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ALTERED EXPRESSION OF THE IRON TRANSPORTER *Nramp1* (*Slc11a1*) DURING FETAL DEVELOPMENT OF THE RETINAL PIGMENT EPITHELIUM IN MICROPHTHALMIA-ASSOCIATED TRANSCRIPTION FACTOR *Mitf^{mi}* and *Mitf^{vitiligo}* MOUSE MUTANTS

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Abstract

Microphthalmia-associated Transcription Factor (*Mitf*) is expressed in neural crest cell-derived melanocytes, and in the retinal pigment epithelium (RPE) during ocular development. Mutations in *Mitf* are associated with auditory/visual/pigmentary syndromes in humans. *Mitf^{mi/mi}* mouse mutants lack pigmentation, and are microphthalmic, while *Mitf^{vit/vit}* mouse mutants display abnormal RPE pigmentation, and progressive retinal degeneration. Microarray analysis was used to identify novel downstream gene targets/pathways in the RPE that are altered by mutations in the transcription factor *Mitf*. Using the Affymetrix platform, gene expression profiles were generated using the eyes of E13.5 mouse fetuses that were wildtype, heterozygous, or homozygous for the *Mitf^{mi}* mutation. In a separate experiment, eyes from E13.5 mouse fetuses homozygous for the *Mitf^{vit}* mutation were compared to eyes from the C57BL/6 control background strain. Statistical analyses were performed using Robust Multiarray Average, mixed-effects ANOVA and random-variance t-tests. Altered expression of genes involved in pigment formation, melanosome biogenesis/transport, and redox homeostasis were observed. 12 genes were commonly mis-regulated in the eyes of both *Mitf* mutants: 10 of these genes were downregulated in both mutants relative to controls, while 2 of the genes (*Nramp1* (*Slc11a1*) and *epoxide hydrolase*) were downregulated in *Mitf^{mi/mi}* mutants, and conversely, upregulated in *Mitf^{vit/vit}* mutants. Quantitative RT-PCR and immunohistochemistry were used to confirm altered gene/protein expression. RPE expression of the Fe⁺² iron transporter *Nramp1* (*Slc11a1*) has not previously been reported. Fe⁺² is an important co-factor utilized by the iron-dependent isomerohydrolase RPE65 in the retinoid visual cycle. However, excess accumulation of Fe⁺² in the RPE has recently been associated with oxidative damage and age-related macular degeneration. Abnormal pigmentation and increased activity of *Slc11a1* in the RPE of *Mitf^{vit}* mice may contribute to the pathology and progressive retinal degeneration observed in these mutants.

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Keywords

retinal pigment epithelium (RPE); microphthalmia-associated transcription factor (*Mitf*); gene expression; *Nramp1* (*Slc11a1*); iron homeostasis; melanosome; vitiligo mouse mutant; retinal degeneration

INTRODUCTION

Microphthalmia-associated transcription factor (*Mitf*) is a myc-family basic helix-loop-helix (bHLH) transcription factor that is expressed in neuroepithelial cells, neural crest cell-derived melanocytes, the retinal pigment epithelium (RPE), and bone marrow-derived mast cells and osteoclasts (Amae et al., 1998). *Mitf* forms homodimers and/or heterodimers with other bHLH transcription factors, and transactivates downstream gene targets through binding an evolutionarily conserved 11-bp sequence termed the M-box which contains a core CATGTG E-box motif (Aksan and Goding, 1998). Although mutations in the *Mitf* gene have been linked to various ocular and neural crest cell deficiencies, the signaling pathways that are disrupted by the different *Mitf* mutations remain largely unknown. In humans, mutations in the *Mitf* gene are associated with Waardenburg Syndrome and albinism-deafness (Tietz) syndrome (Tassabehji et al., 1995). Similar auditory/visual/pigmentary defects are observed in mice with either spontaneous or induced mutations in this gene (Steingrimsson et al., 2004). The current study focuses on the identification of downstream gene targets impacted by the *Mitf*-vitiligo mutation that may play a role in the RPE defects and progressive retinal degeneration phenotype observed in *Mitf*^{vit/vit} mutant mice.

Mitf^{vit/vit} mouse mutants have a G->A transition at base-pair 793 that leads to an Asp222Asn substitution in helix 1, which is predicted to impair the ability of the *Mitf* protein to dimerize or to participate in putative protein-protein interactions. *Mitf*^{vit/vit} homozygotes display extensive spotting at birth, and after 8 weeks of age, mice produce increasing numbers of white hairs with each molt, such that by 6–12 months of age, the mice are nearly entirely white (reviewed in Steingrimsson et al., 2004). *Mitf*^{vit/vit} mutants also exhibit RPE defects that precede a slow, progressive retinal degeneration, the cause of which is not known. In order to complement our analysis of downstream gene targets affected by the *Mitf*^{vit/vit} mutation, we performed a parallel screen for altered gene expression profiles in the eyes of *Mitf*^{mi/mi} mutant mice. The *Mitf*^{mi/mi} mutation corresponds to a 3 base-pair deletion that results in a loss of DNA binding and *Mitf* transcriptional activity (Hodgkinson et al., 1993). *Mitf* is known to regulate several genes involved in pigment formation, including tyrosinase, tyrosinase-related protein, and dopachrome tautomerase, among others (Yasumoto et al., 1997). Loss of *Mitf* transcriptional activity in *Mitf*^{mi/mi} mutant mice therefore results in pigmentation defects, allowing the different genotypes to be distinguished phenotypically by coat color. *Mitf*^{mi} wildtype mice are black, heterozygotes are black with a white belly spot, and homozygotes are completely white. Homozygotes lack pigment in the stria vascularis of the inner ear, are deaf at an early age, have immunological deficits, a deficiency of secondary bone resorption, and often die around the time of weaning (Packer, 1967). Although ocular development is normal in *Mitf*^{mi} heterozygotes, RPE pigmentation is reduced; *Mitf*^{mi/mi} homozygotes, however, are microphthalmic or anophthalmic, and devoid of any pigment in the RPE.

Melanosomes are specialized intracellular organelles that synthesize and store melanin pigment. Premelanosomes are early endosomal organelles lacking pigment that become elongated in shape (ellipsoidal), and form intraluminal striations composed of a proteinaceous fibrillar matrix. This is followed by acidification of the melanosomal compartment, and activation of tyrosinase to form melanin. As the melanosome matures, pigment granules are deposited and polymerized along the fibrillar matrix scaffold (Dell'Angelica, 2003). *Mitf*

appears to be involved in gene regulation at all stages of melanosome biogenesis, fibrillar matrix formation, and pigment synthesis/deposition (Yasumoto et al., 1997).

