Otx but not Mitf transcription factors are required for zebrafish RPE development

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<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
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<tr>
<td>hpf</td>
<td>hours post fertilization</td>
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<tr>
<td>dpf</td>
<td>days post fertilization</td>
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<td>MO</td>
<td>morpholino</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>Mesab</td>
<td>ethyl-m-aminobenzoate methanesulphonate</td>
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Abstract

Otx but not Mitf transcription factors are required for zebrafish RPE development

By Brandon Lane

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Advisor: James Lister, PhD
Human and Molecular Genetics

Mitf and Otx transcription factors have been identified as essential to the development of the retinal pigmented epithelium (RPE), but the relationship between these factors and their specific role in the RPE developmental pathway have not been clearly defined. The role of the two Mitf transcription factors (Mitfa and Mitfb) and two Otx transcription factors (Otx1a and Otx2) in zebrafish RPE development was explored in these experiments. The loss of Mitf activity in mitfa, mitfb, or double mitf null mutant fish lines had no effect on RPE pigmentation or development. The loss of Otx2 activity through morpholino knockdown produced a RPE deficient phenotype in a small percentage of embryos, while the additional knockdown of Otx1a caused widespread and severe RPE developmental abnormalities. Analysis of ocular sections revealed that the retinal layers remain unaffected in mitf mutants, as well as in most RPE-deficient otx morphants. Mitf and Otx combined loss of function experiments suggest that Mitfa and Mitfb may still play a role in zebrafish RPE development. Expression analysis through in situ hybridization has demonstrated that Otx transcription factors are necessary for the proper expression of mitfa and mitfb while Mitf transcription factors are not required for the expression of otx genes. The transcriptional regulation of Mitf by Otx
transcription factors may explain why only Otx transcription factors are necessary for zebrafish RPE development despite the somewhat overlapping functions of Mitf and Otx transcription factors.
Chapter 1

Introduction to zebrafish RPE development

The retinal pigmented epithelium (RPE) is a monolayer of cells that lies between the retinal and choroid and serves a variety of functions in the developed eye. These functions include providing nutritional support to the retinal cells, regulating oxidative stress, and maintaining the outer segment 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 (Beatty et al 1999, Maw et al 1997). The RPE also plays a vital role in early eye development where it is essential to the development of the retinal layers (Raymond and Jackson 1995). 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 (Chow and Lang 2001). Failure of the RPE to develop properly can lead to a gap in the ocular layers known as a coloboma, and in more severe instances can lead to ocular retinal degeneration resulting in microphthalmia or anophthalmia (Scholtz and Chan 1987).

Colobomas arising from incomplete eye development have an incidence of 2.6 out of every 10,000 US births and are present in approximately 5-10 percent of cases of childhood blindness (Verna and Fitzpatrick 2007). Microphthalmia and the more severe anophthalmia conditions are diagnosed in up to 25% of children with severe vision impairments and in approximately 1 of every 3,500 live births (Verma and Fitzpatrick
These developmental ocular disorders are often highly variable as human patients carrying the same mutation can differ with respect to the severity of the eye phenotype. This phenotypic variability is also observed between the eyes of a given patient as well as in animal models of eye development, suggesting stochastic influences on the regulation of eye development and some extent of developmental independence for each eye.

The zebrafish, *Danio rerio*, presents an excellent model to study these ocular disorders due to the similarities between human and zebrafish eye development, cell composition, and morphology (Gross and Perkins 2008). The posterior portions of the zebrafish and human eye are particularly comparable as both contain an RPE surrounding five basic retinal layers. These retinal layers contain the same seven types of retinal cells including Müller glia, ganglion cells, amacrine cells, bipolar cells, horizontal cells, and the rod and cone photoreceptors (Gross and Perkins 2008). The rapid development, embryonic transparency, and external fertilization of a large number of embryos, make zebrafish an attractive developmental model. The eyes of zebrafish larvae are relatively large compared to body size and develop in the first few days following fertilization. The RPE layer begins developing by 19 hours post fertilization (hpf), the retinal layers begin to differentiate around 28 hpf and the eye is functional in free swimming larvae by 72 hpf (Gross et al 2005, Gross and Perkins 2008). A better understanding of the mechanisms controlling RPE development and the interactions between key regulatory factors in lower vertebrates such as zebrafish, may provide valuable insights into human ocular disorders.
β-catenin mediated Wnt signaling is involved in the early anteroposterior patterning of the vertebrate head and nervous system and the specification of the telencephalon, diencephalon, and eye primordium (Mcgrew et al 1995, Woo and Frasier 1995). Activation of the Wnt signaling pathway after the midblastula transition can result in disruptions in head and nervous system development. The role of Wnt inhibitors like TCF3, which acts to bind to Wnt target genes, are critical to the proper development of the anterior brain eye (Kim et al 2000). After the initial patterning of the anterior brain, the specification of the bilateral eye field from the telencephalon, and the evagination of the optic vesicle require the activity of Rx transcription factors (Kennedy et al 2004, Stigholer et al 2005). As eye development progresses, the naïve cells of the optic vesicle differentiate into the optic stalk, retinal, and RPE cells.

Pigmented cells like those found in the retinal pigment epithelium (RPE), are a specialized group of cells that contain a melanin-producing organelle called a melanosome (Marks and Seabra 2001). Unlike the pigmented cells found in the skin and hair, which develop from migrating neural crest cells, the RPE develops from the neuroepithelial cells 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 differentiate into either a RPE or retinal fate (Zuber et al 2003, Martinez-Morales et al 2004). 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 (Nguyen and Arnheiter 2000). Two transcription factors that are upregulated in the prospective RPE cells and are required for proper RPE differentiation in several species are Otx2 and Mitf (Hodgkinson et al 1993, Martinez-Morales et al 2001).
Microphthalmia-associated transcription factor (Mitf) is a member of the basic helix-loop-helix/leucine zipper family of transcription factors. The mammalian Mitf gene contains nine exons and produces numerous isoforms through the use of at least nine different alternative promoters as well as alternative splicing (Bharti et al 2008). Various isoforms of Mitf are expressed in the developing RPE cells including MitfA, MitfH, MitfD, and MitfJ (Bharti et al 2008). Development of the neural crest pigment cells however, is specifically directed by the MITFM isoform (Yajima et al 1999). In mice, mutations that primarily affect the MITFM isoform, result in a reduction of pigmentation in the neural crest derived melanocytes of the skin and coat but a normally pigmented RPE (Yajima et al 1999, Nakayama et al 1998, Planque et al 2004). Mutations that affect the activity of RPE-specific isoforms of MITF can result in the microphthalmia phenotype in mice for which the transcription factor is named (Hertwig 1942). This reduction in eye size is due to retinal degeneration and incomplete closure of the optic fissure, leading to an ocular phenotype that can vary from a simple coloboma to complete anophthalmia (Bharti et al 2006, Bumsted and Barnstable 2000, Yajima et al 2003). The degree of severity of the eye phenotype can be extremely variable not only between animals with the same mutation, but also between eyes of the same animal (Bharti et al 2006, Wyatt et al 2008). A common feature of the ocular mutant phenotype includes the transdifferentiation of dorsal RPE into an additional hyperproliferative retinal layer. Experiments in mice have yet to reveal if the cause of the ocular phenotype is related to the loss of a specific Mitf isoform or the combined loss of Mitf isoform activity in the RPE. Cell culture experiments on avian retinal cells have shown the ability of to
produce RPE-like cells using only the amino terminal portion of Mitf protein, suggesting a level of redundancy within this transcription factor family (Planque et al 2004).

The zebrafish genome, like that of other teleosts, underwent a partial duplication during evolution that has resulted in multiple copies of singular mammalian genes in the zebrafish genome (Amores et al 1998, Postlethwait et al 1998, Gates et al 1999, Woods et al 2000). These gene duplicates often acquire novel functions, lose their now redundant function, or partition the multiple functions of the single mammalian gene between the zebrafish duplicates (Force et al 2009). The first mitf gene identified in zebrafish was mitfa, which spans 9 exons and shares the most similarity with the mouse MITFM isoform (Lister et al 1999). Unlike the neural crest specific MITFM isoform however, mitfa is expressed in both neural crest cells as well as the RPE cells of the developing zebrafish embryo (Lister et al 2001). Despite this dual expression, null mutations in the mitfa gene affect only the neural crest derived melanocytes, leaving the RPE pigmentation unaffected (Lister et al 2001). A further examination of the zebrafish genome revealed a second mitf gene termed mitfb with highly conserved protein domains. The mitfb gene also contains 9 exons but exhibits greater similarity, especially in the amino terminal region, to MITF isoforms present in the developing mouse RPE. (Lister et al 2001). As is the case with the murine RPE orthologues, mitfb is expressed in the developing zebrafish RPE but not in the neural crest derived melanocytes. The onset of mitfb expression in the developing RPE cells at the sixteen somite stage is similar what is observed with mitfa expression, although mitfb appears to be expressed at a slightly lower level (Lister et al 2001). Both genes are capable of inducing expression of pigment and pigmentation related genes when tested in vivo as
well as in vitro (Lister et al 2001). The expression and pigment gene activation potential of mitfb suggest that the RPE and neural crest functions of the single mammalian gene may be partitioned between the duplicate zebrafish mif genes.

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 then restricted to the differentiating RPE (Simeone et al 2002). Complete loss of Otx2 function in mice results in embryonic lethality, while heterozygous mutations have been found to produce RPE mutant phenotypes, particularly when present in an Otx1 null mutant background (Martinez-Morales et al 2001). The ocular phenotype of Otx mutants is similar to what is observed in mouse Mitf mutants with disorganization and degradation of the retinal layers and transdifferentiation of the RPE into a second retinal-like unpigmented layer of cells. (Martinez-Morales 2003, Martinez-Morales et al 2001). However, Otx mutants do not exhibit the dorsal cell proliferation that is observed in Mitf mutants and actually show some retention of the dorsal RPE. Like the Mitf mutant ocular phenotypes, Otx RPE abnormalities vary in severity between individuals with the same mutation, between eyes in the same individual, and within different patches of the same eye (Verma and Fitzpatrick 2007, Wyatt et al 2008). 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 (Wyatt et al 2008).

In zebrafish, the partial genome duplication has resulted in three zebrafish orthologues, 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% identical amino acid composition respectively, though Otx1a does possess some similarities to murine Otx2 as well. (Mercier et al 1995, Li et al 1994, Mori et al 1994). Despite some modest changes in regulatory elements, the expression patterns and knockdown phenotypes suggest that these proteins serve evolutionarily conserved RPE developmental functions in zebrafish. (Foucher et al 2006, Mori et al 1994, Kurokowa et al 2006). Recent morpholino analysis suggests that Otx1a and Otx2 have partially redundant but necessary functions in zebrafish anterior brain and eye development (Foucher et al 2006). Otx1b appears to be a hybrid Otx protein with almost equal similarities in amino acid composition to both murine Otx1 and Otx2. Initial analysis of this gene suggests that it does not play a central role in the development of the RPE but further investigation is required to validate this conclusion and to identify any other function in eye development. (Foucher et al 2006, Mercier et al 1995).

Previous theories have predicted that Otx2 and Mitf act in parallel, or that Otx2 acts upstream of Mitf transcription factors in a RPE developmental pathway due to its slightly earlier expression. Recent analysis of murine Otx2 and Mitf mutants has led to a new theory involving positive feedback regulation between the two factors (Martinez-Morales et al 2003). Analysis of mutant mouse models has revealed that Otx2 expression is eliminated in Mitf mutants and that Mitf expression is severely down-regulated in Otx2 heterozygous mutants (Nguyen and Arnheiter 2000, Martinez-Morales 2001). This feedback relationship was further supported by findings in conditional β-catenin mutants with a RPE deficient phenotype, as both Otx2 and Mitf were present in patches of pigmented RPE cells but absent in the unpigmented regions (Westenskow et
In vitro luciferase assays of both human and mouse RPE transcription factors have revealed that Otx2 and Mitf achieve maximal target gene expression when both proteins are present (Martinez-Morales 2003, Reinisalo et al 2012). Further analysis in human and mouse RPE cells revealed co-localization in the nucleus and the ability of Otx2 and Mitf to form protein complexes through evolutionarily conserved elements (Martinez-Morales et al 2003, Reinisalo et al 2012). Since no target sequences for Mitf or Otx transcriptional activation have been identified in the promoter regions of either factor, the mechanism behind the potential feedback relationship between Otx2 and Mitf and the conservation of this relationship in other species warrants further examination (Martinez-Morales et al 2003).
Figure 1.1: Zebrafish eye at 5 dpf. Cryosections of 5 dpf zebrafish larvae stained with methylene blue. The RPE surrounds 5 basic retinal layers including the outer nuclear layer (ONL) containing the rod and cone photoreceptors, the outer plexiform layer (OPL), the inner nuclear layer containing the amacrine and horizontal cells (INL), the inner plexiform (IPL), and the ganglion cell (GCL) containing the ganglion and Mueller cells.
Chapter 2

Mitf Transcription Factors are not required for zebrafish RPE development

Introduction:

The locus containing the mammalian microphthalmia associated transcription factor gene was first identified in a mutant screen in 1942 and later named for the small eye phenotype that was identified in the mouse mutant (Hertwig 1942). Since then, over 30 Mitf mutations have been identified in mice that produce a variety of phenotypes depending on the Mitf isoforms affected (Bharti et al 2006). Many of these mutations result in abnormalities in neural crest derived melanocytes and the more severe mutant alleles are associated with RPE abnormalities as well. This variable RPE phenotype is most likely due to the multiple isoforms with similar amino terminal composition that are expressed in the mammalian RPE compared to the single MitfM isoform that is required for neural crest derived melanocyte development (Bharti et al 2008). The ocular abnormalities associated with Mitf mutations are characterized by a transdifferentiation of the dorsal RPE into an additional retinal layer, defects in optic fissure closure and folding and degradation of the original retinal layers leading to small eyes or the complete loss of ocular tissue (Bumsted and Barnstable 2008). Mammalian Mitf is also expressed in non pigment-related cells such as osteoclasts, mast cells and natural killer cells as well as in the inner ear and the heart, where its functions are not well understood (Bharti et al 2008). In humans, Mitf mutations have been identified in Waardenburg syndrome and Tietz syndrome, which both are characterized by various

Zebrafish have duplicate *mitf* genes which have been theorized to recapitulate the multiple functions of the original mammalian Mitf gene by partitioning those functions between the duplicate genes (Lister et al 2001). The first zebrafish gene discovered, *mitfa*, is expressed in both the neural crest derived melanocytes as well as the RPE (Lister et al 1999, Lister et al 2001). However multiple *mitfa* mutant alleles display a specific loss of neural crest derived melanocyte pigmentation while leaving the RPE unaffected (Lister et al 1999). Additionally, a temperature sensitive *mitfa* allele can modulate neural crest melanocyte pigmentation when raised at restrictive or permissive temperatures (Johnson et al 2011). A search of the zebrafish genome revealed a second *mitf* gene, *mitfb*, which is expressed in the RPE as well as the epiphysis and olfactory bulb. Mitfa and Mitfb have divergent amino and carboxy termini but are 75 percent homologous to each other at the amino acid level in the main portion of the protein and the key regulatory regions are also highly conserved (Lister et al 2001). Both *mitfa* and *mitfb* begin to be expressed around the 16 somite stage in the dorsal edge of the optic lobe and are capable of activating pigmentation related genes through the evolutionarily conserved target M box sequence (Lister et al 2001). The zebrafish Mitfa transcription factor bears the most resemblance to the murine MitfM isoform responsible for the development of neural crest derived melanocytes. Mitfb is approximately 70 percent identical at the amino acid level to the various murine isoforms that are expressed in the developing RPE. This evidence suggests that Mitfb
may be responsible for the development of zebrafish RPE while Mitfa controls the development of the neural crest derived melanocytes.

