PAX6 Regulates Melanogenesis in the Retinal Pigmented Epithelium through Feed-Forward Regulatory Interactions with MITF

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Abstract
During organogenesis, PAX6 is required for establishment of various progenitor subtypes within the central nervous system, eye and pancreas. PAX6 expression is maintained in a variety of cell types within each organ, although its role in each lineage and how it acquires cell-specific activity remain elusive. Herein, we aimed to determine the roles and the hierarchical organization of the PAX6-dependent gene regulatory network during the differentiation of the retinal pigmented epithelium (RPE). Somatic mutagenesis of Pax6 in the differentiating RPE revealed that PAX6 functions in a feed-forward regulatory loop with MITF during onset of melanogenesis. PAX6 both controls the expression of an RPE isoform of Mitf and synergizes with MITF to activate expression of genes involved in pigment biogenesis. This study exemplifies how one kernel gene pivotal in organ formation accomplishes a lineage-specific role during terminal differentiation of a single lineage.

Introduction
The retinal pigmented epithelium (RPE) is a monolayer of polarized and highly specialized pigmented cells that are located between the outer segments of the photoreceptors and the choroid layer in the eye. This strategic location demands multiple functions of the RPE during the development and homeostasis of the adjacent tissues, the neuroretina and choroid [1]. The RPE is a major component of the blood retinal barrier and it therefore determines the microenvironment of the photoreceptors. RPE cells are also responsible for photoreceptor outer segment phagocytosis and are directly involved in retinoid metabolism [1]. An important and evolutionarily conserved role of the RPE is the absorption of stray light to increase visual acuity and reduce oxidative damage. This latter activity requires functional melanosomes, which contain enzymes that catalyze the production of melanin (e.g. tyrosinase, TYR; tyrosinase-related protein, TYRP1; and dopachrome tautomerase, DCT) [2,3]. Melanosomes accumulate in the RPE during cellular differentiation [4]. Defects in any of these complex functions of the RPE may lead to photoreceptor degradation and, eventually, blindness. Considering the importance of the RPE for ocular physiology and the recent breakthroughs in technologies involving gene transfer and cellular based therapies for treating RPE malfunctions, there is a need to understand the molecular and cellular mechanisms that regulate the acquisition of the various specialized functions of this important tissue.

Most pigmented cells in the body originate from the neural crest. In contrast, the RPE is derived from the neural epithelium of the optic vesicles (OV), which are lateral protrusions of the ventral forebrain. The OV undergo patterning and morphogenesis to give rise to the bilayer optic cup (OC) with an inner layer of retinal progenitor cells and an outer layer populated by the progenitors of the pigmented epithelium. The distal regions of the OC differentiate into the epithelial layers of the ciliary body and iris [5]. The partitioning of the optic neuroepithelium into neuronal and pigmented precursors depends on the activity of extrinsic and intrinsic cues such as transforming growth factor-beta (TGFβ) and WNT ligands, which promote RPE development, and fibroblast growth factors (FGFs), which play a role in inducing neuronal fates [6–13].
Author Summary

It is currently poorly understood how a single developmental transcription regulator controls early specification as well as a broad range of highly specialized differentiation schemes. PAX6 is one of the most extensively investigated factors in central nervous system development, yet its role in execution of lineage-specific programs remains mostly elusive. Here, we directly investigated the involvement of PAX6 in the differentiation of one lineage, the retinal pigmented epithelium (RPE), a neuroectodermal-derived tissue that is essential for retinal development and function. We revealed that PAX6 accomplishes its role through a unique regulatory interaction with the transcription factor MITF, a master regulator of the pigmentation program. During the differentiation of the RPE, PAX6 regulates the expression of an RPE-specific isoform of Mitf and importantly, at the same time, PAX6 functions together with MITF to directly activate the expression of downstream genes required for pigment biogenesis. These findings provide comprehensive insight into the gene hierarchy that controls RPE development: from a kernel gene (a term referring to the upper-most gene in the gene regulatory network) that is broadly expressed during CNS development through a lineage-specific transcription factor that together with the kernel gene creates cis-regulatory input that contributes to transcriptionally activate a battery of terminal differentiation genes. Recently it was shown that a reduction in Pax6 gene dosage leads to development of neuroretina instead of RPE in embryos that are heterozygous for a mutation in Mitf, while embryos with such a mutation and normal Pax6 levels do not exhibit any detectable phenotype [37]. Furthermore, at the OV stage, the redundant activities of Pax6 and Pax2 are required for the early patterning of the OV by regulating Mitf expression [32]. Later in development, Pax6, but not Pax2, is detected in the RPE [32]. These findings establish a role for Pax6 during the RPE specification stage and imply that Pax6 is also important during the differentiation of the RPE, although its role at this stage is still unknown.

