), *mitfb<sup>hu3857</sup> (G), and *mitfb<sup>hu3857</sup>;mitfa<sup>−/−</sup> (H) embryos at the same stage. All mutants were processed simultaneously with approximately 50 larvae examined for each condition and the entire experiment was repeated.

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**Figure 9.** The misexpression of *mitfa* in the developing retina is capable of inducing foci of pigmentation. (A) Schematic of the Tol2 transposon construct expressing EGFP and FLAG-tagged Mitfa, linked by a viral 2A peptide, from a 5×UAS promoter, and containing an alpha crystallin promoter:DsRed-Express transgene marker. (B) 72 hpf larva doubly transgenic for *s1003t* (retinal Gal4-VP16) and UAS:EGFP-2A-FLAG-mitfa, expressing GFP in the developing retina. (C,D) 20 micron cryosections of 28 hpf larvae examined for mitfa expression through in situ hybridization revealed mitfa expression in the developing retina and lens of a GFP expressing embryo (D) but not in an embryo lacking GFP expression (C). (E–G) Cryosections of a 5 dpf *s1003t*; UAS:EGFP-2A-FLAG-mitfa transgenic larva, displaying foci of pigmentation (E) and GFP expression (F) in the retina. (G) Overlay demonstrating extensive overlap of GFP expression and ectopic pigmentation (white arrows). Scale bar indicates 20 micrometers in (E–G). Expression of DsRed-Express is also visible in the lens in (G).

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positive embryos contained scattered patches of pigmented cells throughout the retinal layers in a pattern consistent with the observed GFP expression (Fig 9E–G). This experiment demonstrates that mitfa is capable of inducing pigmentation in the developing zebrafish eye in vivo and provides additional support for the notion that mitfa and mitfb may have a non-essential role in zebrafish RPE development.

**Discussion**

In this study we have demonstrated that in zebrafish, Otx (Otx1α and Otx2, with some contribution from Otx1β) but not Mitf (Mitfa and Mitfb) transcription factors are required for normal RPE development, and that their absence causes a delay in gene expression and pigmentation culminating, in the most severe cases, in significant coloboma. While the role of Otx in eye formation therefore appears to be broadly conserved between zebrafish and other vertebrates [9], we found in contrast that the loss of Mitf transcription factors encoded by the duplicated loci mitfa and mitfb does not create RPE abnormalities in zebrafish, nor the microphthalmia phenotype for which the mouse locus was named.

This surprising result might be simply explained if the mitfa and/or mitfb mutants used in this study retain residual activity. We believe this is unlikely for several reasons. First, three of the four alleles in question (mitfa^w2, mitfb^hu3857 and mitfb^hu3561) create premature stop codons, and one of these (hu3561) lies within the helix-loop-helix (HLH) dimerization domain. The remaining allele, mitfa^b692, is a missense mutation but is also located in the HLH domain, and besides being insufficient for development of neural crest melanocytes, shows no activity in a reporter assay [25]. The exons containing the stop codons in w2 (exon 3) and hu3857 (exon 2) could conceivably be skipped while preserving the reading frame, but we have been unable to detect expression of any such splice forms during embryogenesis. We have in fact identified a second isoform of mitfa with a novel first exon which splices into the third exon of the originally reported isoform, and a promoter capable of driving expression in the RPE (B.M.L. and J.A.L., unpublished results). However, it is predicted that both the mitfa^w2 and mitfa^b692 mutations would affect this isoform as well.

Finally, regarding mitfb loss of function, we have tested several splice-blocking morpholinos against this gene and despite finding more than one that was highly effective as evaluated by RT/PCR, none produced an embryonic phenotype in wildtype or mitfa homozygotes (B.M.L. and J.A.L., unpublished results). We have recently shown that another member of the MiT family, tfec, is also expressed in the developing zebrafish RPE [44]. Although it is apparently insufficient to compensate for loss if Mitf in mammals, Tfec, along with Mitf, was reported to be upregulated in the ocular retardation (or) mouse, in which neural retina is translated to pigmented retina as a result of mutation of the Chx10 gene [41]. Recently, Tfec was shown to rescue the small-eye phenotype of a strong mouse Mitf allele when misexpressed [45]. The zebrafish tfec gene is therefore a strong candidate for another factor that may compensate for the loss of mitfa and mitfb.

![Figure 10. Models for transcriptional regulation of RPE development in mouse versus zebrafish.](https://www.plosone.org/doi/10.1371/journal.pone.0049357.g010)
Another finding from these experiments is the lack of cross-regulation between zebrafish Otx and Mitf transcription factors. This data suggests a model in which a threshold level of gene activation, driven by Otx and Mitf together, is required for proper development of the RPE [Fig. 10]. Under normal conditions, there is sufficient combined target gene activation by the two sets of transcription factors to adequately direct the development of the RPE. In mice, each factor is individually important, and the loss of either Otx or Mitf activity will lead to down-regulation of the other transcription factor [10, 12] [Fig. 10A–B]. In zebrafish, Otx plays a more prominent role, and rather than a reciprocal relationship, Otx regulates Mitf but not vice-versa. Hence, the loss of the Mitf activity in mitf mutants leaves Otx activity unaffected with enough Otx target gene activation to surpass the threshold level required for RPE development, but when Otx activity is knocked down, mitf expression is also decreased, causing the level of RPE target gene expression to drop below the required threshold, leading to a varying degree of RPE abnormalities based on the severity of the decrease in this expression [Fig. 10C–D]. This model of an RPE threshold is supported by the apparent variance in phenotype between eyes of a single animal that is observed in other species with Mitf and Otx mutations [12, 22]. If the level of RPE target gene activation is near the threshold of proper development, small changes may push the phenotype in either direction.