**Materials and Methods**

**Wildtype and Mutant Zebrafish**

Wildtype embryos were obtained through natural matings of adults from the AB strain or AB/WIK hybrids and were staged according to Kimmel et al 1995. Genotyping of \textit{mitfa}^{w2} and \textit{mitfa}^{b692} fish lines was performed as described in Lister et al 1999. \textit{mitfb} mutant alleles hu3857 and hu3561 were obtained from the ZF-MODELS consortium. The mutant lines were sequenced to confirm the base pair changes. Genotyping of mutant fish lines was achieved through restriction enzyme digestion of PCR products as depicted in Figure 1 due to the addition (\textit{mitfb}^{hu3561}) or loss (\textit{mitfb}^{hu3857}) of AlwN1 and Mbo1 restriction sites respectively. Primers for genotyping are \textit{mitfb}^{hu3561}F: AGCATAATAGGTCCCCTTTAA, \textit{mitfb}^{hu3561}R: AATACATGTAACACCTGAAAAGC, \textit{mitfb}^{hu3857}F: AGCGCCCCCAACAGTCCCAGGGCCT, \textit{mitfb}^{hu3857}R: CTGTGGCGACCCCGGATTAATAAAGGGAC. All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Morpholino Knockdown**

The following morpholino oligonucleotides were obtained from Gene tools. Mitfb ATG1: 5' GCACGATCCCGATTCGACTGCAT 3', Mitfb ATG2: 5' GATCCCCGATTCCGACTGCATTTCC 3', Mitfb E1b1b: 5' CGCTGCTTTACCTTCAGCACTCCA 3', Mitfb E2l2: 5'

The efficacy of *mitfb* splice-blocking morpholinos was confirmed through RT-PCR with the primers listed. Mitfb Exon1F: 5’ GCATATCTTTGGATGGAAGC 3’, Mitfb Exon4R: 5’ CGGATTAATAAAGGGACTAAGC 3’.

The control morpholino used in all experiments was designed against the CS2 5’ leader sequence 5’ GATCCTGCAAAAAGAACAAGTAGCT 3’. Morpholino volume was determined by measuring the diameter on a micrometer. 5dpf embryos were anesthetized in MESAB (MS-122) and each eye was examined for phenotype at 5dpf using a SZX12 dissecting stereomicroscope with DP70 camera (Olympus).

**Cryosectioning and analysis**

For histology, fixed specimens were equilibrated in 30% sucrose in PBS, embedded in Tissue-Tek O.C.T (Sakura Finetek, Torrance, CA) and cryosectioned 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 20x zoom. Measurements of the images were made using the ImageJ software. Images were processed using Adobe Photoshop and Helicon Focus software.

**Immunohistochemistry**

Larvae were anesthetized in Mesab at 5dpf and fixed in 4%PFA overnight. Immunohistochemistry was performed as described in (Macdonald et al 1999) except
for the permabilization step which was performed with Proteinase K. The Zpr1 monoclonal antibody (ZIRC) and Alexa 488 goat anti-mouse secondary antibody (Invitrogen) were used at a concentration of 1:1000 each. The larvae were then sectioned at 25 microns and covered with vectashield (Vector laboratories) to prepare for confocal imaging (Leica, TCS SP2 AOBS).

**Gal4 UAS**

The ET(-1.5hsp701:Gal4-VP16)$^{s1003t}$, Tg(UAS-E1b:-Kaede)$^{s1999t}$ transgenic line was acquired from ZIRC. Adult transgenic fish were outcrossed to AB/WIK wildtype fish and embryos with no Kaede fluorescence were selected, raised, and genotyped at 3 months for Gal4-VP16 carrier status. The UAS:Mitfa construct was created using the Tol2 kit (Kwan et al 2007). p3E-2AFLAG-mitfa-pA was combined with p5E-UAS-TP2, pME-GFP-nostop, and pDestTol2cryaa-DsRedpA to create pDestTol2cryaa-DsRedpA-UASP2/EGFP-nostop/2AFLAG-mitfa-pA. The UAS vector was combined in a 1:1 ratio with transposase mRNA for a final concentration of 0.25ng/ul and injected into one to two cell wildtype AB embryos. Embryos with DsRed expression in the lens at 52 hpf were selected and raised to maturity and then mated pair-wise with wildtype AB/WIK fish. Those fish that produced embryos with DsRed expression in the lens at 52 hpf were kept for mating to et19.1 Gal4 lines.

**Statistics**

For ocular section comparisons between genotypes, at least 7 sections from each genotype were averaged for the measurements of RPE thickness, Outer Nuclear
Layer thickness and ventral dorsal eye length. Anova analysis was performed using the online calculator at http://www.physics.csbsju.edu/stats/anova.html.

Results:

Mitfa and Mitfb are not required for zebrafish RPE development

To examine mitfb function in the developing RPE, seven different splice-blocking and translation-blocking morpholinos were injected into one or two cell embryos and examined during the first 5 days of development for any major abnormalities (Fig. 2.1A). The efficacy of the splice-blocking morpholinos was confirmed through RT-PCR analysis of injected embryos (Fig. 2.1B and data not shown). No significant phenotypes were discovered in any of the morphants and any minor phenotypes from higher morpholino concentrations were eliminated when co-injected with a tp53 morpholino, suggesting that these phenotypes were due to morpholino off-target effects. Two mutant lines were then obtained to further examine the phenotypic consequences of loss of mitfb function. The mitfb$^{hu3561}$ 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 (Fig. 2.1A). The mitfb$^{hu3857}$ mutant allele has a nonsense mutation in exon 2 causing a switch from leucine to a stop codon (Fig. 2.1A).

Each of the two mitfb mutant alleles were intercrossed to create homozygous mutants and examined for any ocular developmental phenotype, but no obvious abnormalities in homozygous mitfb mutants were observed (Fig. 2.1 D-F). 5dpf homozygous mutant embryos were then cryosectioned, stained with methylene blue, and photographed (Fig. 2.2). When the dimensions of the eye and the thickness of
several ocular layers were examined, the analysis revealed no significant differences between the \textit{mitfb} mutants and age-matched wildtype embryos in RPE thickness, outer nuclear layer thickness, or overall eye length (Fig. 2.2).

Due to the similarities between \textit{mitfa} and \textit{mitfb} in activation potential and expression in the developing zebrafish RPE, the possibility existed that Mitfa was able to compensate for the loss of Mitf activity in \textit{mitfb} mutants, leading to fully developed RPE. To examine this possibility, two \textit{mitfa} null mutant alleles \textit{mitfa}^{b692} and \textit{mitfa}^{w2} were crossed to the \textit{mitfb}^{hu3561} and \textit{mitfb}^{hu3857} alleles respectively and then bred to obtain homozygous \textit{mitfa; mitfb} double mutant embryos. However, these \textit{mitfa; mitfb} double mutants also did not display any RPE or ocular phenotype when compared to either age-matched wildtype or \textit{mitfa} mutant embryos (Fig. 2.1 G-I and Fig 2.2). Several attempts have been made at verifying the Mitfa and Mitfb protein truncation in these mutant alleles through Western blot analysis but as is the case with many zebrafish proteins, an effective antibody has not been discovered.

The expression of RPE related genes was then examined in \textit{mitfa} and \textit{mitfb} mutants to determine if there any changes to the expression of pigmentation or structural proteins. Dopachrome tautomerase (DCT) is a tyrosinase related protein involved in the production of melanin and can be observed in both the neural crest derived melanocytes as well as the RPE. When \textit{dct} expression was examined in \textit{mitfa} and \textit{mitfb} mutants at 24 hpf, there was a loss of neural crest melanocyte expression in \textit{mitfa; mitfb} double mutants when compared to wildtype and \textit{mitfb}^{hu3857} larvae but the RPE remained relatively unaffected (Fig. 2.3 A-D). There appears to be some possible...
loss of dct expression in the mitfa<sup>b692</sup>, mitfb<sup>hu3561</sup> and mitfa<sup>w2</sup>, mitfb<sup>hu3857</sup> homozygous larvae but this slight expression change is likely due to the lack of neural crest derived melanocyte staining in the eye region of larvae carrying a mitfa null allele. The expression of a structural protein in the premelanosome of the RPE, Silvb, was also examined at 24 hpf in wildtype and mitfa<sup>w2</sup>;mitfb<sup>hu3857</sup> embryos. No change in silvb expression was observed between the wildtype and the mitf double mutant which is consistent with the lack of RPE changes that has been previously observed (Fig. 2.3 E-F).

Since there was no apparent effect on the development of the RPE in mitf mutants, we then examined the photoreceptor layer for abnormalities at 5dpf using the Zpr1 monoclonal antibody that is specific for red/green double cones (Fig. 2.4). There were no changes to the red/green double cones in homozygous mitfa and mitfb single or double mutants when compared to wildtype sections. The rod photoreceptors were also examined through the use of a Zpr3 antibody but the study proved inconclusive due to the presence of auto-fluorescence that obscured the visualization of the stained cells.

**No progressive loss of RPE is observed in zebrafish mitf mutants**

In diseases such as age-related macular degeneration, the RPE develops normally but acquires defects over time that result in vision deficits. Due to the many functions that the RPE serves in the developed eye, the problems created by the loss of RPE function can widely vary. In humans, the most common form of age-related macular degeneration is characterized by defects in RPE function leading to the buildup
of drusen and a progressive loss of central vision. Due to the close relationship between the RPE and the photoreceptors, these cells are often the most severely affected by any disturbances in RPE function. The photoreceptors rely on the RPE for the daily phagocytosis of their outer segments which are consistently replaced near the inner segment.

To investigate any progressive changes to the RPE in zebrafish mitfa and mitfb mutants, cryosections were obtained from fish that have been raised until adulthood. Fish aged between 2 and 2.5 years were fixed and sectioned and the resulting methylene blue stained sections were photographed and compared. Due to the size of the adult fish, only one or two fish from each genotype could be processed at one time which has resulted in a limited number of samples analyzed to date. No consistent obvious abnormalities were observed between homozygous mitf single and double mutants when compared to wildtype sections (Fig 2.5 A-D). One of the three homozygous mitfa\textsuperscript{b692};mitfb\textsuperscript{hu3561} adult fish that were examined had some form of RPE abnormality that resulted in a small area of increased RPE thickness, but this change did not appear to affect the retinal layers. A few additional fish from several mitf mutant genotypes were also analyzed for photoreceptor changes using the Zpr1 red and green double cone specific antibody. No significant differences in Zpr1 expression has been observed in mitfa\textsuperscript{b692};mitfb\textsuperscript{hu3561} and mitfa\textsuperscript{w2};mitfb\textsuperscript{hu3857} homozygous mutant samples (Fig. 2.6). There was some additional Zpr1 staining in the RPE of the only mitfa\textsuperscript{w2};mitfb\textsuperscript{hu3857} homozygous mutant examined to date (Fig. 2.6 D). It was hard to tell if this was due to any RPE abnormality or due some distortion of the sample during the sectioning process. Analysis of additional mitfa\textsuperscript{w2};mitfb\textsuperscript{hu3857} samples will be required.
Due to the limited sample size it is difficult to make any strong conclusions from these experiments but the initial data suggests that in addition to not being required for proper RPE development, Mitf transcription factors are not necessary for the function of the RPE throughout zebrafish adulthood.

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


Using the Gal4-UAS system, we took advantage of the spatio-temporal control of *mitfa* expression to examine the ability of Mitfa to promote an RPE cell fate in developing zebrafish retinal cells. The et19.1 enhancer trap line Et(-1.5hsp701:Gal4-VP16)*1003t* expresses Gal4-VP16 in the developing retina starting around 24 hpf (Fig 2.7A and Scott et al 2007). This retinal specific expression was confirmed though analysis of 3dpf cryosections of et19.1 Gal4-VP16; UAS:Kaede embryos (Fig. 2.7B). A UAS transgenic line was then created with a 5x UAS repeat driving expression of *mitfa* as well as a GFP reporter (Fig. 2.7 C-D). The UAS:*mitfa* construct also contains an
independent alpha crystallin promoter driving expression of DsRed in the developing lens to allow for selection of transgenic embryos carrying the UAS:\textit{mitfa} construct (Fig. 2.7 C-D).

The et19.1 retinal driven Gal4-VP16 line was crossed to germline UAS:\textit{mitfa} transgenic carriers to produce embryos that have GFP expression in the developing retina starting around 24hpf, with robust expression by 28hpf (Fig 2.8 A). GFP expression was not observed in embryos that did not also 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. Cryosections of 5 dpf GFP positive embryos contained scattered patches of pigmented cells throughout the retinal layers in a pattern consistent with the observed GFP expression (Fig 2.8 B). Initial attempts at in situ hybridization of mitfa in et19.1 Gal4-VP16; UAS:\textit{mitfa}, GFP sections has failed due to the inability to effectively process the slides without affecting the integrity of ocular tissue sections. Future experiments will attempt to verify the overlap of mitfa expression with GFP and pigmentation. The overall size of the eye is slightly reduced in the et19.1:Gal4; UAS:\textit{mitfa} embryos (15% reduction in eye to head size ratio) but the basic structure of the retinal layers remains unaffected by the scattered pigmentation. Whether this size reduction is due to the suspected anti-proliferative functions of Mitf or a general disruption of eye development caused by the Gal4-VP16 and UAS constructs will have to be explored further. This experiment demonstrates that \textit{mitfa} is capable of inducing pigmentation in the developing zebrafish eye \textit{in vivo} and provides additional
support for the theory that \textit{mitfa} and \textit{mitfb} may have a non-essential role in zebrafish RPE development.