The goal of this study was to examine the roles of Pax6 during RPE differentiation, after the specification of the RPE is established. We show that during the onset of RPE differentiation Pax6 regulates the expression of Mitf and at the same time Pax6 functions together with MITF to activate the expression of downstream targets that execute melanogenesis in the RPE. Our findings reveal the molecular mechanism through which a single transcription factor, which is expressed in multiple ocular and non-ocular cell types, controls a highly specialized differentiation program of the neuroepithelium-derived pigmented cells of the eye.

Results

PAX6 is required for the pigmentation program of the RPE

Once the optic cup has formed (around E10.5), RPE progenitors begin to accumulate melanin [38,39]. During the initiation of the pigmentation program, the expression of Pax6 is detected throughout the RPE layer (E10-E12.5, Figure 1A). In later stages, the expression of Pax6 is gradually reduced, first in the central and subsequently in the peripheral optic cup (Figure S1A-D). Pax6 is eventually maintained in the pigmented cells of the ciliary body (CB) and iris. To study the role of Pax6 in the RPE after its specification and during the first steps of its differentiation, we generated Pax6loxP/loxP;DctCre mice in which loxP sites are located in exon 4 upstream of the initiator ATG and in intron 6 [5,40,41]. The Det promoter is active in the dorsal side of the OV at E9.5 and by E12.5 its activity is detected in the outer layer of the OC in RPE progenitors [5,41].

Corresponding with DetCre activity and the location of the loxP sequences, the Pax6 paired domain was lost from the optic cup as evident from labeling with an antibody that specifically identifies the N-terminus of Pax6 (E12.5, Figure 1F and Figure S1A-H, red). Nevertheless, a C-terminal fragment of Pax6 was detected in the Pax6loxP/loxP;DctCre mice when using polyclonal antibodies that detect this region of the protein (Figure S1, green). The expression of this variant lacking the paired domain (PD) of Pax6 (Pax6ΔPD) was transient and reliably mimicked that of the full-length Pax6 during development as the Pax6ΔPD gradually disappeared in a central to peripheral pattern and was eventually lost from the RPE at around birth (Figure S1, green).

We further characterized the expression of Pax6ΔPD transcripts in the mutant RPE: a first Pax6ΔPD transcript variant was generated from the P4 promoter and was also detected in control Pax6ΔPD mice in which loxP sites are located in exon 4 upstream of the initiator ATG and in intron 6 (Figure S2A-C). A second Pax6ΔPD variant was generated due to aberrant splicing between exon 3 and 7 (Figure S2B,D). Nonetheless, we did not detect over-expression of exons 7-8, located upstream of the homeodomain (HD), in the mutated RPE by quantitative real-time PCR (QRT-PCR, Figure S2F). Thus, the Pax6loxP/loxP;DctCre mice constitute a genetic model for determining...
the role of the full-length PAX6 protein, while not excluding activities mediated by PAX6 PD.

The phenotype of the Pax6loxP/loxP;DctCre eyes was evident during embryogenesis as the iris and CB progenitors, which are evident at E19.5 (Figure 1C), did not develop in the Pax6loxP/loxP;DctCre OC (Figure 1H) in agreement with a previous report [5]. In addition, reduced pigmentation in the RPE was noted from early stages of RPE differentiation (E12.5, Figure 1B,G) and was evident when viewing the whole eye of Pax6loxP/loxP;DctCre as compared to control litter mates (E19.5, Figure 1C,H) or in flat mount (E19.5, Figure 1K,M).