During fetal ocular development, *Mitf* is highly expressed in the RPE monolayer, but not in any other eye structures. *Mitf* expression in the RPE peaks at E13.5, and remains prominent until E16.5, at which point it begins to decline (Nakayama et al., 1998). Unlike skin and hair melanocytes that continue to express *Mitf* and produce melanin into adulthood, *Mitf* expression and the synthesis of melanin in RPE melanosomes ceases shortly after birth. In humans, melanosome biogenesis in the RPE ceases within the first trimester of gestation, as the cells attain their full complement of melanosomes (Feeney-Burns, 1980; Oguni, 1991). However, polymerization of melanin granules along the fibrillar matrix, and melanosome maturation continues until about 2 years of age (Feeney-Burns, 1980). In wildtype mice, levels of tyrosinase (the rate-limiting enzyme in pigment formation) and *tyrp-1* (tyrosinase-related protein-1) and *tyrp-2* are high at birth, and show a second peak of expression just prior to eye opening (P10–14), but then decline to undetectable levels by P42, indicating that, similar to humans, melanin synthesis continues in RPE melanosomes of mice for a short time after birth (Orlow and Brilliant, 1999).

The morphological and biochemical changes that occur during embryonic and postnatal development of the RPE and neural retina in the *Mitf^{vit/vit}* mutant have previously been described in great detail. RPE defects precede progressive photoreceptor cell degeneration and/or retinal detachment. *Mitf^{vit/vit}* mouse mutants display uneven pigmentation of the RPE and clumping of melanin granules as early as E12–13 (Sidman et al., 1996). Levels of tyrosinase and *tyrp-1* in *Mitf^{vit/vit}* mutants are similar to controls at P2–P4, but reduced at P10 and beyond (Smith et al., 1998). *Mitf^{vit/vit}* RPE cells exhibit abnormal proliferation, and multilayering in parts of the dorsal region with residual cells spreading laterally along a thickened Bruch's membrane (Tang et al., 1996). Short, compact apical microvilli are formed that do not interdigitate with the rod outer segments (Sidman et al., 1996). Melanosomes are absent in the microvilli (Sidman et al., 1996), and reduced numbers of phagosomes are observed between 4–8 weeks of age (Smith et al., 1994). Photoreceptor cells begin to degenerate and undergo apoptosis between 1–2 months postnatally (Nir et al., 1995; Sidman et al., 1996; Smith, 1992), followed by slow, continuous shedding of photoreceptor nuclei into the subretinal space (Kosaras and Sidman, 1996; Smith et al., 1994). Accumulation of rod outer segment debris precedes photoreceptor cell death (Nir et al., 1995). Photoreceptor degeneration follows a radial gradient, being more severe centrally, and more advanced in the superior than in the inferior half of the retina (Sidman et al., 1996). Depigmentation and star patterns are observed in the fundus (Chang et al., 2002) and a central amelanotic patch is present around the optic nerve head (Boissy et al., 1987). By two years of age, almost all of the rods and cones are gone, and the residual neural retina is invaded by heavily pigmented macrophage-like cells (Sidman et al., 1996). Decreased adhesion between the neural retina and RPE is observed, and retinal detachment may occur as early as P30 (Bora et al., 1999). Retinoid metabolism is also perturbed in the *Mitf^{vit/vit}* mutants. Interphotoreceptor retinoid-binding protein and retinyl palmitate levels are elevated in mutant RPE (Smith et al., 1994). Levels of rhodopsin in *Mitf^{vit/vit}* decline significantly from 6–22 weeks of age relative to controls (Smith, 1992). The electroretinogram (ERG) Vmax in *Mitf^{vit/vit}* also decreases slowly over time, corresponding to the progressive loss of photoreceptor cells (Smith et al., 1994; Tang et al., 1997). ERG and histopathological data indicate that the RPE and neural retina are structurally and functionally abnormal from 2 weeks of age onward (Smith et al., 1994).

In order to identify downstream genes regulated by *Mitf* during normal eye development, and further identify those genes that are specifically mis-regulated by the *Mitf^{vit/vit}* mutation, we performed microarray gene expression studies in E13.5 eyes collected from both the *Mitf^{vit/vit}* and the *Mitf^{mi}* mutant mouse strains. Our microarray gene expression analyses

demonstrated altered expression of genes involved in pigment formation, melanosome biogenesis/transport, and redox homeostasis. 12 genes were commonly mis-regulated in the eyes of both *Mitf* mutants: 10 genes were downregulated in both mutants relative to controls, while 2 genes involved in redox homeostasis, (*Nramp1* (*Slc11a1*) and *epoxide hydrolase*, were downregulated in *Mitf^{mi/mi}* mutants, and conversely, upregulated in *Mitf^{vit/vit}* mutants. Because excess iron accumulation has been implicated in several neurodegenerative processes, including retinal degeneration and age-related macular degeneration (Hahn et al., 2003; 2004), we chose to follow up on increased expression of the iron transporter *Nramp1* (*Slc11a1*) in the eyes of the *Mitf^{vit/vit}* homozygotes. The results of the microarray study were further confirmed by quantitative real-time (QRT)-PCR and immunohistochemistry (IHC). Although many proteins involved in RPE iron homeostasis have previously been identified, *Nramp1* expression has not yet been described.

METHODS AND MATERIALS

(1) Experimental Animals

The (B6C3Fea/a-*Mitf^{mi}*/J) and (C57BL/6J-*Mitf^{mi-vit}*/J) mutant mouse strains were obtained from Jackson Laboratories (Bar Harbor, Maine), and breeding colonies were established in the AAALAC-accredited University of Nebraska Medical Center (UNMC) laboratory animal facility. All animal procedures were done in accordance with the ARVO resolution for the Use of Animals in Ophthalmic and Vision Research, and approved by the UNMC Institutional Animal Care and Use committee. Experimental matings between mice heterozygous for the *Mitf^{mi}* mutation were used to generate wildtype, heterozygous, and homozygous offspring for use in these studies. The *Mitf^{vit/vit}* mutants were maintained as homozygotes; C57BL/6 colony mice were used as the control strain for all comparisons with *Mitf^{vit/vit}* mutants. For the gene expression studies, ocular tissue was harvested from E13.5 fetuses of all *Mitf^{mi}* genotypes, as well as from *Mitf^{vit/vit}* mutants and paired C57BL/6 controls. Following confirmation of genotype, total RNA was extracted from the fetal tissue, and turned over to the UNMC Microarray Core Facility for further processing.

(2) Experimental Design

Experiment #1—*Mitf^{vit/vit}* and C57BL/6 mice were placed into timed matings, and pregnant dams sacrificed on E13.5 in order to collect fetal eyes for gene expression analysis. From each of the two groups (*Mitf^{vit/vit}* homozygotes and C57BL/6 controls) 5 fetuses were randomly selected for microarray analysis. For each group of 5 fetuses, 3 of the fetuses were from the same dam, and the 2 additional fetuses each came from a different dam. This allowed for 3 biological replicates within litter and 3 biological replicates between litters for each strain.