Discussion:

The absence of any observable phenotype following the loss of zebrafish \textit{Mitfa} and \textit{Mitfb} activity is a major departure from what has been previously reported in other species. Although the lack of an effective antibody limits our ability to verify the truncation of the zebrafish Mitf mutant protein truncations directly, the use of multiple morpholinos and several mutant lines affords a level of confidence that the activity of this protein has been successfully eliminated. The complicated nature of the mammalian \textit{Mitf} gene which contains a number of alternative splice sites that can result in a variety of different isoforms does raise some concerns that alternate zebrafish isoforms may be responsible for the lack of phenotype. However, the number of morpholinos and mutant lines examined in these experiments does alleviate some concerns. Furthermore, the \textit{mitfb}^{hu3561} allele, despite not producing the severe truncation that is expected in the \textit{mitfb}^{hu3857} allele, the location of the mutation in the helix loop helix domain of the \textit{mitfb}^{hu3561} allele is similar to a murine mutation known to create a null allele (Steingrimsson et al 1994). The evolutionary necessity of this functional domain in the Mitfb protein suggests that it could not be spliced out without severely disrupting the function of the protein.

Degenerate PCR analysis of the zebrafish genome has not revealed any other likely \textit{mitf} genes but another isoform of Mitfa (Mitfa1a) was found to be expressed in the developing zebrafish eye. This Mitfa1a isoform has both a novel promoter and a first
exon upstream of the original mitfa gene and skips over mitfa’s first two exons (Fig. 2.9 A). The promoter region of this mitfa1a locus was cloned into a plasmid in front of a GFP sequence and injected into wildtype embryos (Fig. 2.9 B). The resulting injected embryos displayed GFP in the developing eye, though the exact ocular tissue expression has not been verified (Fig. 2.9 C). However, it is important to note that the existence of this isoform should not change the results of the experiments as both the mitfa w2 and mitfa b692 mutations should create null alleles for this isoform as well.

A closely related protein to Mitf, Tfec, is also expressed in the developing zebrafish RPE (Lister et al 2011). Despite its similarity to Mitf in protein structure and potential functions, decreases in Tfec activity have yet to be linked to pigmentation abnormalities in any species. Early experiments with a tfec translation blocking morpholino in zebrafish wildtype and mitfa/mitfb mutants failed to demonstrate any RPE abnormalities but the effectiveness of this morpholino has not been proven and more experiments will be needed in order to rule out the involvement of Tfec in zebrafish RPE development (Data not shown). mitfa and mitfb double mutants were examined at the 21 somite stage for changes in tfec expression, but the expression of tfec remained consistent with wildtype expression.

In addition to the functions served in early eye development, a few murine Mitf mutant alleles are associated with progressive photoreceptor degeneration and RPE hypopigmentation (Steingrimsson et al 1994). It is not as surprising that zebrafish mitf mutants do not appear to display these progressive RPE and photoreceptor abnormalities due to the reported differences in adult Mitf expression patterns. Murine isoforms that are expressed in the developing RPE are also detected in adult ocular
tissue (Takeda et al 2002). The expression of mitfa and mitfb begins to decrease in the RPE after the start of RPE pigmentation at 24 hpf and by 60 hpf mitfa is no longer expressed in the eye, while mitfb is now expressed in the ciliary margin instead of the RPE (Lister et al 2001).

The ability of zebrafish Mitf transcription factors to promote pigmentation in tissues such as the developing retina is not overly surprising as this is an evolutionarily conserved trait that has been observed in several species. In addition, previous experiments with zebrafish have identified the ability of mitfa and mitfb to direct the activation of tyrosinase related proteins in vitro, and to rescue neural crest derived melanocyte development in vivo (Lister et al 2001).

Despite not being required for zebrafish RPE development, the ability of Mitfa to direct the differentiation of developing retinal cells into a pigmented cell type suggests that zebrafish Mitf proteins are still involved in RPE development. The pigmented cells are not limited to a specific retinal cell layer and this differentiation does appear to be cell autonomous. Rather than developing into the additional pigmented layer in place of most if not all retinal cells, as was observed in mouse retinal Mitf misexpression experiments, the Gal4 UAS transgenic zebrafish eyes exhibit scattered foci of pigmentation coinciding with the mosaic GFP expression in the retinal cells. The lack of a perfect correlation between GFP and pigmentation displayed in the 10 micron z stack images in Figure 3. is likely a result of the thin cryosections used in the analysis. Future studies using thicker sections may provide better evidence of this GFP and pigmentation correlation. Subsequent experiments will also address the correlation between mitfa expression and GFP expression through in situ hybridization analysis.
The scattered mosaic mitfa and GFP expression observed in these Et19.1 Gal4 VP14; UAS:mitfa,GFP embryos is most likely due to the mosaic expression of the original Et19.1 Gal4-VP16 line that is further diluted by combining it with the mosaicism that is most likely present in the transgenic UAS:mitfa,GFP line. The murine Mitf misexpression experiments also took place in a Chx10 mutant background, a retinal specific transcription factor that is also believed to repress Mitf expression in developing retinal cells. It is unknown, whether the zebrafish homologue of Chx10 is capable of inhibiting mitfa or mitfb expression but the ability to produce any pigmentation in the developing retina in a wildtype background through the misexpression of mitfa is a significant result.
Figure 2.1: The elimination of Mitfa and Mitfb activity does not have any noticeable effect on RPE development in zebrafish embryos. (A) Diagram of mitfb gene spanning approximately 77.8 kb with locations of mutations (arrows) and morpholinos (arrowheads). The mitfb<sup>hu3857</sup> allele contains a T>A nonsense mutation that causes a switch from Leucine to a stop codon in exon 2. The mitfb<sup>hu3561</sup> allele has a C<T nonsense mutation that results in a switch from Arginine to a stop codon in exon 7, in the first third of the basic helix loop helix/leucine zipper binding domain. All splice blocking morpholinos are expected to produce a frameshift and early stop codon. (B) The efficacy of splice blocking morpholinos was confirmed through RT-PCR analysis. Images of a gel demonstrating the reduced mRNA transcript size of the exon 2 morpholino injected (left two lanes) compared to uninjected wildtype 24 hpf larvae (right two lanes). (C) Genotyping of mutant alleles was accomplished through restriction enzyme digestion of PCR products. The addition of an AlwN1 restriction site allows for identification of fish carrying the mitfb<sup>hu3857</sup> mutant allele. Lane 1 shows a ladder to determine band size. (D-I) Bright field images depicting the normal RPE pigmentation and development of 5dpf larvae in wildtype (D), mitfb<sup>hu3857</sup> (E), mitfb<sup>hu3561</sup> (F), mitfa<sup>w2</sup> (G), mitfb<sup>hu3857</sup>/mitfa<sup>w2</sup> (H), and mitfb<sup>hu3561</sup>/mitfa<sup>b692</sup> (I) fish lines.
Figure 2.2: No changes to the RPE and retinal layers are observed in cryosections of zebrafish Mitf mutants. (A) Sagittal ocular sections from at least seven equivalent axial sections for each genotype were analyzed using ImageJ software. RPE and Outer Nuclear Layer thickness was measured at the macular area approximated in the figure and the eye length was measured at the proximal point of the lens. Anova analysis revealed no significant differences in RPE thickness (Top, p=0.115), ONL thickness (middle, p=0.156), or total eye length (bottom, p=0.069). All measurement are in pixels and 1 pixel=0.365 um
Figure 2.3   Red-green double cone photoreceptors do not appear to be affected in zebrafish mitf mutants. (A-F) No changes were observed in the number or morphology the red-green double cone photoreceptors in mitf single or double mutants. Representative confocal images of 25 micron sections of 5dpf zebrafish eyes stained with Zpr1 to identify red-green double cones. (A) wildtype, (B) mitfa<sup>w2</sup>, (C) mitfb<sup>hu356</sup>, (D) mitfb<sup>hu3857</sup>, (E) mitfb<sup>hu3561</sup>/mitfa<sup>b692</sup>, and (F) mitfb<sup>hu3857</mitfa<sup>w2</sup>. Scale bars in A-F are 20 micrometers.
Figure 2.4: The loss of Mitf does not noticeably affect the expression of RPE related genes. (A-D) The expression of Dopachrome Tautomerase (DCT) was examined at 24 hpf and representative images are displayed for wildtype (A), mitfb\textsuperscript{hu3857} (B), mitfb\textsuperscript{hu3561}/mitfa\textsuperscript{b692} (C), and mitfb\textsuperscript{hu3857}/mitfa\textsuperscript{w2} (D). DCT is expressed in both neural crest derived melanocytes and RPE (A-B) except in mitfa mutants where the neural crest expression is lost (C-D). Due to the presence of neural crest melanocytes in the eye region of wildtype and mitfb mutants, it is difficult to tell if the RPE specific DCT expression is decreased slightly in mitfa/mitfb double mutants. (E-F) The expression of a RPE specific melanosome structural protein, Silvb, was also examined in wildtype (E) and mitfb\textsuperscript{hu3857}/mitfa\textsuperscript{w2} (F) at 24 hpf and no significant changes were observed.
Figure 2.5: No progressive loss of RPE or change to retinal lamination is observed in zebrafish Mitf mutants. (A-D) 25 micron sections from adult fish aged between 1.8-2.5 years were stained with methylene blue and analyzed for any major RPE or retinal abnormalities. No consistent abnormalities were observed in the limited number of sample examined in (A) wildtype (1.8-2.3 years old, N=2), (B) mitfb<sup>hu3857</sup> (2.5 years, N=1), (C) mitfa<sup>b692</sup>;mitfb<sup>hu3561</sup> (2-2.2 years, N=3), mitfa<sup>w2</sup>;mitfb<sup>hu3857</sup> (2.5 years, N=2). Scale bars are 40 micrometers.
Figure 2.6: The red green double cone photoreceptors appear unaffected in adult mitf mutants. (A-D) Representative confocal images of 25 micron sections from adult fish aged between 2-2.5 years and stained with Zpr1 antibody. No consistent abnormalities were observed in the limited number of sample examined in (A) wildtype (2.3 years old, N=1), (B) mitfa$^{w2}$ (2 years, N=2), (C) mitfa$^{b092}$/mitfb$^{hu3661}$ (2-2.2 years, N=2), mitfa$^{w2}$/mitfb$^{hu3857}$ (2.5 years, N=1). Scale bars are 50 micrometers.
Figure 2.7: The Gal4-UAS system. (A) Mitfa expression in developing retinal cells was achieved using the Gal4-VP16 UAS system. Visualization of Gal4 activity was achieved through the use of a UAS Kaede reporter and is highly expressed by 28 hpf. (B) Cryosections of 3 dpf embryo eyes demonstrate the expression of Kaede in all 5 basic retinal layers in the et19.1 Gal4-VP16 fish line. (C) The UAS:mitfa transgenic line was created with a 5x UAS element driving expression of mitfa and GFP. (D) The selection of embryos carrying the UAS:mitfa vector was achieved through the use of an independent alpha-crystallin promoter driving expression of DsRed in the developing lens at 48hpf. Scale bar indicates 25 micrometers in (B)
Figure 2.8: The misexpression of Mitfa in developing retinal cells is capable of inducing foci of pigmentation in the retina. (A) The offspring generated from crossing the et19.1 Gal4-VP16 line to the UAS:Mitfa transgenic line express GFP in the developing retina. GFP expression begins around 24hpf and is easily visible in the 3dpf embryo. (B) Methylene blue stained cryosections of 5 dpf et19.1 Gal4-VP16; UAS:Mitfa,GFP larvae exhibit scattered pigmentation throughout the retinal layers. (C-F) Confocal microscopy analysis of et19.1 Gal4-VP16; UAS:Mitfa,GFP cryosections at 5 dpf depicting pigmentation (C), GFP expression (D), and DsRed expression in the lens (F). An overlap of the three z-stack images (H) demonstrates the overlapping of GFP expression and pigmentation in the retina (white arrows). Scale bar indicates 20 micrometers in B and 10 micrometers in (C-F)
Figure 2.9: An additional isoform of Mitf is expressed in the eye and capable of directing GFP expression in the developing eye. (A) An additional isoform of Mitfa (Mitfa1a) is expressed in the zebrafish eye. Mitfa1a uses an alternative promoter approximately 20kb upstream of the original mitfa gene. It has a novel first exon and skips over the first two exons of the original mitfa gene. (B) To examine the timing and expression pattern of the Mitfa isoform, the mitfa promoter was cloned into a vector to drive expression of GFP. (C) When injected into one cell embryos, GFP began to show mosaic GFP expression in the eye around 24 hpf and highly expressed in the 48 hpf fish.
Chapter 3

Otx transcription factors are required for zebrafish RPE development

Introduction:

Orthodenticle related transcription factors are members of the bicoid subfamily of homeodomain transcription factors. The mammalian Otx1 and Otx2 genes have been identified as necessary for anterior brain and head development (Simeone et al 2002). Null mutations of Otx1 result in brain abnormalities but do not disturb the development of the murine eye or RPE (Martinez-Morales et al 2003). Homozygous null mutations of Otx2 are embryonic lethal in mice, likely due to its vital role in the anterior head development (Martinez-Morales et al 2003). Heterozygous mutations of Otx2 in mice have been associated with a small percentage of embryos with RPE and eye developmental abnormalities (Martinez-Morales et al 2003). When heterozygous Otx2 mutants are raised in a homozygous Otx1 null mutant background, almost all of the resulting embryos display severe RPE abnormalities leading to microphthalmia or an+ophthalmia eye phenotypes (Martinez-Morales et al 2003). These RPE deficits occur primarily in the ventral portion of the eye and can leave the dorsal most RPE unaffected. Depending on the severity of the RPE deficits, these ocular phenotypes are often associated with folding and subsequent degradation of the original retinal layers. The cells fated to become RPE cell layers in these Otx mutants remain unpigmented and display markers and characteristics of an additional retinal layer.