Although pigmentation was reduced, the fate of the RPE was maintained in the Pax6-mutant RPE based on the expression of the transcription factors Otx2 and Sox9 (Figure S3). Consistent with maintenance of RPE fate, transmission electron microscope (TEM) analysis conducted on E15.5 control and Pax6loxP/loxP;DctCre eyes (Figure 1D,E,I,J) demonstrated that the typical RPE morphology of a single layer was preserved despite reduction in pigmentation. The adjacent structures of the choriocapillaris and neuroretina maintained normal morphology despite Pax6 loss in the RPE (Figure 1D,I). We next examined actin distribution by phalloidin staining in flat mounts of the RPE and observed that the typical polygonal morphology of the RPE was maintained (Figure 1L,N). Moreover, using QRT-PCR analysis we did not detect significant differences between control and Pax6-deficient RPE in the levels of mRNAs encoding the intercellular junction proteins ZO-1, Connexin-43 and P-cadherin (E15.5, Figure 1O).

These findings reveal a role for PAX6 in execution of the pigmentation program, although its absence does not alter the fate and morphology of the RPE at the OC stage.

Figure 1. PAX6 expression is essential for proper pigment accumulation in the RPE but dispensable for RPE polygonal and single layer morphology. (A-N) RPE of (A,E,K,L) Pax6loxP/loxP and (F,J,M,N) Pax6loxP/loxP;DctCre mice analyzed for (A,F) PAX6 expression, (B,C,G,H,J,K,M) pigment accumulation and (D,L,N,O) morphology and specification. (A,F) Paraffin sections of E12.5 eyes were stained for PAX6 N-terminus and (B,G) viewed by differential interference contrast imaging. Scale bar is 100 µm. (C,H) Whole eye images of E19.5 mice. (D,E,I,J) Transmission electron microscope images of E15.5 eyes. Dashed lines mark the apical and basal membranes of the cells; arrowheads indicate melanosomes. Scale bar is 2 µm. (K-N) RPE flat-mount views of E19.5 eyes (K,M) using bright field or (L,N) stained for actin. Scale bar is 100 µm. (O) Relative transcript levels of connexin-43 (a gap junction marker), P-cadherin (an adherens junction marker) and ZO-1 (a tight junction marker) from control and Pax6-deficient E15.5 RPE fractions determined using QRT-PCR (n = 6). Abbreviations: CB, ciliary body; CC, choriocapillaris; N, nucleus; PR, photoreceptors. doi:10.1371/journal.pgen.1004360.g001
PAX6 is required for the expression of key melanogenic genes

To determine the global change in gene expression following Pax6 loss in the OC we determined the transcript profile in control and Pax6<sup>loxP/loxP</sup>;DctCre E15.5 RPE, using Affymetrix GeneChip Mouse Gene 1.0 ST arrays. Of the 28,853 genes represented on the microarray, levels of 100 transcripts were significantly altered in mutant RPE, compared to the wild-type (fold change greater than 1.5, p<0.05, Table S1). The expression of 73 of these genes was reduced in the Pax6-deficient RPE. In agreement with the observed phenotype, analysis of enrichment in GO categories revealed significant representation of melanogenic genes (p<0.05; using the ToppGene Suite algorithm; [42]) as summarized in Table 1. The identified pigmentation genes encode key enzymes of melanogenesis (Tyr and Tyrp1), as well as factors involved in melanosome biogenesis (Si, Mlana, and Gpr143) or melanosome transport (RAB27a) and factors implicated in melanosome biogenesis (Gpmb, Slc45a2, Slc24a5 and Slc3a2). Corresponding to the phenotype observed, the transcriptome analysis indicates an arrest in the melanogenesis program following Pax6 loss.