Experiment #2—*Mitf^{+/mi}* heterozygote mice were placed into timed matings, and pregnant dams sacrificed on E13.5 in order to collect embryonic eyes of each genotype for gene expression analysis. 3 separate litters were harvested, and two *Mitf^{+/+}*, *Mitf^{+/mi}*, and *Mitf^{mi/mi}* embryos from each litter were randomly selected for use in the microarray experiment.

(3) RNA Isolation and Microarray Gene Chip Hybridization

Total RNA from each pair of E13.5 fetal eyes was isolated using the Arcturus PicoPure kit (Mountain View, CA) with an on-column DNase treatment, and checked for integrity using a Bioanalyzer NanoChip (Agilent Technologies, Palo Alto, CA). For the microarray experiment, 100 ng of total RNA was reverse-transcribed, and the Affymetrix two-cycle target labeling protocol was used to generate biotin-labeled cRNA probe. cRNA probes were hybridized to the Affymetrix (Santa Clara, CA) Mouse 430 2.0 genome chip, which profiles the expression of 45,101 unique transcripts. Chips were scanned on a GeneChip Scanner 3,000 6G in the UNMC Microarray Core Facility, and images were analyzed using GCOS imaging

software. Quality metric parameters including noise level, background, and the efficiency of reverse transcription were ascertained for all hybridizations. Data sets passing the stringent quality recommendations were normalized using the GCOS software, and the raw intensity values exported for further analysis.

(4) Microarray Data Processing

Analyses were conducted with BRB ArrayTools v3.1 developed by Dr. Richard Simon and Amy Peng Lam. Low-level analysis which converts probe level data to a gene level expression data was done using Robust Multiarray Average (RMA), implemented using the *rma* function of the *Affymetrix* package of the Bioconductor project (<http://www.bioconductor.org/>) in the R programming language (Irizarry et al., 2002; Irizarry et al., 2004; R Dev Core Team, 2004). The RMA background correction method corrects the perfect match (PM) probe intensities by using a model based on the assumption that the observed intensities are the sum of signal and noise. Quantile normalization was used to normalize the PM probes and the calculation of summary expression measures was done using the median polish method, which fits a multichip linear model to the data, and gives the expression on the log₂ scale. Prior to statistical analysis a filter was applied to reduce the number of genes examined. A gene was excluded if less than 20% of the expression data had at least a 1.5-fold change in either direction from the gene's median value.

(5) Statistical Analysis of Differential Gene Expression

For each gene, a mixed-effects ANOVA model was used to determine if there was a significant difference in expression between the 3 groups for that gene (Wright and Simon, 2003). The mixed-effects ANOVA model included a fixed effect for strain and a random effect for dam. The random effect takes into account that data from embryos nested within dam could be correlated. In addition, random-variance t-tests were conducted to compare the groups, ignoring the possible correlation, and the results were compared to the mixed-effects ANOVA models. P-value adjustment for multiple comparisons was done with the false discovery rate (FDR) of Benjamini-Hochberg (Benjamini and Hochberg, 1995). A false discovery rate (FDR) of 10% was allowed for significant gene selection for both the mixed-effects models and for the t-tests, and a significance level of 0.001 for the adjusted univariate test was used to select differentially expressed genes.

(6) Quantitative Real-Time PCR (RT-PCR) Validation

The SuperScript™ III First-strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize first-strand cDNA from total RNA. Quantitative RT-PCR was performed using the Stratagene MX3000P with MxPro v 3.00 software for Comparative Quantitation (Stratagene, La Jolla, CA). Controls included no-template controls and “minus reverse transcriptase” controls. All samples were run in triplicate. Two target genes were used for data normalization: Dctn6 (Dynactin 6) and Ppil2 (peptidylprolyl (cyclophilin)-like isomerase 2), and a calibrator template, Mouse Universal RNA (from mouse tissues, Stratagene, La Jolla, CA) was also used throughout the course of the assays. Ct values were determined using the MxPro software (Stratagene, La Jolla, CA). Relative levels of mRNA expression between the different *Mitf^{mi}* genotypes, or between the *Mitf^{vit/vit}* mutants and C57BL/6 controls were calculated using the Relative Expression Software Tool (REST v1.9.12) (Pfaffl et al., 2002). This software provides proper error propagation and robust statistical analysis by using a random reallocation algorithm with 50,000 iterations. Normalization for each experimental sample used the geometric means of the relative concentration of each normalizer gene (Dctn6 and Ppil 2) (Vandesompele et al., 2002).

(7) Ingenuity Pathway Analysis

The list of significantly altered genes identified in the microarray analysis was examined for the presence of interacting gene networks using Ingenuity Pathway Analysis software (www.Ingenuity.com). A data set containing gene identifiers and their corresponding expression values was uploaded as an Excel spreadsheet using the template provided in the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. For our data set, the Affymetrix probe set ID was used as an identifier, and an expression value cutoff of 1.5 was set to identify genes whose expression was significantly differentially regulated. These genes were then used as the starting point for generating biological networks. Biological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base.

(8) Immunohistochemistry

The distribution and abundance of Nramp1 protein was characterized immunohistochemically (IHC) in the RPE of E13.5 *Mitf*^{+/+}, *Mitf*^{mi/mi}, *Mitf*^{vit/vit} and C57BL/6 mice. E13.5 embryos were fixed overnight in fresh 4% paraformaldehyde, paraffin-embedded, and sectioned at 10µm. Sections were immunostained using primary antibody to Nramp1 (Santa Cruz, CA) optimized at 1:100, followed by biotin-conjugated secondary antibody, streptavidin-HRP and diaminobenzidine (DAB) solution until brown staining was visible. Slides were counterstained with methyl green. Alternatively, the Vectastain Alkaline Phosphatase Rabbit IgG ABC kit (Vector AK5001) and Vector Red Alkaline Phosphatase Substrate kit (Vector SK5100) were used for visualization. Sections were viewed under a Nikon Eclipse E800 microscope, photographed using an Optronics® (Goleta, CA) camera and AnalySIS® (Soft Imaging Systems Corp., Lakewood, CA) software, and images processed in Adobe Photoshop. **HMB45 IHC and Confocal Microscopy:** Primary antibody to HMB45, (a marker for Stage I premelanosomes), (Zymed, San Francisco, CA) was optimized at a concentration of 1:10. Fetal tissues were processed as previously described, and sections through the eyes of E13.5 C57BL/6 control, *Mitf*^{vit/vit}, *Mitf*^{+/+} and *Mitf*^{mi/mi} mice were stained using fluorescent immunohistochemistry, and observed by confocal microscopy for expression of HMB45.

(9) Transmission Electron Microscopy

Samples were fixed in 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% acrolein in 0.1M Sorensen Phosphate buffer, and post-fixed in aqueous 1% osmium tetroxide. Following post-fixation, samples were dehydrated in an ethanol series, and soaked overnight in a 1:1 Araldite/propylene oxide mixture. Final embedding was in fresh Araldite and polymerized at 65°C. Ultrathin sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate and examined with a Philips 410LS transmission electron microscope (TEM) operated at 60Kv.