Similar to what is observed with zebrafish mitf genes, the original mammalian Otx1 gene has undergone a duplication event in the zebrafish genome that has resulted
in two Otx1-like genes, otx1a and otx1b. Like the mammalian Otx genes, these zebrafish genes contain 3 exons and are involved in anterior brain and head development. The zebrafish Otx1a and Otx2 proteins are highly conserved when compared to the murine orthologues (78% and 96% respectively) but Otx1b appears to be a hybrid protein with 63 percent homology to murine Otx1 and 62% homology to murine Otx2 (Mercier et al 1995). Otx1a and Otx1b also display similarities in timing and expression patterns to murine Otx2. A previous analysis of these three otx genes by Foucher et al used translation blocking morpholinos to examine the effects of this protein knockdown on zebrafish brain development and was able to verify the effectiveness of these morpholinos using a pan Otx antibody (Foucher et al 2006). Using various combinations of the three otx morpholinos, this study found that the combination of otx1a and otx2 morpholinos altered the cerebellar fate of zebrafish larvae. This suggests a level of redundancy in developmental functions between Otx1a and Otx2 with Otx1b having less of a developmental impact. Ocular abnormalities were also observed in these otx1a/otx2 morphants but were not described and the individual contributions of these zebrafish Otx transcription factors requires further analysis.

**Methods:**

**Wildtype and Mutant Zebrafish**

Wildtype embryos were obtained through natural matings of adults from the AB strain or AB/WIK hybrids and were staged according to Kimmel et al 1995. The otx2 mutant line was obtained from the Sanger Institute and genotyped with the following primers Otx2F: 5’ ATATTTTCATGCAGGAG 3’, Otx2R: 5’
GACACTTTGCCCTTCGGTCTT 3’. All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Morpholino Knockdown**

Morpholinos were obtained from Gene tools. *Otx1a* and *Otx2* morpholinos are described in Foucher et al 2006. *Otx1b* MO: 5' TAACATATAGCCTACCTGAACTCGG 3’. The efficacy of the otx1b splice blocking morpholino was confirmed thought RT-PCR analysis with Otx1bF: 5’ ATGGACCTACTACACCCGTG 3’ , Otx1bR: 5’ GTGCTGAGCCTGGAGAAA TG 3’. The control morpholino used in all experiments was designed against the CS2 5'leader sequence. The volume of morpholino dispensed was measured using oil and a micrometer to calculate the diameter of the morpholino droplet. 5 dpf embryos were anesthetized in Mesab and each eye was examined for phenotype at 5dpf using a SZX12 dissecting stereomicroscope with DP70 camera (Olympus).

**Cryosectioning and analysis**

For histology, fixed specimens were equilibrated in 30% sucrose in PBS, embedded in Tissue-Tek O.C.T (Sakura Finetek, Torrance, CA) and cryosectioned 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 20x zoom. Measurements of the images were made using the ImageJ software. Images were processed using Adobe Photoshop and Helicon Focus software.
Statistics

For ocular section comparisons between genotypes, at least 7 sections from each genotype were averaged for the measurements of RPE thickness, Outer Nuclear Layer thickness and ventral dorsal eye length. Anova analysis was performed using the online calculator at http://www.physics.csbsju.edu/stats/anova.html.

Immunohistochemistry

Larvae were anesthetized in Mesab at 5dpf and fixed in 4%PFA overnight. Immunohistochemistry was performed as described in (Macdonald et al 1999) except for the permabilization step which was performed with Proteinase K. The Zpr1 monoclonal antibody (ZIRC) and Alexa 488 goat anti-mouse secondary antibody (Invitrogen) were used at a concentration of 1:1000 each. The larvae were then sectioned at 25 microns and covered with vectashield (Vector laboratories) to prepare for confocal imaging (Leica, TCS SP2 AOBS).

Results:

Otx1a and Otx2 transcription factor activity is required for zebrafish RPE development

Previous analysis of zebrafish otx genes suggests that in addition to anterior brain abnormalities, the combined knockdown of Otx1a and Otx2 transcription factors can result in eye abnormalities (Foucher et al 2006). Using the previously characterized translation-blocking morpholinos for otx1a and otx2 as well as a new splice-blocking
morpholino designed against *otx1b*, we investigated the phenotypic effect of the individual and combined loss of function for Otx transcription factors. Similar to what was previously reported, the knockdown of any of the Otx factors by themselves did not result in noticeable RPE pigmentation changes. 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 produced a significant delay in pigmentation in the developing RPE when examined at 35hpf, leaving the neural crest derived melanocytes unaffected (Fig. 3.1 A-B). RPE pigmentation began to recover around 72 hpf, possibly due to the transient knockdown produced by the morpholino.

At 5 dpf, the *otx* morphant phenotype was extremely variable both between embryos and between eyes of a single embryo (Fig 3.1 C-D). Each eye from 5dpf embryos was examined and scored as having either 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. 3.2 A-C). Similar to what is observed in murine *Otx* mutants and in the zebrafish pigmentation phenotypes at 35 hpf, the single knockdown of Otx1a, Otx1b, or Otx2 had little effect on eye phenotype (0%, 3% and 6% minor phenotype respectively). 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 (Fig 3.2 D). The combination of *otx1a* and *otx2* morpholinos however, resulted in defects in nearly all embryos (95%) and most embryos (64%) having major disruptions of the developing
RPE (Fig. 3.2 D). These results suggest that Otx1a and Otx2 have the major, though partially redundant roles in zebrafish RPE development. Otx1b appears to have a role in zebrafish RPE development though its functions seem to be almost completely redundant to Otx1a and Otx2, as 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 factors creates 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 the 2.4 ng/embryo that is present in the otx1a/otx2 morpholino combination (Fig. 3.2 E).

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 the gene encoding 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. 3.3 A-B). The analysis of the RPE specific silvb gene at 24 hpf produced similar results, with a small area of absent expression in the ventral portion of the eye in a small percentage of single otx1a, otx1b, or otx2 morphants (Fig 3.4 A-D). The combination of otx1a/otx2 morpholinos produced significant ventral silvb expression deficits and these deficits were even greater in the otx triple morphants (Fig 3.4 E-F).

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 phenotypic morphants does not have any
noticeable effect on the retinal layers (Fig. 3.5A). Major loss of RPE causes a loss of the cup shape in the ventral half of the developing eye with the some loss of ventral ocular tissue and the remaining retinal layers often extending proximally instead of distally (Fig. 3.5 B). A rotation of the entire eye up to 90 degrees is also observed in many of the more severe RPE deficient zebrafish larvae. Despite the rotation and missing tissue, the retina appears to be well organized in areas of the eye lacking RPE. Otx morphants also do not display any microphthalmia except for the occasional 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 RPE layer immediately adjacent to the affected ventral area. (Fig. 3.5 C-E)

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. 3.5 A-B). In addition to examining retinal lamination, 5dpf larvae were assessed using the monoclonal Zpr1 antibody that is specific for red/green double cone photoreceptors (Fig. 3.6 ). 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. Initial attempts to examine any possible transdifferentiation of ganglion cells in place of the absent RPE using a Zn8 antibody have been unsuccessful. Further experimentation is needed to determine the mechanisms responsible for the missing RPE. The lens does not appear to be affected by the RPE deficits, though some minor displacement or rotation can be observed in the most severe cases.
Recently, an *otx2* null mutant line has been obtained and initial experiments have revealed a minor, albeit variable, coloboma phenotype in homozygous *otx2* mutants (Figure 3.7). This phenotype provides support for the results of the *otx* morpholino experiments and suggests that Otx1a activity is not sufficient to direct complete RPE development. However no conclusions can be drawn from the phenotype until the mutant line has been outcrossed several times to a wildtype strain in order to reduce the probability of a phenotypic effect from another gene mutation in the *otx2* mutant line.

**Discussion**

The function of Otx transcription factors appears to be evolutionarily conserved between zebrafish and mice despite the existence of an additional zebrafish *otx* gene. Based on the results of the individual and combined knockdown of zebrafish Otx factors, the development of the RPE appears to depend on the activity of Otx1a and Otx2, with Otx1b activity mostly overlapping the functions of the other two Otx transcription factors. This is supported by the finding that *otx1b* morpholino knockdown did not show any appreciable effect on the eye phenotype of zebrafish larvae. The combined knockdown of *otx1b* with either *otx1a* or *otx2* failed to produce the increased phenotype severity that is observed in *otx1a/otx2* morpholino knockdown, whereas the most severe RPE deficits were identified when all three otx genes were targeted. The increased severity of *otx1a/otx2* morphants compared with *otx1a/otx1b/otx2* morphants was apparent even when the individual concentration of the three morpholinos was reduced to achieve the same total concentration as the *otx1a/otx2* morpholino experiments. This suggests an overlap in the function of Otx1b with Otx1a and Otx2
which is not surprising when considering the similarities in amino acid composition of Otx1b with both Otx1a and Otx2 transcription factors. It is unknown why the previous analysis of Otx transcription factors by Foucher et al failed to identify any RPE developmental abnormalities with Otx1b knockdown, but this is most likely due to their concentration on brain-specific phenotypes.

Despite the similarities in the necessity of Otx transcription factors in RPE development across species, the phenotype of zebrafish otx morphants differs from what has been observed in mice. Both Mitf and Otx murine mutants experience a transdifferentiation of the RPE into an additional retinal layer with folding and degradation of the original retinal layer. The retinal layers appear largely unaffected in even the most severe RPE deficient zebrafish otx morphants and shows no folding up to 10 dpf. The RPE of zebrafish otx morphants does not appear to transdifferentiate into another retinal layer when examined for Zpr1 stained photoreceptors or through basic observation of methylene blue stained sections. The ability to examine the RPE deficits in zebrafish otx morphants is hampered by the position of the ventral RPE right alongside the oral cavity. It is unknown if these ocular positioning differences between species could have some effect on the development of the retinal layers or the transdifferentiation of the RPE layer.

Attempts have been made to create a transgenic line expressing a UAS:otx2, GFP construct. However, when mated to the Et.19.1 Gal4-VP16 carrier line, the resulting embryos failed to produce pigmentation in the developing eye despite a similar pattern of GFP expression to what is observed in UAS:mitfa, GFP experiments. Additional GFP expression is again observed in scattered tissues including the heart,
but unlike the mitfa transgenic embryos, this GFP expression in the heart is not accompanied by additional pigmentation and circulation defects. Future in situ hybridization experiments will be required to determine if this GFP expression is reflective of Otx2 expression or if new attempts at the creation of a UAS:otx2 transgenic line are needed.
Figure 3.1: Zebrafish Otx genes. (A-C) The zebrafish genome contains three otx genes (Otx1a, Otx1b, and Otx2) corresponding to the two mammalian Otx1 and Otx2 genes. (A) Representative depiction of the three zebrafish otx genes which all contain three coding exons which result in a protein containing a central homeodomain surrounded by amino and carboxyl terminal activation domains. (B) Alignment of human, mouse and zebrafish Otx2 amino acid sequences showing a 96% similarity between the zebrafish and mammalian sequences. Homeodomain is depicted in red and asterisks depict changes in amino acid composition between species. (C) Alignment of human and mouse Otx1 with zebrafish Otx1a and Otx1b amino acid sequences depicting a 78% and 63% similarity between the mouse sequence and Otx1a and Otx1b respectively. Otx1b also is 62% identical to murine Otx2 in amino acid composition. The homoeodomain is depicted in Red for all species. There were no changes to the homoeodomain sequences between species between the Otx1 and Otx2 orthologues.
Figure 3.2: Otx combined knockdown causes a decrease in RPE pigmentation and an incomplete closure of the optic fissure. (A-D) Embryos were injected with *otx1a* (1.25 ng/embryo), *otx1b* (1.25 ng/embryo), *otx2* morpholinos (1.25 ng/embryo), as well as the various combination of these morpholinos (1.25 ng/embryo each) (A-B) Control morpholino (C) and *otx1a*/*otx2* morpholino (D) injected at embryos photographed at 35 hpf. White arrows depict the unaffected neural crest derived melanocytes in *otx1a*/*otx2* morphants and black arrows identify the loss of RPE pigmentation in the developing RPE of *otx1a*/*otx2* morphants. (C-D) Ventral view of 5 dpf control morpholino (F) and *otx1a*/*otx2* morpholino (G) injected embryos. The eyes of *otx1a*/*otx2* morphants displayed an eye phenotype that can vary in severity between eyes of the same embryo (E) with one eye displaying a minor phenotype (black arrow) and the other eye displaying a major RPE deficit phenotype (white arrow).
Figure 3.3: Otx morpholino knockdown results in a variable degree of RPE deficits in zebrafish. (A-C) Eye phenotypes were scored at 5dpf and defined as normal when the RPE displayed no visible defects (A), minor when the eye had a coloboma but there was still a connection between the anterior and posterior RPE (B), and major when the anterior and posterior RPE were completely separated (C). (D) When injected at a concentration of 1.25 ng/embryo of each morpholino, the knockdown of otx1a (n=108), otx1b (n=230), or otx2 (n=122) had little effect on RPE development and the combined knockdown of otx1a/otx1b (n=222) and otx1b/otx2 (n=256) produced a modest increase in the percentages of minor phenotypes (15.8% and 25.6% respectively). The combined knockdown of the otx1a/otx2 (n=214) produced RPE deficits in almost all embryos (95%) with most of the eyes exhibiting a major RPE deficit (64%). The loss of RPE was even more severe in the otx1a/otx1b/otx2 triple morphants (n= 178) with 96.6% of larvae displaying major RPE deficits and the rest having minor deficits (3.4%). (E) To determine if the increase in phenotypic severity between otx1a/otx2 and otx1a/otx1b/otx2 morphants is due to the increased total morpholino concentration, morphants from each condition were examined at 5 dpf at a total concentration of 2.4 ng/embryo. The otx1a/otx1b/otx2 morphants (n=296) again displayed a significant increase in the percentage of eyes with major RPE deficits when compared to otx1a/otx2 morphants (n= 156) (92.2% compared to 57.5%, p< 0.001).
Figure 3.4: Otx knockdown causes a decrease in DCT expression in the developing RPE. DCT mRNA expression was analyzed through in situ hybridization at 24 hpf in Otx morphants. The expression of DCT in the RPE was only reduced slightly in a small percentage of otx1a (B), otx1b (C), and otx2 (D) morphants when compared to control morphants (A). DCT was found to be significantly decreased in the ventral RPE of otx1a/otx2 morphants and even further decreased in otx1a/otx1b/otx2 morphants (F) when compared to control morphants (A). DCT expression in neural crest melanocytes was unaffected in all morphants. (black arrows point to RPE expression and white arrows point to neural crest expression).
DCT expression