To validate the microarray results, six melanogenic genes were analyzed by QRT-PCR (Figure 2A). In agreement with the microarray results, transcript levels of Tyr, Tyrp1, Si and Mlana were significantly reduced in the mutant RPE as compared with control, whereas the level of the mRNA encoding the enzyme DCT, which is involved in melanin synthesis, was slightly reduced, and the level of Myo7a mRNA, which encodes a protein involved in cellular transport of melanosomes in the RPE [43], was similar to wild-type. The reductions of Si transcript (Figure 2B,E) and of TYR and TYRP1 proteins (Figure 2C,D,F,G) were validated by in situ hybridization and antibody labeling, respectively. These findings support a role for PAX6 in the proper expression of key melanogenic genes in the RPE.

PAX6 is required for the expression of D-Mitf in the developing RPE

The transcription factor Mitf is considered the master regulator of all melanin-bearing pigment cells and several melanogenic genes are direct targets of MITF [44,45]. Out of the 10 melanogenic genes found to be down-regulated following Pax6 loss, seven are known direct targets of MITF: Tyr and Tyrp1 [46], Si and Mlana [47], Gpmb [48], RAB27a [49] and Gpr143 [50]. Mitf has previously been found to be regulated by PAX6 and PAX2 at the OV stage [32]. We therefore wanted to investigate whether Mitf expression is dependent on Pax6 after RPE specification, when Pax2 is not expressed in the pigmented epithelium [32].

The Mitf gene encodes a family of isoforms generated from a common gene. The isoforms that are predominantly expressed in the RPE are A, H and D [18] (Figure S4A,B). The average response of all the Mitf probes in the GeneChip array revealed a reduction in the transcript levels by 1.37 fold (p = 0.069) in Pax6-deficient RPE compared to the wild-type. By indirect immunofluorescence (IF) analysis we indeed detected reduced levels of MITF protein in the RPE of Pax6<sup>loxP/loxP</sup>;DctCre embryos as compared with control Pax6<sup>loxP/loxP</sup> Cre<sup>+</sup> embryos at E12.5 and at E15.5 (Figure 3A-D). We next determined the expression levels of the specific Mitf isoforms by QRT-PCR of RNA extracted from control and Pax6-deficient RPE (E15.5). This analysis revealed a significant reduction in the expression of D-Mitf, which was over 3-fold lower in the mutants, and slight elevations in levels of A-Mitf and H-Mitf in the mutant RPE, compared to wild-type (Figure 3E).

Quantification of a downstream amplicon that is common to all Mitf isoforms revealed a significant 1.43-fold reduction in pan-Mitf transcripts, consistent with the microarray results. In situo analysis of the upstream regulatory region of D-Mitf revealed three putative binding sites for Pax6 PD and four for Mitf (Table S2); all are located within the 1200bp preceding the D-Mitf transcript start site (TSS) (Figure 3E). An electrophoretic mobility shift assay (EMSA) revealed that Pax6 binds two of the three sites in vitro (Figure 3G). This binding was specific, as it was competed by a cold probe (Figure 3G). Luciferase reporter assay on regulatory sequences of D-Mitf (between -1,153 and +6 relative to the TSS) was performed using different combinations of Pax6, Pax6APD and Mitf expression vectors (Figure 3H). This analysis revealed synergistic transactivation of the D-Mitf promoter by Pax6 and Mitf. The co-transfection of Mitf with Pax6APD failed to produce the same result.

In order to identify the regulatory sequence required for Pax6 and Mitf transcriotional activity, a series of D-Mitf truncated promoters was analyzed (Figure 3H). The critical regulatory sequence for Pax6 and Mitf transactivation is located between –310 and –180 relative to the TSS. This region contains only a Pax6 binding site (site 3: –212 and –194 relative to the TSS) but no known Mitf binding site. Taken together, these results suggest that the transactivation of D-Mitf promoter by Pax6 and Mitf depends on Pax6 PD and requires the PD binding site. These

Table 1. Melanogenic genes down-regulated in the Pax6-deficient RPE.