RESULTS

(1) Gene Expression

(a) *Mitf*^{vit/vit} vs. C57BL/6 Gene Expression Data—The mixed-effects ANOVA identified 126 probe sets as being differentially expressed between the *Mitf*^{vit/vit} mutants and the C57BL/6 controls with a false discovery rate (FDR) of 10%. The random-variance t-tests identified 105 genes as being differentially expressed with a FDR of 10%. When the results were compared, 100 probe sets overlapped as being differentially expressed on both lists. Of the differentially expressed probe sets identified in the mixed-effects ANOVA, several represent alternative transcripts for the same gene. Taking this into consideration, 103 unique genes were identified as differentially expressed in the microarray analysis: 77 of these represent known genes, while 26 are ESTs or unknowns. The 77 known genes differentially

expressed in the microarray analysis of the *Mitf^{vit/vit}* vs. C57BL/6 embryos ($p < 0.001$) are provided in a table as supplemental data. The genes have been categorized according to gene ontology groups, and the file contains the accession number, gene symbol, gene name, and the highest mean fold change for the expression level of each gene. The fold change represents the value for the comparison of level of gene expression in the *Mitf^{vit/vit}* relative to the C57BL/6 control embryos; positive numbers represent relative increases in gene expression, whereas negative numbers represent relative decreases (mean fold change) in gene expression.

(b) *Mitf^{mi/mi}* vs. *Mitf^{+/mi}* vs. *Mitf^{+/+}* Gene Expression Data—The mixed-effects ANOVA comparing the 3 genotypes of *Mitf^{mi}* mutants identified 152 probe sets as being differentially expressed between at least 2 of the groups with a 10% FDR. Of the differentially expressed probe sets identified in the mixed-effects ANOVA, several represented alternative transcripts for the same gene. Taking this into consideration, 123 unique genes were identified as differentially expressed between at least 2 of the genotypes in the microarray analysis: 108 represent known genes, while 15 are ESTs or unknowns.

A total of 12 common (overlapping) genes were identified that demonstrated altered expression in both the *Mitf^{vit/vit}* mutant (exp. #1) and the *Mitf^{mi/mi}* mutant (exp. #2) relative to their respective controls. Only 2 of these genes in the *Mitf^{vit/vit}* mutants were pigment-related genes: *Mlana* (melanoma antigen) and *Gpnmb* (iris pigment dispersion). The geometric means of intensities of the 2 pigment genes, and the other 10 common (overlapping) genes that are differentially expressed in E13.5 eyes of both the *Mitf^{vit/vit}* and the *Mitf^{mi}* mutants are shown in TABLE 1. Of the known genes that were commonly mis-regulated in both *Mitf* mutants, *Nramp1* and *Epoxide Hydrolase* were both downregulated in the microarray comparison of *Mitf^{mi/mi}* vs. *Mitf^{+/+}*, while conversely upregulated in the microarray comparison of *Mitf^{vit/vit}* vs. C57BL/6. We chose to further investigate the altered expression of *Nramp1* in the RPE of *Mitf* mutants.

(2) Gene Ontology Groups

A pie chart showing the relative distribution (percentage) of the gene ontology groups identified in the microarray analysis of the *Mitf^{vit/vit}* mutants vs. the C57BL/6 control embryos is shown in FIGURE 1. The largest ontology group of known genes that was altered included signal transduction (13.5%), followed by transcription factors (12.7%), extracellular matrix (11.1%), cell adhesion/cytoskeleton (6.3%), ion homeostasis (4.8%), and pigmentation genes (1.6%).

(3) Ingenuity Pathway Analysis

The 12 overlapping genes (shown in Table 1) commonly mis-regulated in the eyes of both *Mitf* mutants were submitted for Ingenuity Pathway analysis. Nine of these twelve genes appeared in the resulting network. A subset of this network highlighting the connections between *Mitf* and iron-regulatory genes is shown in FIGURE 2. *Myc* forms a hub for the regulation of genes involved in iron homeostasis (highlighted in yellow). *c-myc* represses H-ferritin, and stimulates the expression of IRP-2, thereby increasing the intracellular iron pool (Wu et al., 1999), consistent with its role in cell proliferation and transformation. *Myc* and *PIAS2* (*Miz1*) cooperate to activate or repress expression of *Nramp1* (Bowen et al., 2002; Lapham et al., 2004). Although previous literature reports have not linked *Mitf* directly to the regulation of cellular iron homeostasis, *Mitf* has been shown to interact with *PIAS3* (Levy et al., 2003; Sonnenblick et al., 2004), and to regulate expression of HIF-1 α (Busca et al., 2005), a transcription factor that functions in oxygen sensing, and cellular adaptation to iron deficiency (Le and Richardson, 2002; Lee et al., 2006).

(4) Quantitative RT-PCR Validation

The Genes of Interest (GOI) that were selected for detailed analysis of gene expression in the quantitative RT-PCR experiments are shown in TABLE 2. In addition to the genes identified as significantly altered on our microarray analysis (*Nramp1*, *Ephx1*, *Gpnmb*, *Mlana*), we chose to further investigate the expression of other genes involved in iron uptake, storage, metabolism or export in the RPE, including: *Nramp2* (*Slc11a2*), *Transferrin*, *Transferrin-Receptor*, *Ferritin*, *Ferroportin* (*Slc40a1*), *Frataxin*, *Ceruloplasmin* (*Cp*), and *Hephaestin* (*Heph*).

(a) Iron Homeostasis—Our quantitative RT-PCR validation of the microarray gene expression results confirm that *Nramp1* is downregulated in the E13.5 *Mitf^{mi/mi}* mutant eyes relative to heterozygous and wildtype littermates, and conversely, upregulated in the *Mitf^{vit/vit}* mutant eyes relative to C57BL/6 controls (FIGURE 3). *Nramp2* message detected in the eyes of E13.5 *Mitf^{vit/vit}* mutants was significantly lower than levels detected in C57BL/6 controls. Quantitative RT-PCR analysis detected low levels of message for *Frataxin* and *Hephaestin* in E13.5 eyes, although there were no statistical differences in expression between mutants and controls. There were also no differences detected in the expression of *Transferrin* (*Trf*), *Ferritin* (*Fth1*), and *Nramp2* (*Slc11a2*). However, the analysis of additional genes involved in iron homeostasis demonstrated significant downregulation of *Transferrin Receptor* (*Tfrc*), which is involved in iron uptake into the cell, and conversely, significant upregulation of genes involved in iron export out of the cell: *Ceruloplasmin* (*Cp*), and *Slc40a1* (*Ferroportin*). (see FIGURE 4). Although the differences in expression of these genes were statistically significant in the RT-PCR analysis (* $p < .05$, ** $p < .01$), these genes were probably not detected in the microarray analysis because they fell just below or very close to the 1.5-fold change cutoff determination.