In addition to the differences between zebrafish and mice in the transcription factors required for RPE development, there are also differences in the ocular phenotype. Murine Mitf and Otx mutants experience the formation of a hyperpluriparative, second inverted retinal layer in place of the RPE cells [12, 23]. Preliminary histology and analysis of double cone photoreceptors did not reveal any duplication of the retinal layers in zebrafish otx morphants. As the missing prospective RPE cells do not appear to transdifferentiate into retinal cells in zebrafish otx morphants, it is possible that they instead experience migration defects, fail to proliferate, or simply die off early in development. The eyes of most zebrafish otx1a/otx2 morphants also do not show any reduction in size, and the retinal layers remain intact and organized in the unaffected dorsal regions of the eye. Previous evidence exists in zebrafish for the orderly lamination of the retina in the absence of RPE [46]. The morpholino paradigm used in our experiments has been reported to deplete embryos of detectable Otx2 during late somitogenesis stages [20], but we cannot definitively say to what degree the potential recovery of Otx activity in zebrafish during late somitogenesis stages [20, 47], but we cannot evidence exists in zebrafish for the orderly lamination of the retina organized in the unaffected dorsal regions of the eye. Previous reduction in size, and the retinal layers remain intact and provide a better understanding of the regulatory pathways that may also control human RPE development. Similar to what has been observed in zebrafish, mutations in OTA2 but not MITF have been identified in cases of severe human RPE developmental abnormalities such as microphthalmia and anophthalmia [15]. The phenotypic variance observed between the eyes of zebrafish is similar to what is observed in human patients with OTA2 mutations. Finally, it appears that zebrafish may be similar to humans in the lack of dependence on Mitf for proper eye morphogenesis, as in contrast to mice the most severe human MITF mutations result in Tietz syndrome, a condition which involves relatively minor eye abnormalities relative to the effect on neural crest melanocytes [47].

Supporting Information

Figure S1 Otx knockdown does not affect lamination or dorsal eye size. Histograms showing measurements of the RPE (A), Outer nuclear layer (B) and the length of the dorsal half of the eye (C), comparing otx1a/otx2 morphants and control (uninjected) larvae at 5 dpf. Equivalent sections from 15 different eyes were averaged and no significant differences were observed between the two groups (P>0.05).

Figure S2 Otx knockdown leads to a decrease of key RPE related genes. (A–F) Expression of sibb was examined in otx morphants at 24 hpf and representative images are shown. At 24 hpf, a small percentage of the single otx1a [B], otx1b [C] and otx2 [D] morphants displayed a slight loss of sibb expression in the ventral eye when compared to controls (A). Combined otx1a/otx2 morphants showed a more severe reduction (E) and expression was almost completely eliminated in otx1a/otx1b/otx2 morphants (F).

Figure S3 No changes to the RPE and retinal layers are observed in zebrafish Mitf mutants. (A) Equivalent sagittal sections from at least seven individual specimens for each genotype were analyzed using ImageJ software. RPE and outer nuclear layer thickness were measured at the central retina and eye length was measured at the proximal point of the lens. ANOVA analysis revealed no significant differences in RPE thickness (A, P=0.115), ONL thickness (B, P=0.156), or total eye length (C, P=0.069).

Figure S4 Results of individual trials for otx1a/otx2 knockdown in mitf mutants. The results from otx1a/otx2 knockdown in mitf mutants were too variable to interpret as a single group but all demonstrate a significant difference between phenotypic outcomes in mitf1a2b double mutants to wildtype and single mitf mutants. The phenotypic variability in phenotype-to-genotype correlations between trials is likely the result of the potency of the morpholino combination and the difficulty in replicating exact injection conditions between trials. On the left are tabulated the raw eye phenotype data for the three additional trials and on the right are displayed the same data as phenotype percentages for each genotype. The mitf1a2b double mutants displayed a significantly higher percentage of eyes with major phenotypes when compared to wildtype and single mitf mutants in all trials (p<0.0001).

Figure S5 Single morpholino knockdown of otx1a or otx2 in mitf mutants. Injection of otx1a (A) or otx2 (B) morpholinos individually at a concentration of 2 ng/embryo produced a significantly greater percentage (P<0.0001) of eye defects in mitf1a2b;mitf1a2b double mutants compared to injections of wildtype and single mitf mutants. (A) wildtype, N=476; mitf1a, N=372; mitf1b, N=464; mitf1a2b, N=472. (B) wildtype, N=368; mitf1a, N=330; mitf1b, N=220; mitf1a2b, N=274.

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Author Contributions
Conceived and designed the experiments: BML JAL. Performed the experiments: BML JAL. Analyzed the data: BML JAL. Contributed reagents/materials/analysis tools: BML JAL. Wrote the paper: BML JAL.

References