| Gene symbol | Gene name | Fold change | p value | References |
|-------------|-----------|-------------|---------|------------|
| Gpmb        | Glycoprotein (transmembrane) mbm | −5.73 | 0.00020 | [48,87] |
| Mlana       | Melan-A   | −4.17 | 0.00018 | [47] |
| Gpr143      | G protein-coupled receptor 143 | −2.83 | 0.00267 | [88,89] |
| Tyrp1       | Tyrosinase-related protein 1 | −1.91 | 0.00297 | [46] |
| Slc45a2     | Solute carrier family 45, member 2 | −1.90 | 0.00630 | [90,91] |
| Rob27a      | Member RAS oncogene family | −1.81 | 0.00530 | [92] |
| Slc24a5     | Solute carrier family 24, member 5 | −1.68 | 0.03025 | [93] |
| Slc3a2      | Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 | −1.66 | 0.00025 | [94] |
| Si          | Silver    | −1.60 | 0.00721 | [47,95] |
| Tyr         | Tyrosinase | −1.58 | 0.04166 | [46] |

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results also indicate that a self-sustaining PAX6-dependent feedback loop controls Mitf expression.

D-Mitf is dispensable for melanogenesis in the RPE

The abrogated melanogenesis in the Pax6-deficient RPE and the corresponding reduction in the D-Mitf isoform suggested that the pigment depletion in the Pax6-deficient RPE could be mediated by D-Mitf. Recently mice with specific deletion of D-Mitf were generated by ablation of 0.2 kb downstream to exon D, exon D and the 5.6 kb preceding sequence (Mitf<sup>DD</sup>, Figure S4C). In these mice, a slight reduction in pigmentation was observed at E11; however, at later stages the pigmentation was comparable to normal, in contrast to the depigmentation observed in the Pax6<sup>loxP/loxP;DctCre</sup> mutants. D-Mitf transcript level was completely abolished while A-Mitf and H-Mitf expression levels were elevated (Figure 4E). In addition, transcript quantification of the six melanogenic genes examined in the Pax6-deficient mutants revealed minor reductions in the levels of Tyrp1, Si and Myo7a, but only the reductions of the latter two were significant (Figure 4F). The normal phenotype of Mitf<sup>DD/DD</sup> mice is probably due to redundant activity of the Mitf isoforms expressed in the RPE. Together, the above results reveal that while PAX6 is required for normal levels of expression of D-Mitf, the reduced levels of D-Mitf following Pax6 loss cannot account for the observed arrest in melanogenesis in the Pax6<sup>loxP/loxP;DctCre</sup> mutants. Moreover, the dramatic loss of pigmentation, while levels of Mitf are partly maintained, indicates that PAX6 has other functions in melanogenesis of the RPE in addition to the regulation of Mitf levels.

PAX6 enhances the transcriptional activity of MITF on downstream melanogenic factors

The findings above reveal that PAX6 plays a pivotal role in the pigmentation program that goes beyond regulation of D-Mitf expression. This is reminiscent of the activity of the Pax3 gene in melanocyte precursors, where it regulates the onset of Mitf expression.

Figure 2. PAX6 is required for the expression of several melanogenesis genes. (A) Relative levels of Tyr, Tyrp1, Si, Mlana, Dct and Myo7a transcripts in RPE of control Pax6<sup>loxP/loxP</sup> and mutant Pax6<sup>loxP/loxP;DctCre</sup> E15.5 mice determined using QRT-PCR. *p<0.05, **p<0.005, ***p<0.0005, (n = 5). (B-G) Control and mutant RPE (B,E) cryo-sections showing the distal OC subjected to in situ hybridization for Si and (C,D,F,G) paraffin sections labeled with antibodies against TYR and TYRP1. Scale bar is 50 μm in B and E and 25 μm in C,D,F,G.