(b) Redox—*Epoxide hydrolase 1 (microsomal)*: Quantitative RT-PCR analysis of the microarray results confirmed that *Ephx1* gene expression was significantly upregulated in the eyes of *Mitf^{vit/vit}* mutants ($p < .001$).

(c) Pigment Formation—*Glycoprotein (membrane bound)*, *nmb*, (*Gpnmb*) was highly significantly downregulated in the *Mitf^{vit/vit}* mutant eyes relative to C57BL/6 controls ($p < .001$). Although *Silver* (*Pmel17*) did not appear significantly altered in our microarray analysis, we examined its expression in *Mitf^{vit/vit}* mutants by quantitative RT-PCR because of its homology to *Gpnmb* and association with *Mlana*. However, the slight decrease observed in expression of *Silver* in the quantitative RT-PCR analysis was not significant. Nevertheless, *Mlana* (*MART1*), which forms a complex with *Silver* (*Pmel17*), was downregulated on the microarray analysis, and was also very significantly downregulated in *Mitf^{vit/vit}* mutant eyes in our quantitative RT-PCR studies ($p < .001$).

(5) Immunohistochemistry: NRAMP1 Protein Expression

The pinkish-red (alkaline phosphatase) or brown staining (DAB) indicate positive expression of Nramp1 protein in the surface ectoderm, as well as in the rim of the developing optic cup in C57BL/6 control mice (FIG. 5A). In agreement with the quantitative RT-PCR gene expression data, staining for Nramp1 protein is significantly reduced or absent in the homozygous *Mitf^{mi/mi}* mutants (FIG. 5B). In contrast, expression of Nramp1 protein in the *Mitf^{vit/vit}* mutants appears more intense and more wide-spread throughout the RPE (FIG. 5C), which concurs with the increase in gene expression observed in the RT-PCR experiment. Expression of Nramp1 protein in C57BL/6 controls (FIG. 5D) diminishes in cross-sections through the RPE in a gradient that is most intense in cells at the rim of the optic cup, and appears to decrease in intensity along the RPE as you move proximally towards the optic stalk. In *Mitf^{vit/vit}* mutants, however, Nramp1 protein continues to be highly expressed throughout the entire length of the RPE (FIG. 5E). The smaller, wedge-shaped RPE cells at the rim of the optic

cup that label strongly for Nramp1 protein in wildtype fetuses represent a less differentiated stem cell-like population than the RPE cells closer to the optic nerve [Lord-Grignon et al., 2006]. Continued expression of Nramp1 protein throughout the RPE of *Mitf^{vit/vit}* mutants may therefore indicate delayed differentiation of cells in the RPE monolayer. Alternatively, altered protein-protein interactions as a result of the *Mitf^{vit/vit}* mutation may result in a gain-of-function or a failure to repress *Nramp1* as the RPE cells closer to the optic nerve become fully differentiated.

(6) Premelanosome Formation (HMB45 IHC)

Both *Mlana* and *Silver* (*Pmel17*) have been shown to be transcriptionally regulated by *Mitf* (Du et al., 2003). *Silver* (*Pmel17*) is present in early stage ‘premelanosomes’, prior to pigment deposition, and the protein can be recognized by antibody to HMB45. To assess the presence, number, and location of premelanosomes in the RPE of mutants and controls, sections through the eyes of E13.5 C57BL/6 control and *Mitf^{vit/vit}* and *Mitf^{mi/mi}* embryos were stained using fluorescent immunohistochemistry, and observed by confocal microscopy for expression of HMB45. The results are shown in FIGURE 6. Premelanosomes labeled with HMB45-Texas Red are visible on the basolateral side of the RPE in C57BL/6 control embryos (FIG. 6A). No staining for HMB45 was observed in the RPE of *Mitf^{mi/mi}* mutants (FIG. 6B). Premelanosomes are present in the *Mitf^{vit/vit}* mutants, albeit fewer in number, and their distribution is disorganized (FIG. 6C) relative to C57BL/6 controls. Premelanosomes are visible on the basolateral side of the *Mitf^{vit/vit}* RPE in cells expressing pigment (white arrowheads), but in those cells devoid of pigment (arrows), HMB45-positive staining is visible on the apical side of the cell (FIG. 6C).

(7) Melanosome Ultrastructure (TEM)

Melanosome ultrastructure was evaluated in the RPE of E13.5 C57BL/6 and *Mitf^{vit/vit}* embryos using transmission electron microscopy (TEM). Normal Stage IV mature melanosomes are present in the E13.5 RPE of C57BL/6 embryos (FIG. 7A); melanosomes in the *Mitf^{vit/vit}* embryos demonstrate a disorganized, fragmented fibrillar matrix, and abnormal clumping of melanin granules (FIG. 7B, 7C).

DISCUSSION

Our comparative gene expression analysis of downstream target genes affected by mutations in the transcription factor *Mitf* identified altered expression of not only known *Mitf*-regulated pigment-related genes (*Gpnmb*, *Mlana*), but also of novel candidate genes involved in iron transport (*Nramp1*) and redox homeostasis (*Ephx1*) that may play a role in the RPE pathology observed in *Mitf^{vit/vit}* mice. Decreased expression of *Gpnmb* and *Mlana*, and abnormal pigmentation in RPE melanosomes were observed in *Mitf^{vit/vit}* mutants. Expression of the iron transporter *Nramp1* and the redox-active enzyme *Ephx1* are upregulated in *Mitf^{vit/vit}* mutants. Excess accumulation of Fe⁺² leads to the production of reactive intermediates, predisposing cells to oxidative damage. Melanin plays an important role in protecting cells from oxidative damage and inhibiting iron-induced lipid peroxidation (Zadlo et al., 2007). Hypopigmentation and altered expression of *Mitf* and *Mitf*-regulated downstream gene targets have been observed following experimental induction of oxidative stress (Jimenez-Cervantes et al., 2001). Iron overload has been implicated in the pathogenesis of age-related macular degeneration (ARMD) in human patients and in mouse models (Chowers et al., 2006; Dentchev et al., 2005; Dunaief et al., 2005; Hahn et al., 2004), and polymorphisms in microsomal epoxide hydrolase (*Ephx1*) have also been associated with ARMD (Kimura et al., 2000). Altered expression of *Gpnmb*, *Mlana*, *Nramp1*, and *Ephx1* may therefore be involved in the RPE pathology observed in *Mitf^{vit/vit}* mutants.