A. Control MO
B. Otx1a MO
C. Otx2 MO
D. Otx1b MO
E. Otx1a + Otx2 MO
F. Otx1a + Otx1b + Otx2 MO

24 hpf
Figure 3.5: Otx knockdown causes a decrease in silvb expression in the developing RPE. (A-F) Expression of the RPE specific silvb gene 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 silvb expression in the ventral eye when compared to wildtype (A). The combined otx1a/otx2 morphants (E) and displayed a significant loss of ventral silvb expression in most larvae and this expression was even further decreased in otx1a/otx1b/otx2 morphants (F).
Silvb expression

A 24 hpf  Wildtype

B 24 hpf  Otx1a MO

D 24 hpf  Obx1b MO

C 24 hpf  Otx2 MO

E 24 hpf  Otx1a + Otx2 MO

F 24 hpf  Otx1a + Otx1b + Otx2 MO
Figure 3.6: The loss of ventral RPE in otx morphants has little effect on the retinal layers. (A-B) Methylene blue stained sagittal ocular section of otx1a/otx1b/otx2 morphants with a minor (A) and major (B) RPE deficits at 20x magnification. The basic structure of the eye and the retinal layers remain relatively unaffected despite the loss of RPE. A closer examination reveals that the RPE appears to be absent rather than transdifferentiated into the additional retinal like layer that is observed in murine Otx mutants. (C-E) Measurements of the RPE (C), Outer nuclear layer (D) and the length of the dorsal half of the eye (E) were compared between otx1a/otx2 morphants and wildtype embryos. Equivalent sections from 15 different eyes for each condition were averaged and no significant differences were observed between the otx1a/otx2 morphants and wildtypes eyes (p > 0.05).
Figure 3.7: The loss of RPE in Otx morphants does not result in deficits in red green double cone photoreceptors. (A-D) Zpr1 staining of 25 micron sagittal cryosections reveals that the red-green double cone photoreceptors remain relatively unaffected in otx morphants. The cone photoreceptor expression did not show any deficits despite the loss of RPE in minor phenotypes (A). There was some loss of the original ventral ocular tissue in some of the more severe RPE deficient morphants phenotypes (B) as well as rotation of the eye (C-D) but this did not appear to affect the development of the red green cone photoreceptors despite the close developmental relationship between RPE and the photoreceptors. Scale bar is 25 micrometers in A-C and.
Figure 3.8: Otx2 homozygous mutants display a minor loss of ventral pigmentation and RPE development. (A) Otx2 homozygous mutants examined at 35 hpf display a loss of ventral pigmentation. (B-C) When examined at 5 dpf, Otx2 homozygous mutant larvae display a variable minor loss of RPE phenotype (B) but are otherwise phenotypically normal (C). (D-E) When DCT expression is examined at 24 hpf, otx2 homozygous larvae (E) exhibit a lack of ventral DCT expression when compared to wildtype larvae (D).
Chapter 4

The relationship between Mitf and Otx transcription factors

Introduction:

The relationship between Mitf and Otx transcription factors is complex and has been examined in several different species. These two families of transcription factors have an evolutionarily conserved ability to act as homodimers or to combine and form Otx/Mitf heterodimers, though the exact regulatory elements essential to these heterodimer formations is still under debate. Experiments in human and murine RPE cells have established that the transcription of pigmentation related genes are significantly increased when both factors are present, than with high concentrations of either factor alone (Martinez-Morales 2003, Reinisalo et al 2012). In addition to acting together to direct the development of pigmented cells, these transcription factors are suspected of regulating the expression each other through a feedback loop. In murine mutants of both Otx and Mitf, the expression of both factors is lost in the prospective RPE cells (Nguyen and Arnheiter 2000, Martinez-Morales 2001). Mitf and Otx expression is observed in the patches of RPE found in murine conditional B-catenin mutants but the expression of both factors is absent in the areas of the eye lacking RPE (Westenskow et al 2009). Zebrafish mutants of the upstream developmental factor Rx3 also lose both Mitfb and Otx2 expression in the prospective RPE cells (Rojas- Munoz et al 2005). It is unclear if this loss of Mitf and Otx expression in zebrafish Rx3 mutants is reflective of any feedback regulation or due to the loss of a common upstream factor.
and complete loss of ocular tissue. Cell culture experiments and analysis of the promoter regions of Otx and Mitf genes in several species suggests that this transcription feedback regulation occurs through unknown distal elements or intermediary molecules (Martinez-Morales 2003, Reinisalo et al 2012). Analysis of this potential Mitf and Otx feedback regulation in zebrafish may help validate some of the conclusions made in other species or uncover important differences in the zebrafish developmental pathway.

Methods:

Wildtype and Mutant Zebrafish

Wildtype embryos were obtained through natural matings of adults from the AB strain or AB/WIK hybrids and were staged according to Kimmel et al 1995. Primers for genotyping are listed in Chapter 2. All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Morpholino Knockdown

Morpholinos were obtained from Gene tools. The otx morpholinos are described in Chapter 3. The control morpholino used in all experiments was designed against the CS2 entry element GATCCTGCAAAAAAGAACAAGTAGCT. 5 dpf embryos were anesthetized in Mesab and each eye was examined for phenotype at 5dpf using a SZX12 dissecting stereomicroscope with DP70 camera (Olympus).
**In-situ hybridization**

Digoxigenin-labeled RNA probes were generated from the following plasmid templates: pCR2.1-otx1a (nt30050-31137), linearized with Xho1 and transcribed with T7 RNA polymerase; pCRII-otx2, linearized with Xho1 and transcribed with SP6 RNA polymerase. Probes for mitfa and mitfb and DCT were described previously (Lister et al 1999). In situ hybridization was carried out as described previously (C. Thisse & B. Thisse, 2008). When comparing the RNA expression between morphants and mutants, all embryos were processed at the same time to ensure equal exposure to the probes and coloration solution. Representative images were chosen for each condition based on the relative expression levels of the other morphants and mutants. All comparisons were performed 3 times except for the expression of otx1b.

**Quantitative PCR**

Total RNA was extracted from 50 embryos at 24hpf from matings of wildtype and homozygous mitfb\textsubscript{hu3561}, mitfb\textsubscript{hu3857}, mitfa\textsubscript{b692}, mitfb\textsubscript{hu3561}, and mitfa\textsubscript{w2}, mitfb\textsubscript{hu3857} larvae using Trizol reagent. This collection was repeated to obtain 3 independent samples for each genotype. The purified RNA concentration was measured using a spectrophotometer. Two different sets of primers were designed for each gene of interest as well as the B-actin control and tested for efficacy and specificity using absolute quantification analysis. Primers were designed against the exon boundaries to avoid possible DNA contamination of the results. The best primer set for Otx1a, Otx2, and β-actin were selected, combined with the samples, and run in triplicate for each of the 3 genotype samples. Analysis was performed using the Fast 7500 Q-PCR machine.
(Applied Biosystems). Primers used: B-actinF-cgagctgtcttcccatcca, B-actinR-tcaccaacgtagctgtttctg, otx1aF-cgacctgccaaaagaagag, otx1aR-atagaggctgggtccaaat, otx2F- gtgcattaagcccaagagc, otx2R- atacgcattcacgacccttc.

**Statistics**

Mitf and Otx combined knockdown data were first analyzed for significant interactions between the genotype and trials based on phenotype using R software. There was a significant interaction in the otx1a/otx2 combination morpholino experiments and these had to be analyzed on a trial by trial basis. Further analysis was performed through the use of Fisher’s exact T test.

**Results**

**Mitf transcription factors may still have role in RPE development**

Due to the evolutionarily conserved ability of Mitf protein to activate pigment related genes, the combined knockdown to Otx and Mitf transcription factors was explored to determine if mitfa and mitfb may still have a non-essential role in RPE development. To accomplish this, otx1a and otx2 morpholinos were injected into wildtype, mitfa\textsuperscript{w2}, mitfb\textsuperscript{hu3857}, and mitfa\textsuperscript{w2}; mitfb\textsuperscript{hu3857} homozygous mutant embryos to determine if the loss of Mitf activity would increase the severity of RPE deficits in otx morphants. Due to the potency of the combination of otx1a/otx2 morpholino injections even at the reduced concentration of 2ng total morpholino concentration/embryo, there was too much phenotypic variability between trials to analyze the multiple trials as a single group. However, each trial on its own had a significant increase in the
percentage of \textit{mitfa}^{w2}; \textit{mitfb}^{hu3857} double mutant embryos eyes with major RPE deficit phenotypes and a significant decrease in the percentage of eyes with a normal phenotype when compared to wildtype, \textit{mitfa}^{w2}, and \textit{mitfb}^{hu3857} mutant embryos (Fig 4.1). The same experiment performed in the \textit{mitfb}^{hu3561} mutants lines and also produced a high amount of phenotypic variability between trials (Fig 4.2). 3 of the 4 trials in these experiments produced a significant increase the percentage of eyes with a major RPE deficit phenotype in the \textit{mitfa}^{b692}, \textit{mitfb}^{h3561} morphants as well as a significant decrease in the percentage of normal eyes when compared to the wildtype, \textit{mitfa}^{w2}, and \textit{mitfb}^{hu3561} morphant embryos (Fig. 4.2).

The single injections of either \textit{otx1a} or \textit{otx2} morpholinos into \textit{mitfb}^{hu3857} mutants had less variability over 3 trials and produced a significant increase in both the percentage of embryos with a phenotype, as well as the severity of those phenotypes in \textit{mitfa}^{w2}; \textit{mitfb}^{hu3857} homozygous double mutants when compared to the other genotypes. (Fig 4.3) In addition to the increased phenotypic severity, the combined knockdown of Otx1a and Otx2 also produced a significant decrease in DCT expression in \textit{mitfa}^{w2}; \textit{mitfb}^{hu3857} double mutants when compared to wildtype \textit{otx} morphants and uninjected \textit{mitfa}^{w2}; \textit{mitfb}^{hu3857} mutants (Fig. 4.4). Taken together, the evidence suggests that although \textit{mitfa} and \textit{mitfb} are not necessary for RPE development, they do have a role in zebrafish RPE development. The increase in severity in \textit{mitfa};\textit{mitfb} double mutants when compared to single \textit{mitfa} and \textit{mitfb} mutants suggests that \textit{mitfa} and \textit{mitfb} have overlapping RPE developmental functions.

\textbf{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. In zebrafish, mitfa and mitfb expression was examined though in situ hybridization at 21hpf in otx1a, otx1b, and otx2 single morphants as well as the 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. 4.5). Expression of mitfb in the RPE but not in the epiphysis was also significantly decreased in otx1a, otx1b, and otx2 morphants (Fig. 4.6). The reduction in mitfa and mitfb expression varied somewhat between embryos, but otx1a morphants have consistently displayed a greater reduction in expression when compared to otx1b and otx2 morphants. Interestingly, when the expression of tfec was examined in otx morphants, the single morpholino knockdown of otx2 and otx1b but not otx1a resulted in a significant decrease in tfec expression when compared to wildtype embryos at the 21 somite stage.

The expression of otx1a and otx2 was also examined through in situ hybridization at 21hpf in mitfa^w2 and mitfb^hu3857 single and double mutants. There was no observable change in otx1a or otx2 expression in any of the mitfa^w2 and mitfb^hu3857 single or double homozygous mutants when compared to wildtype embryos (Fig. 4.7). To verify these results, total RNA was collected from 24 hpf wildtype, mitfb^hu3561, mitfb^hu3857, mitfa^b692, mitfb^hu3561, and mitfa^w2; mitfb^hu3857 homozygous mutants on three separate occasions. After verifying the specificity of the primers, Q-PCR analysis was performed with 3 replicates of the three separate samples for each condition. Relative quantification of otx1a and otx2 changes in each sample were examined against the B-
actin control levels. The results of the analysis showed a small and inconsistent decrease in Otx1a and Otx2 expression in mitf mutants (Fig 4.8). The expression of otx1a was significantly decreased to about 4/5 of its normal expression levels in mitfb^{hu3857}, mitfa^{b692};mitfb^{hu3561}, and mitfa^{w2};mitfb^{hu3857} samples (Fig 4.8). However, when Otx2 expression was examined, a significant decrease in expression was observed in the mitfb^{hu3857} samples but not the mitfa^{b692};mitfb^{hu3561}, and mitfa^{w2};mitfb^{hu3857} samples (Fig 4.8). The mitfb^{hu3561} samples actually display a slight insignificant increase in both Otx1a and otx2 expression levels. The inconsistencies between single and double mutant genotypes and in the direction of otx expression level changes suggest that there may have been some human errors involved in the sample collection. These errors could be a result of the difficulty in timing the extraction of RNA from embryos of 5 genotypes at the same exact time-point. Due to the rapid development occurring around 24 hpf, minor changes in timing between samples could result in significant changes in the expression of various factors and may explain the inconsistent results.

Taken together with the lack of changes that were observed in the in situ hybridization analysis, these experiments suggest that if there is any regulation of Otx expression by Mitf transcription factors, this regulation is very minor. Initial attempts have been made to obtain head or eye specific RNA for Q-PCR for a more quantitative analysis of mitfa and mitfb expression in otx morphants since the expression of mitf is not limited to the ocular tissue, but these attempts have proved unsuccessful. The unidirectional regulation of Mitf by Otx transcription factors uncovered in these
experiments is a major difference from what is observed in mice and may explain why Mitf transcription factors are not necessary in zebrafish RPE development.

Discussion

The data from the combined loss of Mitf and Otx experiments support previous findings reported in Chapter 2 that zebrafish Mitf transcription factors may still be involved in RPE development. The observation that the significant increase in phenotype severity occurred only in the mitfa;mitfb double mutants suggests that these transcription factors have overlapping functions in zebrafish RPE development. To what extent these mitf transcription factors actually contribute to the development of the RPE is unclear. However, the data from the loss of combined otx1a/otx1b and the otx1b/otx2 morpholino experiments in wildtype embryos was very similar to the phenotypes produced through the injection of otx1a or otx2 morpholinos into mitfa;mitfb double mutants. These findings suggest that Mitfa and Mitfb together may contribute to the development of the RPE at a similar level to Otx1b and provides further support for theory that Mitf and Otx transcription factors may have largely overlapping functions in zebrafish RPE development.

The observed variability between trials in the otx1a/otx2 combination knockdown in mitf mutants is not overly alarming despite the seemingly conflicting data when comparing the wildtype and single mitfa or mitfb mutants. The process of injecting the embryos has many variables that are difficult to control. The microinjections are accomplished using a glass needle that is broken somewhat arbitrarily to allow for the passage of liquid through the needle tip. This needle variability combined with the
variability associated with measuring the volume of the dispensed morpholino using the diameter of a drop of liquid on a micrometer make perfect phenotypic comparisons between trials occurring on different days using different needles rather difficult. For this reason, all genotypes for each individual trial were injected with a single needle over a span of several hours and the needle was examined after each genotype injection to ensure that no change to the volume was occurring through needle breakage or any blockage that can occur if embryonic material becomes lodged in the needle opening. This allowed for some control of variation between genotypes in a single trial, but each trial used a different needle that may have caused some variation. With all of these variables it is not surprising that the combination of otx1a and otx2 morpholinos, which are capable of producing a variable eye phenotype between eyes of a single embryo, would encounter some variability in the phenotypic percentages between trials. The relative variation between the three wildtype and single mitf mutant genotypes among the different trials was somewhat difficult to explain. The single cell of the newly fertilized embryos were injected in roughly the same location but minor alterations in the needle positioning could also affect the phenotypic outcome.

The unidirectional control of the expression of mitfa and mitfb genes by Otx transcription factors was a very surprising outcome considering the previous experiments in mice that have demonstrated a positive feedback relationship between the two families of transcription factors. To ensure the reliability of these expression change results and the relative decreases of mitf expression caused by the different otx morpholinos, both the otx and mitf in situ hybridization analysis was performed at least twice. The decrease in mitfa and mitfb expression in otx1a morphants was significantly
greater than either the $otx1b$ or $otx2$ morphants. Interestingly, this situation was reversed when $tfec$ expression was examined, as $otx2$ morphants were the most significantly decreased. In both cases, the $otx1b$ morphants displayed an intermediate amount of expression loss when compared to $otx1a$ and $otx2$ morphants. This provides further support for the theory that Otx1b is a hybrid protein whose functions overlap those of Otx1a and Otx2.

The major surprise that emerged from the expression analysis experiments was the lack of any change in $otx1a$ or $otx2$ expression in zebrafish $mitf$ mutants. Expression changes in $otx1b$ have not been examined to date due to the relatively minor phenotypes created in morpholino knockdown experiments. The relatively Mitf-independent expression of Otx transcription factors reveals that unlike zebrafish Otx combination morphants as well as murine Mitf and Otx mutants, zebrafish Mitf mutants retain high expression levels of transcription factors capable of directing RPE development. This may explain the lack of phenotype observed in zebrafish Mitf loss of function experiments.
Figure 4.1: The combined knockdown of Otx and Mitf transcription factors create a more severe phenotype suggesting that Mitf transcription factors may still play a role in zebrafish RPE development. (A) The phenotypic effect of Otx knockdown was compared between wildtype, mitfa<sup>w2</sup>, mitfb<sup>hu3857</sup>, and mitfa<sup>w2</sup>/mitfb<sup>hu3857</sup> fish lines to determine if Mitf transcription factors contribute to RPE development. All four genotypes were injected in a random order with a single needle. Each eye of the injected embryos was evaluated at 5dpf and scored as normal, minor RPE deficits and major RPE deficits. The phenotypic percentage of morphants eyes in each genotype in the first of four trials is depicted in a graph (A). (B) The injection of otx1a/otx2 morpholinos produced an extremely variable phenotype between repeated experiment trials. However, all four otx1a/otx2 morpholino trials produced a significant increase in the percentage of severe RPE deficits phenotype in the mitfb<sup>hu3857</sup>/mitfa<sup>w2</sup> fish eyes when compared to wildtype and single mitfa and mitfb mutant fish lines (p < 0.001). All four trials also displayed a significant reduction in the percentage of eyes with a normal phenotype in the mitfb<sup>hu3857</sup>/mitfa<sup>w2</sup> larvae when compared to the other genotypes (p < 0.001). The raw eye phenotype numbers and percentages are listed for all four trials.
### Graph A

![Bar Graph](image)

#### Wildtype vs. Mutant Conditions

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#### Statistical Analysis

- **Wildtype**: N = 252
- **Mitfa<sup>w2</sup>**: N = 196
- **Mitfb<sup>hu3857</sup>**: N = 158
- **Mitfa<sup>w2</sup> ; Mitfb<sup>hu3857</sup>**: N = 170

*P* value < 0.0001
Figure 4.2: The knockdown of Otx1a and Otx2 into mitfb<sup>hu3651</sup> mutants produces similar results to what is observed in the mitfb<sup>hu3857</sup> experiments. (A) The phenotypic effect of Otx knockdown was compared between wildtype, mitfa<sup>w2</sup>, mitfb<sup>hu3561</sup>, and mitfa<sup>b692</sup>/mitfb<sup>hu3561</sup> fish lines to determine if Mitf transcription factors contribute to RPE development. All four genotypes were injected in a random order with a single needle. Each eye of the injected embryos was evaluated at 5dpf and scored as normal, minor RPE deficits and major RPE deficits. The phenotypic percentage of morphants eyes in each genotype in the first of four trials is depicted in a graph (A). (B) Similar to what was observed in the mitfb<sup>hu3857</sup> experiments, the injection of otx1a/otx2 morpholinos produced an extremely variable phenotype between repeated experiment trials. However, 3 of the four otx1a/otx2 morpholino trials produced a significant increase in the percentage of severe RPE deficits phenotype in the mitfa<sup>b692</sup>/mitfb<sup>hu3651</sup> fish eyes when compared to wildtype and single mitfa and mitfb mutant fish lines (p < 0.001). The same three trials also displayed a significant reduction in the percentage of eyes with a normal phenotype in the mitfa<sup>b692</sup>/mitfb<sup>hu3561</sup> larvae when compared to the other genotypes (p < 0.001). The third trial is the one trial that does not correlate well with the other trials. The raw eye phenotype numbers and percentages are listed for all four trials.
### Table 1: Gene Expression Analysis

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
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<td><strong>wildtype</strong></td>
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<td>90.9</td>
<td>20.8</td>
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<td>6.4</td>
<td>79.5</td>
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<td>6.6</td>
<td>8.1</td>
<td>78.5</td>
<td>79.4</td>
</tr>
</tbody>
</table>

### Figure 1: Otx1a/Otx2 MO

- **wildtype**
- **mitfa<sub>b692</sub>**
- **mitfb<sub>hu3561</sub>**
- **mitfa<sub>b692</sub>;mitfb<sub>hu3561</sub>**
Figure 4.3 The single injection of Otx1 or Otx2 results in a consistent increase in phenotype severity in mitfa/mitfb double mutants when compared to wildtype or single mitf mutants. The single injection of *otx1a* (A) or *otx2* (B) morpholinos produced a significant increase in the percentage of mitfb$^{hu3857}$/mitfa$^{w2}$ double mutant embryos with RPE deficits (p< 0.001) as well as the severity of these deficits (p< 0.001) when compared to wildtype, mitfa$^{w2}$, or mitfb$^{hu3857}$ homozygous larvae.
Figure 4.4: The combined loss of Mitf and Otx activity causes a significant decrease in DCT expression. (A-D) DCT mRNA expression was examined at 24hpf in control morphants (A), otx1a/otx2 combination morphants (B), mitfb\textsuperscript{hu3857}/mitfa\textsuperscript{w2} mutants (C), and the combination otx1a/otx2 morphants in a mitfb\textsuperscript{hu3857}/mitfa\textsuperscript{w2} background (D). The combined loss of Otx and Mitf activity results in an increased loss of DCT expression (D), suggesting that Mitf does promote pigmentation in the RPE.
**DCT expression**

(A) Control MO

(B) otx1a + otx2 MO

(C) mitfa<sup>W2</sup>; mitfb<sup>hu3857</sup>

(D) otx1a + otx2 MO into mitfa<sup>W2</sup>; mitfb<sup>hu3857</sup>
Figure 4.5: The expression of *mitfa* is decreased in *otx* morphants. (A-D) *mitfa* expression was examined through in situ hybridization at the 21 somite stage in embryos injected with a control morpholino (A), an *otx1a* morpholino (B), an *otx1b* morpholino (C) or an *otx2* morpholino (D). Representative images of the morphants are shown. Analysis revealed a decrease in mitfa expression in the developing RPE cells (black arrows) but not in the neural crest cells (white arrows) of *otx1a* (A), *otx1b* (B), and *otx2* morphants (C), when compared to control morphants (A). Though the severity of RPE specific *mitfa* expression decreases varied slightly between morphant embryos, *otx1a* morphants consistently displayed a greater loss of *mitfa* expression when compared to *otx2* morphants. The loss of *mitfa* expression in *otx1b* morphants was somewhere in between what is observed in *otx1a* and *otx2* morphants. All single morpholinos were injected at a concentration of 1.25 ng/embryo and the combination *otx1a/otx2* morpholinos were injected at 1.25ng each/embryo.
mitfa expression

A

Control MO

21 hpf

B

otx1a MO

21 hpf

C

otx1b MO

21 hpf

D

otx2 MO

21 hpf
Figure 4.5: The expression of mitfb is decreased in otx morphants. (A-D) mitfb expression was examined through in situ hybridization at the 21 somite stage in embryos injected with a control morpholino (A), an otx1a morpholino (B), an otx1b morpholino (C) or an otx2 morpholino (D). Representative images of the morphants are shown. Mitfb expression was also reduced specifically in the RPE (black arrows) of otx morphants (B-D) when compared to the control morphants (A), leaving epiphysis expression (white arrows) unaffected. otx1a morphants again displayed an increased reduction of mitfb expression when compared to otx1b and otx2 morphants. The expression changes in otx1b morphants was again intermediary between otx1a and otx2. All single morpholinos were injected at a concentration of 1.25 ng/embryo and the combination otx1a/otx2 morpholinos were injected at 1.25ng each/embryo.
mitfb expression

A  Control MO

B  otx1a MO

C  otx1b MO

D  otx2 MO

21 hpf
Figure 4.6: In situ hybridization experiments reveal that *otx1a* and *otx2* expression is not altered in *mitfa* and *mitfb* mutants. (A-D) *otx1a* expression was examined at the 21 somite stage through in-situ hybridization was examined in wildtype (A), *mitfa^w2* (B), *mitfb^hu3857* (C), and *mitfb^hu3857;mitfa^w2* (D) fish lines. (E-H) *otx2* expression was examined at the 21 somite stage through in-situ hybridization was examined in wildtype (E), *mitfa^w2* (F), *mitfb^hu3857* (G), and *mitfb^hu3857;mitfa^w2* (H) fish lines. No consistent change was observed in any of the mutant alleles for either *otx1a* or *otx2*. 
wildtype

mitfa

mitfa w2

mitfa /mitfb

mitfa w2 /mitfb

otx2 probe

otx1a probe
Figure 4.7: Q-PCR analysis of otx1a and otx2 expression in mitf mutants reveals only minor and inconsistent deficits in expression. (A-B) Total RNA was collected from 24 hpf from wildtype, mitfb<sup>hu3561</sup>, mitfb<sup>hu3857</sup>, mitfa<sup>b692</sup>/mitfb<sup>hu3561</sup>, and mitfa<sup>w2</sup>/mitfb<sup>hu3857</sup> embryos and analyzed for changes in expression of otx1a (A), and otx2 (B) when compared to the internal B-actin control.
Figure 4.8: Otx but not Mitf transcription factors regulate the expression of the Mitf-related transcription factor, Tfec. (A-F) Expression of tfec was examined at the 21 somite stage. (A-B) No change in tfec expression was observed between wildtype (A) and mitfa<sup>w2</sup>/mitfb<sup>hu3857</sup>. (C-F) In a reversal of what is observed in mitf expression analysis, otx2 morphants (E) experienced the largest decrease in tfec expression when compared to wildtype embryos. The knockdown of Otx1a (C) slightly decreased tfec expression and once again, otx1b morphants (D) displayed an intermediate amount of loss when compared to otx1a and otx2 morphants. The combined knockdown of otx1a and otx2 produced a dramatic loss of tfec expression (F).
Control MO

mitfa /mitfb

Otx1b MO

Otx1a MO

Otx2 MO

Otx1a + Otx2 MO

w2

hu3857

Otx1a + Otx2 MO
Chapter 5

Final Discussion

The regulatory pathway controlling the development of zebrafish retinal pigment epithelium appears to have some major differences compared to what has been reported in other species (Fig 8A-B). Particularly surprising is the observation that the loss of Mitf transcription factors does not appear to cause RPE abnormalities in zebrafish or the microphthalmia phenotype for which the locus is named. There are a few theories regarding or this major difference between species in the developmental pathways controlling RPE development. The first possibility that arises is the question of whether the Mitfa and Mitfb proteins are actually eliminated in these experiments due to the alternative splicing that is prevalent in the mammalian gene. As addressed in Chapter 1, the number of mitfb morpholinos, and location of the mutations in the mutant alleles suggest that the elimination of mitfa and mitfb is achieved despite the inability to directly confirm the protein knockdown. A second possibility is the presence of another mitf gene, isoform, or closely related bHLH / leucine zipper transcription factor that is expressed in the RPE and is able to compensate for the loss of mitfa and mitfb. As addressed in Chapter 1, the genome has been searched previously for additional Mitf related proteins. One alternative mitfa isoform that is expressed in the developing eye has been identified, but this isoform should be eliminated by both of the mitfa mutant alleles used in these experiments. The closest related Mitf protein that has been discovered in the developing RPE to date is Tfec. Initial attempts at knocking down Tfec activity in mitf mutants have not uncovered any altered RPE phenotypes which is
consistent with the lack of evidence for the requirement of tfec activity for RPE
development in other species.