In our microarray analysis and quantitative RT-PCR validation of gene expression, the pigment-related genes *Glycoprotein (membrane bound)*, *nmb*, (*Gpnmb*) and *Mlana* (*melanoma-associated antigen*) were significantly downregulated in *Mitf^{vit/vit}* mutant eyes relative to C57BL/6 controls. *Gpnmb* encodes an endosomal/melanosomal-specific membrane glycoprotein that is involved in melanin synthesis in the RPE and iris (Bachner et al., 2002), and shares sequence homology to the melanosomal protein *Silver* (*Pmel17*). Mutations in *Gpnmb* are associated with iris pigment dispersion syndrome, a condition that has been implicated in pigmentary glaucoma (Anderson et al., 2002). Although *Silver* (*Pmel17*) did not appear significantly altered in our microarray analysis, we examined its expression in *Mitf^{vit/vit}* mutants by quantitative RT-PCR because of its homology to *Gpnmb* and association with *Mlana*. *Silver* (*Pmel17*) plays an essential role in formation of the fibrillar matrix that is deposited in early stage melanosomes (Hoashi et al., 2006); formation of the fibrillar matrix scaffold is necessary for the polymerization of melanin granules in later stage maturing melanosomes (Dell'Angelica, 2003). However, the slight decrease observed in expression of *Silver* in the quantitative RT-PCR analysis was not significant. Nevertheless, *Mlana* (*MART1*), which was downregulated on the microarray analysis, was also very significantly downregulated in *Mitf^{vit/vit}* mutant eyes in our quantitative RT-PCR studies. *Mlana* forms a complex with *Silver* (*Pmel17*), and plays a critical role in its expression, stability, trafficking, and processing (Hoashi et al., 2006).

In contrast to the observed downregulation in expression of the pigment genes *Gpnmb* and *Mlana*, expression of the iron transporter *Nramp1* is significantly upregulated in *Mitf^{vit/vit}* mutants. *Nramp1* (*Slc11a1*), also known as *Natural Resistance Associated Macrophage Protein 1* encodes a divalent cation transporter expressed in lysosomes, and phagosomes that plays a role in iron transport, redox homeostasis and the innate immune response (Blackwell et al., 2003; Canonne-Hergaux et al., 1999). *Nramp1* functions as a pH dependent antiporter, and plays a role in phagosomal acidification (Hackam et al., 1998). Its activity has been best characterized in macrophages and cells of myeloid lineage (Jabado et al., 2000). In macrophages, *Nramp1* regulates metabolism and release of iron acquired by phagocytic, but not transferrin-mediated iron uptake (Mulero et al., 2002). Some studies in macrophages suggest that *Nramp1* transports Fe^{+2} from the cytosol into the phagosome, resulting in an increase in the amount of intraphagosomal Fe^{+2} available for participation in the Fenton reaction (Goswami et al., 2001). The generation of highly toxic hydroxyl radicals is thought to facilitate a bactericidal activity of the macrophage on the engulfed bacteria. Other studies in macrophages, however, suggest that *Nramp1* pumps Fe^{+2} from the phagosome into the cytosol, and the resulting metal-ion depletion of the phagosomal lumen becomes a rate-limiting factor for growth of the engulfed bacteria (Mulero et al., 2002). Iron is an essential element for carrying out numerous biological reactions. However, tight control of cellular iron homeostasis is necessary to avoid an excess of “free” iron, because trace amounts of iron can catalyze the production of highly toxic free radicals via the Fenton reaction, leading to oxidative damage. Several proteins are involved in the regulation of cellular uptake, transport, storage and export of iron (Sargent et al., 2005) (see FIGURE 8). Iron circulates in the blood bound to transferrin. The principal known effectors of iron uptake and transport into the cell are the transferrin receptor (TfR) (De Freitas and Meneghini, 2001) and divalent metal transporter 1 (*Nramp2*) (Canonne-Hergaux et al., 1999). *Nramp2* shares structural and functional homology to *Nramp1* (Vidal et al., 1995), and plays a role in transport of Fe^{+2} out of the early endosome and into the cytosol (Andrews, 1999; Canonne-Hergaux et al., 2001; Gruenheid et al., 1999). Fe^{+3} in the blood is bound to transferrin; this complex binds the transferrin receptor on the basolateral surface of the RPE (Hunt et al., 1989), and is internalized by endocytosis. As the internalized endosome undergoes acidification, Fe^{+3} is released from transferrin, and reduced to Fe^{+2} by endosomal ferric reductase. Although *Nramp2* expression/function has not yet been characterized in the eye, ferrous ion (Fe^{+2}) is very likely transported out of the endosome and into the cytosol in RPE cells by *Nramp2*, and the transferrin receptor complex recycled back

to the basolateral cell surface (Sargent et al., 2005). Cytosolic ferrous ion entering the labile iron pool can be utilized for several critical cellular functions. Fe^{+2} is a critical co-factor for the enzymatic isomerohydrolase activity of RPE65 in the visual cycle (Moiseyev et al., 2006), and also forms Fe-S clusters for utilization by the mitochondria in redox reactions (Gakh et al., 2006). Fe^{+2} in the labile iron pool may be bound by ferritin, an intracellular iron-sequestering protein that functions in iron storage and the protection of cells from iron toxicity. Alternatively, iron may be exported through the RPE apical membrane to the neural retina by the divalent iron ion exporter ferroportin (IREG1) (Donovan et al., 2000). In macrophages, Fe^{+2} is transported into or out of phagosomes by the iron transporter *Nramp1* (Nevo and Nelson, 2006). However, the role of *Nramp1* in the RPE with respect to transport of Fe^{+2} into or out of subcellular organelles (ie. melanosomes, lysosomes and/or phagosomes) has yet to be determined.

The expression of *Nramp1* has previously been reported only in macrophages, and hematopoietic cells of the myeloid lineage (Jabado et al., 2000). RPE cells possess macrophage-like properties, and have been shown to express hematopoietic cell markers in culture (Elner et al., 1992; Limb et al., 1997). Progressive retinal degeneration in *Mitf^{vit/vit}* mutants is accompanied by a massive accumulation of 'macrophage-like' cells in the subretinal space between P21 and P60 (Boissy et al., 1987; Sidman et al., 1996), but it is not known if these cells are of monocyte-macrophage lineage, or alternatively, if they are RPE cells that have detached. Damage caused by Fe^{+2} at the blood-retinal barrier includes destruction of anionic sites at the basement membrane of the RPE. Oxidative damage to Bruch's membrane and RPE cells might alter the integrity of the blood-retinal interface, thereby compromising ocular immune privilege, and allowing the invasion of immune cells. In addition to its role in iron transport, *Nramp1* is involved in macrophage activation (Blackwell et al., 2000). It is therefore interesting to speculate that increased expression of *Nramp1* in *Mitf^{vit/vit}* mutants may result in Fe^{+2} accumulation and oxidative damage to the RPE, coupled with enhanced migration of macrophages to the site of injury.

RPE defects in *Mitf^{vit/vit}* mutants precede the onset of retinal degeneration. RPE cells interact closely with photoreceptors of the neural retina, and play an important role in the maintenance of visual function (Strauss, 2005). Iron is necessary in the RPE for numerous enzymatic and metabolic functions, yet it is also highly reactive, and if improperly regulated, can lead to oxidative damage and cell death. Human polymorphisms in the ferroxidase gene *Ceruloplasmin*, are associated with iron overload and macular degeneration (Dunaief et al., 2005), and disruption of both *Ceruloplasmin* and *Hephaestin* in a mouse model results in iron overload and retinal degeneration with features of ARMD (Hahn et al., 2004). Increased expression of ferritin and ferroportin has also been observed in the retinas of patients with ARMD (Chowers et al., 2006; Dentchev et al., 2005). Although the expression/function of numerous proteins involved in iron metabolism in the RPE has previously been reported (Hahn et al., 2004; Martin et al., 2006; Yefimova et al., 2000), our knowledge regarding the complete network of proteins and regulatory mechanisms that control iron homeostasis in the RPE is still unfolding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Gene Ontology Groups

A pie chart showing the relative distribution (percentage) of the gene ontology groups identified in the microarray analysis of the *Mitf^{vit/vit}* mutants vs. the C57BL/6 control embryos is shown in Figure 1. The largest ontology group of known genes that was altered included signal transduction (13.5%), followed by transcription factors (12.7%), extracellular matrix (11.1%), cell adhesion/cytoskeleton (6.3%), ion homeostasis (4.8%), and pigmentation genes (1.6%).

FIGURE 2. Ingenuity Pathway Analysis

Nine of the twelve overlapping genes mis-regulated in the eyes of both *Mitf* mutants appeared in the same network following Ingenuity Pathway Analysis. A subset of that network highlighting the connections between *Mitf* and iron-regulatory genes is shown in Figure 2. The networks are displayed graphically as nodes (genes/gene products) and edges (the biological relationships between the nodes). Human, mouse, and rat orthologs of a gene, are stored as separate objects in the knowledge base, but are represented as a single node in the network. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes highlighted in yellow represent genes involved in iron homeostasis. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed as a direct interaction (solid line) or as an indirect/unknown interaction (dashed line). The nodes have also been arranged to show the location of the gene/gene product with respect to cellular or subcellular localization (e.g. plasma membrane, cytoplasm, nucleus, etc.). *Myc* forms a hub for the regulation of genes involved in iron homeostasis.

Mitf Eyes, NRAMP1 (Slc11a1) Relative expression

C57/Vit Eyes, NRAMP1 (Slc11a1) Relative expression

FIGURE 3. Quantitative RT-PCR Analysis of Nramp1 Gene Expression

Quantitative RT-PCR validation of the microarray gene expression data confirm that *Nramp1* is downregulated in the E13.5 *Mitf^{mi/mi}* mutant eyes relative to heterozygous and wildtype littermates (top panel), and conversely, upregulated in *Mitf^{vit/vit}* mutant eyes relative to C57BLJ/6 controls (bottom panel). [*p<.05, **p<.001].

FIGURE 4. Quantitative RT-PCR Analysis of Gene Expression

Quantitative RT-PCR expression analysis of additional genes involved in iron homeostasis demonstrates significant downregulation of the *Transferrin Receptor* (*Tfrc*), which is involved in iron uptake into the cell, and conversely, significant upregulation of *Ceruloplasmin* (*Cp*), and *Slc40a1* (*Ferroportin*), which are involved in iron export from the cell. *Epoxide hydrolase* (*Ephx1*) was significantly upregulated in *Mitf^{vit/vit}* mutants relative to C57BL/6 controls, while the pigment-related genes *Gpnmb* and *Mlana* were significantly downregulated (**p*<.05, ***p*<.001). Numbers above 1.00 on the y-axis represent the relative fold-change increase or *upregulation* in gene expression, while numbers below 1.00 on the y-axis represent the relative fold-change decrease or *downregulation* in gene expression in *Mitf^{vit/vit}* mutants relative to C57BL/6 controls. ***Ephx1*** (*epoxide hydrolase*); ***Slc11a1*** (*Nramp1*); ***Slc40a1*** (*Ferroportin*); ***Cp*** (*Ceruloplasmin*); ***Fxn*** (*Frataxin*); ***Heph*** (*Hephaestin*); ***Si*** (*Silver; Pmel17*); ***Trf*** (*Transferrin*); ***Fth1*** (*Ferritin, heavy chain*); ***Slc11a2*** (*Nramp2*); ***Tfrc*** (*Transferrin Receptor*); ***Gpnmb*** (*Glycoprotein, membrane bound*); ***Mlana*** (*MART-1; Melanoma antigen*).

FIGURE 5. Immunohistochemical Expression of Nramp1 in RPE

The brown staining (DAB) indicate expression of Nramp1 protein in the ectoderm, as well as in the rim of the developing optic cup in E13.5 C57BL/6 control mice (A). In agreement with the quantitative RT-PCR gene expression data, staining for Nramp1 protein is significantly reduced or absent in the RPE of homozygous *Mitf^{mi/mi}* mutants, although expression persists in the surface ectoderm (B). In contrast, expression of Nramp1 protein in the *Mitf^{vit/vit}* mutant appears more intense and widespread (C), which concurs with the increase in gene expression observed in the RT-PCR experiment. The pinkish-red (alkaline phosphatase) stain demonstrates that expression of Nramp1 protein in C57BL/6 controls (D) diminishes in cross-sections through the RPE as you move proximally away from the ectoderm towards the optic stalk. In *Mitf^{vit/vit}* mutants, however, Nramp1 is widely expressed throughout the RPE (E) and continues to be expressed proximally towards the optic nerve stalk.

FIGURE 6. Immunohistochemical Expression of HMB45 (premelanosomes) in RPE

Silver (Pmel17) is present in early stage ‘premelanosomes’, prior to pigment deposition, and the protein can be recognized by antibody to HMB45. Premelanosomes fluorescently labeled with HMB45-Texas Red were visualized by confocal microscopy on the basolateral side of the RPE in E13.5 C57BL/6 controls (white arrowheads) (A). No staining for HMB45 was observed in the RPE of *Mitf^{mi/mi}* mutants (B). Premelanosomes are present in the *Mitf^{vit/vit}* mutants, albeit fewer in number, and their distribution is disorganized (C) relative to controls. Premelanosomes are visible on the basolateral side of the *Mitf^{vit/vit}* RPE in cells expressing pigment (white arrowheads), but in those cells devoid of pigment (yellow arrows), HMB45-positive staining is visible on the apical side of the RPE cells. Images show fluorescent immunostaining superimposed on the corresponding Nomarski image (100x magnification). The red measure bar indicates 10μm, and the blue bracket indicates the RPE cell monolayer. NR (neural retina).

FIGURE 7. RPE Melanosome Ultrastructure

Ultrastructural analysis of Stage I-IV melanosomes was evaluated in RPE of E13.5 C57BLJ/6 and *Mitf^{vit/vit}* embryos using transmission electron microscopy (TEM). Normal Stage IV mature melanosomes are present in the E13.5 RPE of C57BL/6 embryos (A), while melanosomes in the RPE of E13.5 *Mitf^{vit/vit}* embryos demonstrate a disorganized fibrillar matrix, and abnormal clumping of melanin (B, C).