An alternative theory has emerged from the experiments which revealed the lack
of a reciprocal feedback relationship between zebrafish Otx and Mitf transcription
factors (Fig. 8B). While the loss of either Otx or Mitf activity in mice leads to the
elimination of both transcription factors in the eye, zebrafish only exhibit reduced
expression of both factors when Otx activity is eliminated (Nguyen and Arnheiter 2000,
Martinez-Morales 2001) (Fig. 8A-B). This new evidence combined with the phenotypic
variability associated with RPE deficits has inspired the theory that there exists a
threshold level of Mitf and Otx mediated RPE gene activation required for proper RPE
development in vertebrates (Fig 8C-D). If the level of RPE target gene activation is near
the threshold required for proper development, slight changes in the microenvironment
can push the phenotype in either phenotypic direction. Under normal conditions, there
is sufficient combined target gene activation between the two sets of transcription
factors to direct the complete development of the RPE due to their similar
developmental functions. In mouse development, the loss of either Mitf or Otx activity
will cause a decrease in both factors, leading to insufficient RPE gene activation.
Similarly, when the expression of zebrafish Otx factors is knocked down, mitf
expression is also decreased. This causes the level of RPE target gene expression to
dip below the required threshold, leading to a varying degree of RPE abnormalities.
However, loss of Mitf activity in zebrafish mitf mutants will leave Otx activity unaffected,
and this activity is sufficient to activate RPE target genes and drive complete RPE
development. Further experiments may reveal which Mitf and Otx transcription factors
and combinations create sufficient RPE target gene activation and exactly which target genes are necessary to drive complete RPE development. This theory of a RPE threshold or even multiple thresholds is supported by the apparent stochastic effects in the regulation of RPE development that is observed in other species with Mitf and Otx mutations, as suggested by the variance in phenotype between eyes of a single animal.

In addition to differences between in the transcription factors required for RPE development in zebrafish and mice, there are also differences in the ocular phenotype created by abnormal RPE development. Murine Mitf and Otx mutants experience the formation of a hyperproliferative second inverted retinal layer in place of the RPE cells. Preliminary histology and analysis of double cone photoreceptors did not reveal any noticeable duplication of the retinal layers in zebrafish otx morphants. Since the missing RPE cells do not appear to transdifferentiate into retinal cells in zebrafish, future experiments will explore the fate of prospective RPE cells in otx morphants to determine if they experience migration defects or simply die off early in development. Initial immunostaining experiments in otx morphants did not reveal any increase in the staining of activated capase 3, suggesting that these RPE deficits may not be due to apoptosis (data not shown). The eyes of most 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. This phenotypic change may be due to the incomplete loss of RPE in zebrafish otx morphants compared to mouse Mitf and Otx mutants. Whether this partial loss of RPE is due to incomplete knockdown of Otx transcription factors, the transient nature of otx morpholino knockdown experiments, or functional differences between murine and zebrafish Otx proteins remains to be seen.
Previous evidence supports the development of the retinal layers in zebrafish despite a lack of RPE development, thus the lack of retinal abnormalities in otx morphants could be due to differences in the dynamics of ocular cell development between mice and zebrafish (Rojas-Munoz et al 2005).

Especially fascinating are the differential patterns of RPE deficits that occur in murine Mitf and Otx mutants. RPE deficits associated with Mitf mutations are commonly reported to occur preferentially in the dorsal portion of the eye despite associated abnormalities in the closure of the ventral optic fissure. The murine Otx mutants have been reported to display deficits in the ventral RPE with the occasional patch of dorsal RPE remaining unaffected. This specific loss of ventral RPE has now been confirmed in zebrafish otx morphants as well as the otx2 mutants. Differences in the regional requirement of transcriptional activity between Mitf and Otx transcription factors has not been widely discussed in previous publications. Given that the RPE-specific genes that have been identified as targets of one transcription factor have also been confirmed to be activated by the other transcription factor as well, it would be interesting to determine if these regional RPE differences arise from unknown target genes or if they are due to interactions with other molecules in the eye. These other factors could be involved in the dorsal ventral patterning of the eye or could display greater regional specificity. Future experiments can examine the change in expression of molecules involved in dorsal-ventral patterning like sonic hedgehog as well as ventral and dorsal specific genes. The modulation of genes like gas1, which has been implicated in the closure of the optic fissure, may also uncover some functional differences in zebrafish Mitf and Otx transcription factors. These specific changes in
gene expression can be examined through in situ hybridization, while additional genes can be identified through microarray analysis of zebrafish wildtype embryos and mitf and otx mutants.

In spite of the drastic change in the necessity of Mitf transcription factors in zebrafish RPE development when compared to previous experimental models, these experiments could provide a better understanding of the regulatory pathway controlling human RPE development. Regardless of the evolutionary distance between zebrafish and humans, zebrafish may present a better representation of the human eye than mice due to similarities such as the cone dominance that occurs in the photoreceptor layer. Zebrafish and humans are both diurnal species that rely heavily on cones for detailed vision, while nocturnal species such as mice and rats are more reliant on the dim light vision provided by rods. Similar to what has been observed in zebrafish, mutations in Otx2 but not Mitf have been identified in cases of severe human RPE developmental abnormalities such as microphthalmia and anophthalmia. The phenotypic variance observed between the eyes of human patients with the same Otx2 mutations is also similar to what is observed in zebrafish and other species. In humans, Mitf mutations are responsible for pigmentation disorders, such as those seen in Waardenburg syndrome, but even minor RPE abnormalities are generally not associated with these mutations. It is conceivable that human embryos with severe Mitf mutations may not survive to term or have yet to be identified. Another possibility is that like zebrafish, human Mitf may function to promote pigmentation in the developing RPE but is not necessary for its development.
Figure 5.1: Current model of the zebrafish RPE developmental pathway. (A)
Analysis of Mitf and Otx mutant gene expression in other organisms inspired the
theoretical developmental pathway in which Mitf and Otx transcription factors operate a
similar hierarchal level in RPE development with the expression of each factor
dependent on the activity of the other. (B) The recent experimental evidence obtained
in this study suggests a developmental pathway in which Otx transcription factors play a
more fundamental role in RPE development. In zebrafish RPE development, the
expression of Mitf transcription factors is dependent on Otx activity but Otx expression
does not depend on Mitf activity. (C-D) The variability in eye phenotype associated with
Mitf and Otx deficiencies in all species suggest a threshold level of gene activation
needed for proper RPE development. Once near the threshold, small changes in the
environment can create major differences in the RPE deficient eye phenotype. In other
species such as the mouse, loss of either Otx or Mitf transcription factors results in a
decrease in both factors and insufficient pigment gene activation(C). Due to the
regulatory differences from other species, the loss of Otx factors in zebrafish decreases
the total pigment gene activation below the required developmental threshold due to the
subsequent loss of Mitf activity (D). Mitf mutations however do not eliminate the activity
of Otx transcription factors, which are then capable of activating the required threshold
of pigment genes to drive complete RPE development in zebrafish.
Previously theorized pathway of RPE development

Proposed zebrafish RPE development pathway

Murine Threshold

Zebrfish threshold

Mitf driven gene activation

Otx driven gene activation
Appendix

To investigate the possible factors acting upstream of Mitf and Otx transcription factors, the Rx3 and TCF3 transcription factors were examined for their role in zebrafish RPE development. Many of these experiments are incomplete as the focus of more recent experiments has concentrated on Mitf and Otx transcription factors, but they do provide some interesting insights into earlier ocular tissue specification.

Part 1: Rx3

Rx3 Introduction:

Members of the Rx family are homeodomain-containing transcription factors function in anterior brain and eye development. Mammalian genomes carry a single Rx gene compared to the 3 rx genes found in teleost fish such as the zebrafish. Murine Rx null mutants fail to develop an optic cup and present deficits in forebrain and midbrain development. Teleost fish have 3 rx genes corresponding to the single mammalian gene, \( rx1 \), \( rx2 \) and \( rx3 \). \( rx1 \) and \( rx2 \) are involved in the development of the retina and lens in zebrafish. The Rx3 transcription factor is the earliest of the teleost Rx transcription factors to be expressed and is required for the specification of the eye field from the telencephalon and the evagination of the optic lobe. In medaka, \( rx3 \) null mutants display an eyeless phenotype while overexpression of \( rx3 \) results in the formation of ectopic RPE and retinal cells.

Previous examination of zebrafish Rx3 has taken advantage of multiple mutant alleles in the \( chokh (chk) \) locus that encodes the Rx3 protein to identify the
consequences of the loss of Rx3 function. The more severe rx3 mutations result in a phenotype in which zebrafish larvae develop without any discernible ocular tissue (Kennedy et al. 2004). Two identified missense mutations in the rx3 gene display a less severe phenotype, in which the zebrafish larvae have a lens and laminated retina but the RPE fails to develop properly (Rojas-Munoz et al. 2005). These eye phenotypes are variable and are altered by the genetic background of the zebrafish. The lens and retinal tissue, despite being laminated, appear to be severely displaced from the normal ocular position (Rojas-Munoz et al. 2005). rx1 expression is slightly increased in the presence of Rx3 while the expression of rx2 completely lost in rx3 null mutants (Kennedy et al. 2004, Rojas-Munoz 2005). Injection of rx1 and rx2 DNA into rx3 mutants restores some lens and retinal tissue but does not affect RPE development (Rojas-Munoz et al. 2005). This data suggests that in addition to its earlier role in the specification of the eye field, Rx3 also required for RPE development. The expression of mitb, otx2, and dct is reduced in rx3 partial function mutants and completely lost in null allele mutants (Rojas-Munoz et al. 2005). The promoter regions of these genes do not appear to contain target sequences for Rx3 activation, so it is unknown if this activation is achieved through more distal enhancer elements or through an intermediary molecule.

Methods:

Wildtype and Mutant Zebrafish

Wildtype embryos were obtained through natural matings of adults from the AB strain or AB/WIK hybrids and were staged according to Kimmel et al. 1995. Primers for genotyping chkw29 allele was previously described in Kennedy et al. 2004. The mitfa
mutant allele was described previously by Lister et al 1999. All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Microarray**

Total RNA was collected from 30 embryos at 24 hpf using Trizol reagent. The concentration of the RNA was determined using a spectrophotometer. Microarray analysis was accomplished in the VCU Nucleic Acids Research Facilities. RNA was analyzed using the Affymetrix Genechip Zebrafish Genome Array was used in the Affymetrix Microarray System. The results of the experiment were analyzed using the Affymetrix GCOS and data mining software.

**Results:**

**Chk\textsuperscript{w29} mutant line**

Previous analysis of zebrafish \textit{chk} mutants had reported the loss of \textit{mitfb}, \textit{otx2}, and \textit{dct} expression through in situ hybridization analysis (Kennedy et al 2004, Rojas-Munoz 2005). To examine these and other interactions, a \textit{chk}\textsuperscript{w29} fish line was obtained from Zirc that has been previously described by Kennedy et al (2004). This mutant allele contains an early stop codon in exon 2 which eliminates the last two thirds of the homeodomain element of the Rx3 protein and is reported to lose all ocular tissue. Fish lines carrying the \textit{chk}\textsuperscript{w29} mutant allele were intercrossed to confirm the loss of ocular tissue phenotype that is associated with this null allele. Homozygous \textit{chk}\textsuperscript{w29} larvae do not survive longer than a few weeks. 5 dpf larvae displayed the occasional spot of pigmentation in the ocular cavity and to determine if this pigmentation was neural crest...
derived the $chk^{w29}$ line was crossed to the $mitfa$ null mutant $w2$ line and the raised until adulthood (Fig A1.1). The resulting $chk^{w29}+/+; mitfa^{w2}/+$ fish were then intercrossed to obtain homozygous $chk^{w29}/chk^{w29}; mitfa^{w2}/mitfa^{w2}$ larvae. These 5 dpf double mutant larvae still displayed the small patch of pigmentation that is sometimes also accompanied by iridiphores. The ocular area still appears devoid of ocular tissue and this pigmentation is highly variable and is likely a result of the genetic background effects that have been reported in other $chk$ mutant alleles (Rojas-Munoz et al 2005). This variable eye phenotype, even between eyes of the same embryo supports a similar theory in of stochastic regulation that was described for RPE development that requires a threshold level of transcriptional activity.

The potential downstream targets of the Rx3 transcription factor in the zebrafish RPE developmental were then explored through the use of microarray analysis. Since the only $chk^{w29}$ carriers available at the time of the experiment also contained the $mitfa^{w2}$ mutant allele, the only genotype that could be definitively identified based on the appearance of in an intercross of $chk^{w29}/+; mitfa^{w2}/+$ fish were the $chk^{w29}/chk^{w29}; mitfa^{w2}/mitfa^{w2}$ larvae. To identify the changes to gene expression when Rx3 activity is eliminated, 30 $chk^{w29}/chk^{w29};mitfa^{w2}/mitfa^{w2}$ 24 hpf larvae and 30 $mitfa^{w2}/mitfa^{w2}$ 24 hpf larvae from a separate fish mating were collected and the total RNA was extracted from each genotype. The RNA was then analyzed using the Zebrafish microarray chip and the resulting output was evaluated to select the genes with significant decreases in the $chk^{w29}/chk^{w29};mitfa^{w2}/mitfa^{w2}$ samples when compared to $mitfa^{w2}/mitfa^{w2}$ samples (Fig. A1.2). Several expected genes were among the targets that exhibited the largest decrease in gene fold expression. Among these were RPE specific genes such as $dct$,
retinal specific genes such as pax6, rx1, and rx2, as well as several crystallin related genes involved in lens development. There were also several genes with unknown functions in eye development. It is unknown if these genes are actually involved in eye development or if they are a result of using total RNA instead of eye or rx3 cell specific RNA. The mitfa<sup>w2</sup>/mitfa<sup>w2</sup> line was also compared to 24 hpf wildtype total RNA samples in the same microarray experiment to analyze changes in mitfa-dependent genes (Fig A1.3). Further analysis of this microarray data has shifted to acquiring more tissue specific RNA.

**Frx3**

To investigate the role of Rx3 transcription factors in zebrafish RPE development, the fugu genome was investigated due to its comparatively condensed genome to allow for easier cloning manipulations. The fugu rx3 (frx3) promoter region spans 1275 bp compared to over 4000 bps in zebrafish. The fugu peptide is approximately 67 percent identical to the zebrafish protein while displaying a high degree of conservation in key regulatory elements. To test the efficacy of the fugu rx3 promoter in zebrafish, it was combined with 879 bps of the zebrafish rx3 open reading frame (ORF) to create a vector with the frx3 promoter driving expression of the zebrafish rx3 ORF along with GFP (Fig A1.4A). This plasmid was injected at a concentration of 0.25 ng/embryo into offspring obtained from the intercross of chk<sup>W29/+;</sup> mitfa<sup>w2</sup>+ fish lines. The injected embryos displayed a mosaic pattern of GFP fluorescence with expression in and around the developing eye. This mosaic expression is typical of DNA injections. Any increased pigmentation caused by the expression of rx3 was difficult to identify in the non-phenotypic wildtype and
heterozygous chk\textsuperscript{w29} carriers but a few of the small number of injected chk\textsuperscript{w29} homozygous mutants showed a small degree of RPE rescue (Fig. A1.4B-C). This suggests that the cloned fugu promoter and zebrafish \textit{rx3} ORF are functional in the zebrafish embryo.