FIGURE 8. Iron Homeostasis in the RPE

Proteins known to be involved in cellular iron uptake, storage, metabolism and export, and their proposed role in iron homeostasis in the RPE are illustrated. Fe^{3+} bound to Transferrin (Trf) circulates in the blood vessels on the basal side of the RPE (choriocapillaris) and binds to the Transferrin Receptor expressed on the RPE cell surface; this complex is internalized by endocytosis. Fe^{3+} is converted to Fe^{2+} , exits the endosome into the cytosolic labile iron pool (LIP) via Nramp2 (hypothesized), and the endosome is recycled back to the cell surface. Once in the LIP, Fe^{2+} may be stored (Ferritin), incorporated into Fe-S clusters in the mitochondria (Frataxin), or be utilized as a cofactor by RPE65 in the retinoid visual cycle. Fe^{2+} from the LIP may also be converted to Fe^{3+} by the ferroxidase Ceruloplasmin, followed by export to the retina via Ferroportin. In macrophages, Nramp1 functions to transport Fe^{2+} into or out of phagosomes, although its role in the transport of iron into or out of subcellular organelles in the RPE has yet to be determined.

Trf	(Transferrin)	Nramp2	(Slc11a2; Dmt1)
TfRc	(Transferrin Receptor)	Ferroportin	(Slc40a1; IREG)
LIP	(Labile Iron Pool)	Fxn	(Frataxin)
Cp	(Ceruloplasmin)	Nramp1	(Slc11a1)

TABLE 1

Comparison of Affymetrix Mouse 430_2 Microarray Results

	Gene	Gene Symbol	Geometric Mean of Intensities on Microarray				
			+/+	+/mi	mi/mi	C57	vit/vit
Pigment Genes	tyrosinase	Tyr	1162.5	992.7	20.7	nd	nd
	tyrosinase-related protein 1	Tyrl	1273.5	626.7	24.7	nd	nd
	dopachrome tautomerase	Det	2394.0	1531.0	956.8	nd	nd
	silver	Si	6908.9	4090.1	46.1	nd	nd
	pink-eyed dilution (oculocutaneous albinism)	POCA2	656.4	643.0	203.6	nd	nd
	Ocular Albinism	OAl (Gpr143)	87.9	24.7	18.8	nd	nd
	Slc45a2 (underwhite)	Slc45a2	252.1	189.9	91.5	nd	nd
	melan-A	Mlana	495.3	167.4	34.0	629.8	50.2
	glycoprotein (transmembrane) nmb	Gpnmb	738.3	259.8	69.9	738.8	427.8
	NRAMP1 (slc11a1)	Slc11a1	246.5	153.3	70.4	191.2	1383.7
Redox	epoxide hydrolase 1, microsomal	Ephx1	127.7	85.2	48.9	191.8	1321.2
ECM	procollagen, type VIII, alpha 1	Col8a1	1521.8	1102.3	608.2	283.5	141.8
	RIKEN cDNA E130010M05 gene	Col4a4	224.6	124.3	31.8	226	76.3
	procollagen, type VI, alpha 1	Col6a1	891.4	461.3	704.9	734	437.7
	S 100 calcium binding protein A1	SI00a1	233.7	95.2	110.7	276.8	117.6
Ion	Transient receptor potential cation channel subfamily M, member 1 (melastatin)	Trpm1	1553.8	1507.8	560.2	392.7	194.2
Other	tetraspanin 10 (oculospanin)	Tspan10	242.8	161.2	31.2	240.1	131
	transferrin	Tfr	393.8	94.6	54.9	1099.3	466.9
	sclerostin domain containing 1	Sostdc1	141.0	121.5	79.1	287.4	145

TABLE 2

IRON HOMEOSTASIS, PIGMENT FORMATION AND REDOX QRT-PCR PRIMER/PROBE LIST

Gene Symbol	Gene	Applied Biosystems	TaqMan [®]	Description/Function
<i>Slc11a1</i>	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Mm00443045_m1	GOI	Transports Fe ²⁺ into or out of phagosomes (macrophages); expression not previously reported in the eye
<i>Slc11a2</i>	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	Mm00435363_m1	GOI	Transports Fe ²⁺ out of the endosomal compartment and into the cytosolic labile iron pool
<i>Tfr</i>	Transferrin	Mm00446708_m1	GOI	Binds Fe ³⁺ in the blood; important for iron uptake into the cell
<i>Tfrc</i>	Transferrin receptor	Mm00441941_m1	GOI	Transferrin-Fe ³⁺ complex binds the transferrin receptor on the basolateral surface of the RPE; internalized via endocytosis (Yefimova, 2000)
<i>Fth1</i>	Ferritin heavy chain 1	Mm00850707_g1	GOI	Iron storage; H subunit has ferroxidase activity
<i>Slc40a1</i> (<i>Ferroportin</i>)	Solute carrier family 40 (iron-regulated transporter), member 1	Mm00499837_m1	GOI	Exports iron (Fe ³⁺) out of the cell; co-localizes with ceruloplasmin and hephaestin in RPE and Muller cells
<i>Fxn</i>	Frxatin	Mm00784016_s1	GOI	Mitochondrial iron chaperone; participates in the synthesis of Fe-S clusters; mutations associated with Friedreich's Ataxia; retinitis pigmentosa, optic neuropathy
<i>Cp</i>	Ceruloplasmin	Mm00432654_m1	GOI	Ferroxidase; converts Fe ²⁺ to Fe ³⁺ for export out of the cell; polymorphisms associated with iron overload and retinal degeneration (ARMD) (Dunaief, 2005)
<i>Heph</i>	Hephaestin	Mm00515970_m1	GOI	Ferroxidase; disruption of ceruloplasmin and hephaestin in mouse model results in iron overload and retinal degeneration (ARMD)
<i>Gpmb</i>	Glycoprotein (transmembrane) nmb	Mm00504347_m1	GOI	Shares sequence homology with Silver (Pmol17); endosomal/melanosomal compartment-specific membrane glycoprotein expressed in the Iris and RPE; mutations associated with Iris pigment dispersion syndrome and/or pigmentary glaucoma
<i>Mlana</i>	Melan-A (MART1)	Mm01195780_m1	GOI	Forms a complex with Silver (Pmol17); critical role in expression, stability, trafficking, and processing of Silver
<i>Sl</i> (<i>Pmol17</i>)	Silver	Mm00498996_m1	GOI	Expressed in early (non-pigmented) melanosomes, essential role in formation of the fibrillar matrix/scaffold necessary for the polymerization of melanin granules
<i>Ephx1</i>	Epoxide hydrolase 1 (microsomal)	Mm00468752_m1	GOI	Deloxifies reactive epoxide intermediates, polymorphisms implicated in the exudative form of age-related macular degeneration
<i>Ppil2</i>	Peptidylprolyl isomerase (cyclophilin)-like 2	Mm00713185_m1	HCK	Protein folding and ubiquitination; housekeeping gene