**Fr\textit{x3} GFP**

To obtain more RPE and eye specific cells for microarray analysis of \textit{Rx3}, the \textit{frx3} promoter was cloned in front of a GFP reporter element and injected in a 1:1 ratio with transposase RNA at a total concentration of 0.25 ng/ul (FigA1.5A). The embryos with appropriate GFP expression in the eye were then raised and tested for germline transmission once the fish reached adulthood. The \textit{frx3} driven GFP in the transgenic line was expressed in the developing eye as well as the midbrain-hindbrain boundary of the transgenic embryos (FigA1.5B). The GFP expression in this line can then be used to select cells for future \textit{Rx3} and other eye related microarray analysis experiments. This line was also crossed to the carriers of the chk\textsuperscript{w29} mutant allele. The resulting embryos were examined for GFP expression and genotyped for selection of \textit{Frx3}:GFP; chk\textsuperscript{w29}/+ larvae. The \textit{chk\textsuperscript{w29}/chk\textsuperscript{w29}} GFP positive embryos still retained the midbrain-hindbrain boundary expression but the eye expression found in the heterozygous and wildtype siblings was present instead in the forebrain (FigA1.5 C-D). This supports previous report by Stigloher et al of the telencephalic fate of presumptive eye cells in \textit{rx3} mutants (2006). This also provides further support for the similar function of the \textit{frx3} promoter in zebrafish.

**Fr\textit{x3} Gal4**
The previously created frx3 promoter element was combined Gal4-VP16 to create a vector to facilitate future Gal4 UAS experiments with frx3 driven Gal4 expression (Fig. A1.6A). To examine the ability of the downstream factor Mltfa to rescue eye or RPE development in chk\textsuperscript{w29} homozygous mutants, a UAS:mitfa,GFP was co-injected with Frx3: Gal4 into and intercross of chk\textsuperscript{w29}/+ carriers. The injected embryos all contained additional GFP and pigmentation in the head and eye region as well as some sporadic pigmentation throughout the body (Fig. A1.6 B). The injected homozygous chk\textsuperscript{w29} mutants displayed GFP along with pigmentation in the developing forebrain as opposed to the eye region (Fig. A1.6C-F). However, no obvious noticeable eye or RPE rescue was observed. This is not surprising despite the evidence of the rx3 regulation of mitf expression in the RPE because of Rx3’s additional role in the specification of the eye field.

The frx3 promoter driven Gal4-VP16, GFP vector was then combined with transposase mRNA in a 1:1 ratio at a total concentration of 0.25 ng/ul and injected into wildtype embryos to create a transgenic line. However, several attempts at producing transgenic lines failed to produce fish with germline transmission of the Gal4 vector. To increase germline transmission, a new plasmid was then created with frx3: Gal4-VP16 and a separate heart specific promoter driving expression of GFP for selection of transgenic embryos. Injection of this vector accompanied by transposase mRNA resulted in germline transmission of the heart specific GFP, however this transgenic line failed to activate UAS expression when injected with UAS:GFP or when crossed to UAS:mCherry carrier lines.
**Cmv Gal4; UAS Rx3**

To further examine the ability of Rx3 to induce ectopic pigmentation and/or ocular tissue when broadly expressed, a CMV promoter driving expression of Gal4-VP16 was used (Fig. A1.7A). When co-injected into 1 cell embryos from an intercross of $chk^{w29}$ carriers at a 1:1 ratio with a UAS:rx3,GFP plasmid, the injected embryos displayed widespread GFP expression (Fig. A1.7B). This GFP expression throughout the body was confirmed to correlate with $rx3$ expression through whole mount in situ hybridization (Fig. A1.7C). The CMV driven expression of $rx3$ resulted in increased pigmentation throughout the body the occasional RPE and ocular tissue deficits (Fig. A1.7D). The ocular abnormalities were occasionally accompanied by the development of possible ectopic ocular tissue in the anterior dorsal midline (Fig A1.7E-G). The increased $rx3$ expression also led to the increased $mitfb$ and $silvb$ expression in patches throughout the body (Fig A1.8). In a few larvae, a concentrated patch of $silvb$ expression was also observed in the dorsal region of the head where the possible additional ocular tissue is observed (Fig A1.8B). $Silvb$ is a melanocyte specific type 1 transmembrane glycoprotein that is required for the structural organization of the premelanosome of RPE cells. Since these embryos were not sectioned or analyzed for expression of other ocular markers, it is hard to make any definitive conclusions about the ability of Rx3 to direct ectopic eye development. However it does provide evidence that $mitfb$ is a downstream target of Rx3 and that Rx3 can induce pigmentation and structural components of the RPE in extraocular tissues.

Despite the ocular abnormalities and possible ectopic ocular tissue that are created by the broad expression of $rx3$, the homozygous $chkw29$ mutants did not show
any obvious rescue of eye or RPE development. This is not entirely surprising as the mosaic expression achieved through DNA injections and non-specific expression of rx3 are not ideal conditions to replicate the timing and expression patterns required for proper eye development.

Part 2: TCF3

TCF3 Introduction

The early anteroposterior patterning of the developing zebrafish brain relies on the activity of Wnt signaling pathways such as Wnt8 (Mcgrew et al 1995, Woo and Frasier 1995). After the midblastrula transition, the inhibition of Wnt signaling is necessary for the development of the anterior brain and eyes. The complete loss of TCF3 activity as a repressor of Wnt signaling targets in zebrafish has been reported to cause headless embryos, but the expression of a dominant negative form of TCF3 that lacks the B-catenin binding domain has been associated with the ectopic expression of RPE in the developing embryos (Kim et al 2000).

Results:

To examine the ability of a dominant negative TCF3 element (deltaTCF3) to influence the development of zebrafish RPE, the dominant negative TCF3 construct was cloned in front of a 5x UAS sequence and co-injected in one cell wildtype and mitfaw2 homozygous embryos with the CMV driven GAL4 plasmid to achieve a broad expression of deltaTCF3 in the developing embryo (Fig A2.1A). The injected embryos
displayed disruptions in the pigmentation of the RPE, though the development of the eye does not appear to be affected by the loss of pigmentation (Fig A2.1B-D). Cryosectioning was not undertaken for these fish and so it is hard to make any definitive statements about the nature of the loss of pigmentation or the effect it has on the retinal layer in the injected embryos. This loss of pigmentation was also not specific to the ventral portion of the eye as seen in otx morphants. The expression of deltaTCF3 also created patches of RPE-like pigmentation around the eye (Fig A2.1E). It is unknown if this pigmentation is a result of the displacement of the original RPE or if it is an additional area of RPE differentiation from extra-ocular tissue.
Figure A1.1 The chkw29 mutant allele causes a variable loss of ocular tissue. (A) Embryos that are homozygous carriers of the chkw29 mutant allele do not develop optic lobes at 10 somite stage. (B-D) The eye phenotype and anterior head development of homozygous chkw29 mutants are variable depending on the genetic background with some larvae displaying a patch of RPE-like pigmentation in the ocular cavity.
Figure A1.2: Top negative expression changes in chk\textsuperscript{w29} microarray experiment. The total RNA from 30 24 hpf mitfa\textsuperscript{w2}/mitfa\textsuperscript{w2} and chk\textsuperscript{w29}/chk\textsuperscript{w29}, mitfa\textsuperscript{w2}/mitfa\textsuperscript{w2} embryos was collected and analyzed on Affymetrix zebrafish microarray chips. The top negative expression change results are listed with the corresponding gene.
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<td>Dr.84900</td>
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Figure A1.3: Top negative expression changes in mitfa\textsuperscript{w2} microarray experiment. The total RNA from 30 24 hpf wildtype and mitfa\textsuperscript{w2}/mitfa\textsuperscript{w2} embryos was collected and analyzed on Affymetrix zebrafish microarray chips. The top negative expression change results are listed with the corresponding gene if known.
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<td>Dr.16249</td>
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Figure A1.4: The Frx3 driven expression of the zebrafish rx2 ORF is capable of partial rescue of the chk$^{w29}$ homozygous mutant phenotype. (A) A plasmid was created that contains the Frx3 promoter driving expression of the zebrafish ORF (A). (B-C) The injection of this plasmid into embryos from a intercross of chk$^{w29}$ carriers resulted in mosaic expression of GFP and a partial rescue of the RPE and possibly ocular tissue in several of the homozygous chk$^{w29}$ larvae (B-C).
Figure A1.5: The fugu promoter is capable of directing GFP expression in the developing zebrafish eye. (A-B) When the 1275 bp fugu promoter was cloned into a plasmid with a GFP reporter (A) and injected into wildtype embryos it resulted in GFP expression the eye. This plasmid was then co-injected with transposase RNA to create a transgenic line that has strong expression of GFP in the developing eye (B). (C-D) When this transgenic line was combined with the chk\textsuperscript{w29} mutant allele and intercrossed, the chk\textsuperscript{w29} homozygous carriers (D) displayed increased GFP expression in the forebrain instead of the ocular region when compared to siblings (C).
A: Diagram of pT2Kmin Fxr3-GFP 6660 bp

- ampicillin
- Fugu rx3 pr
- a2B2
- intron
- exon
- Repeat
- SV40 polyA
- Repeat

B: Image at 48 hpf

C: Images at 24 hpf

D: Images at 24 hpf
Figure A1.6: The expression of mitfa is not able to rescue RPE development in chk^{w29} homozygous mutants. (A) A frx3 driven Gal4-VP16 plasmid (A) was co-injected with a UAS:Mitfa plasmid into 1 cell embryos from a chk^{w29} intercross. All injected embryos displayed increased pigmentation in the anterior portion of the head (B). (C-F) The injected homozygous chk^{w29} larvae displayed a mosaic pattern of GFP expression (C) and increased pigmentation in the anterior head region (D-F) but no apparent RPE or ocular tissue rescue.
Figure A1.7: CMV driven Rx3 expression can induce ectopic pigmentation and the possible development of additional eye tissue. (A) A plasmid containing a Cmv promoter directing the expression of Gal4-VP16 was co-injected with a plasmid containing UAS driven rx3 expression into 1 cell embryos from a chk^{w29} intercross. (B) The injected embryos displayed a mosaic pattern of GFP expression throughout the body which correlated well with rx3 expression that was observed through in situ hybridization analysis. (D-G) The injected homozygous chk^{w29} larvae displayed increased pigmentation throughout the body (D) but no apparent RPE or ocular tissue rescue. Injected heterozygous carriers or wildtype siblings display some RPE defects (E-F) and the possible formation of additional ocular tissue in the anterior dorsal midline (E-G).
Figure A1.8: The CMV driven expression of rx3 can result in ectopic expression of silvb and mitfb. (A-C) The expression of silvb was examined in CMV:Gal4/UAS:rx3 injected and un-injected embryos from a chk\(^{w29}\) intercross. Un-injected wildtype and heterozygous chk\(^{w29}\) carriers display silvb expression in the developing RPE (A left) but this expression is lost in chk\(^{w29}\) homozygous larvae (A right). Injected chk\(^{w29}\) homozygous larvae (B) and their wildtype or heterozygous siblings displayed scattered pigmentation throughout the body and large patches of silvb expression in the anterior dorsal midline. This expression is consistent with the possible additional ocular tissue observed in Figure A1.7. (D-G) The increased expression of rx3 also caused an increase in the downstream mitfb gene in wildtype and heterozygous carriers (D-E) as well as chk\(^{w29}\) homozygous larvae (F-G).
Figure A2.1: CMV driven deltaTCF3 expression can alter the expression of RPE pigmentation.  (A) The Cmv:Gal4 plasmid was co-injected with a UAS:deltaTCF3, GFP plasmid (A) into wildtype and mitfa^{w2} homozygous embryos.  (B-E) Injected embryos displayed scattered GFP expression throughout the body and head (B).  The expression of deltaTCF3 resulted in a variable amount of RPE deficits but the development of the eye did not appear to be affected when examined at 35 hpf (C-D).  (E) In addition to RPE deficits, the expression of deltaTCF3 caused the ectopic expression of RPE-like pigmentation in the head of injected embryos.
References:


Brandon Marc Lane was born in Baltimore, MD on January 18th 1982 and attended Calvert Hall College High School until his graduation in 2000. He continued his education at the University of North Carolina at Chapel Hill and graduated with a Bachelor of Science in Biology in December of 2004. During his undergraduate studies, Brandon pursued research in a variety of labs at UNC as well as an internship during two summers at Johns Hopkins Hospital in the lab of Dr. Gary Cutting, performing Cystic Fibrosis research. After graduation, took a research technician position at the UNC Chapel Hill in the lab of Dr. A. Leslie Morrow in January of 2005 to investigate the effect of acute and chronic ethanol exposure on GABA receptors in the rat brain. After a year and a half, Brandon left the lab to pursue a PhD in the Human and Molecular Genetics at Virginia Commonwealth University in August of 2006. He first did a rotation in the lab of Dr. Grotewiel, performing Drosophila research before joining the lab of Dr. James Lister. During his time in the Lister lab, Brandon was able to attend several zebrafish related conferences and meetings. In December of 2012, Brandon was invited to present his recent research at the Mid Atlantic Regional Zebrafish meeting held at Johns Hopkins Hospital. During his career at VCU, he also presented his research in a poster presentation at the annual Watts Day Symposia in 2010 and 2011 as well as the VCU Graduate Student Research Symposium in the spring of 2010. He was also selected to give an oral presentation at the VCU Forbes Day honors colloquium in the spring of 2011.

Publications:

Lane BM, Lister JA. Otx, but not Mitf transcription factors are required for zebrafish RPE development. Manuscript in preparation.

Kumar S, Lane BM, Morrow AL. Differential effects of systemic ethanol administration on protein kinase C epsilon, gamma, and beta isoform expression, membrane translocation, and target phosphorylation: reversal by chronic ethanol exposure. J Pharmacol Exp Ther. 2006 Dec;319(3):1366-75. PMID: 16997974