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PAX6 regulates pigmentation program in mouse eye

PAX6 enhances the transcriptional activity of MITF on downstream melanogenic factors.
expression as well as the expression of Mitf target genes like Tyrp1 [51]. In order to examine the ability of PAX6 to trans-activate known targets of MITF we performed luciferase reporter assays using the regulatory regions of three pigmentation genes: mTyrp1, hTyr and mMlana (see Tables S3-S5 for details on MITF and PAX6 putative binding sites). We also examined the transcriptional activity of PAX6\textsuperscript{PD}, which was detected in PAX6\textsuperscript{loxP/loxP,DctCre} mice (Figure S1 and Figure S2), on these promoters. On the mMlana promoter there was additive cooperation between the two transcription factors (PAX6: 2.25 fold change, \( p = 0.02 \); MITF: 5.03 fold change, \( p = 0.004 \); MITF + PAX6: 14.9 fold change, \( p \leq 0.04 \); \( n = 3 \); Figure S5). In contrast to PAX3, PAX6 by itself failed
to activate the $mTyrp1$ promoter either in pigment producing cell lines such as ARPE19 and UACC.62, or in HEK-293T, NIH-3T3 and HeLa cells (Figure 5A and data not shown). However, in the presence of MITF, PAX6 cooperatively and synergistically trans-activated the $mTyrp1$ promoter (MITF: 5.6 fold change, $p = 0.006$; MITF + PAX6: 51.9 fold change, $p = 0.002$; n = 4; Figure 5A). A similar synergistic transactivation pattern was observed using the hTyr promoter (MITF: 12.6 fold change, $p = 0.001$; MITF + PAX6: 71.3 fold change, $p = 0.003$; n = 3; Figure 5B). Chromatin immunoprecipitation (ChIP) confirmed the association of PAX6 with the $hTyrp1$ promoter region in RPE cells derived from human embryonic stem cells [52]. We observed more than 4-fold enrichment of PAX6 in the $hTyrp1$ proximal promoter compared to a region 2 kb downstream (data not shown and Table S6).

We next examined the contributions of the putative binding sites of PAX6 and the binding sites of MITF (M- and E-box) to the transactivation of the promoters of $mTyrp1$ and $hTyr$ by PAX6 and MITF. Interestingly, deletion or point mutations in the MITF binding sites dramatically reduced the transactivation observed when MITF and PAX6 were co-expressed. While the wild-type $mTyrp1$ promoter was trans-activated 51.9 fold in the presence of both factors, compared to their absence, a promoter carrying a deletion or mutations in the M-box was trans-activated only 3.5 fold (Figure 5A). Similarly, the wild-type $hTyr$ promoter was trans-activated 71.3 fold by both PAX6 and MITF, whereas promoters carrying mutations in the M- and E-box sequences were trans-activated 43.3 fold and 13.3 fold, respectively (Figure 5B). In contrast, deletion of the putative binding site for PAX6 in $mTyrp1$ promoter did not significantly alter the transactivation by MITF and PAX6 (Figure 5A). To examine whether the M-box is sufficient to enhance MITF activity by PAX6, we performed a luciferase reporter assay with PAX6, MITF or both using a reporter with four consecutive M-box elements. As shown in Figure 5C, PAX6 alone did not activate the promoter, MITF alone enhanced the activity by 6.6 fold, and PAX6 and MITF together enhanced the reporter activity by 11.9 fold ($p = 0.046$, n = 3). These findings suggest that in tissue culture, the MITF binding sites are essential and sufficient for the transactivation of $mTyrp1$ and $hTyr$ promoters by PAX6 and MITF.

The reporter assays revealed that PAX6 transactivation effects are largely dependent on MITF expression and on its binding sites. This mode of action suggests a physical interaction between PAX6 and MITF. We therefore conducted co-immunoprecipitation assays (co-IP) to evaluate this possibility. First, a reciprocal co-IP experiment was performed in ARPE19 cells that endogenously express both Pax6 and Mitf [53]. MITF antibodies co-precipitated PAX6, and immunoprecipitation with PAX6 antibodies resulted in precipitation of MITF (Figure 5D). The enrichment of MITF in the PAX6 immunoprecipitate was very significant as MITF expression was almost below detection in the input sample (Figure 5D, right panel, lane 5). These results support an association of MITF and PAX6 in ARPE19 cells.

To determine whether the PAX6APD variant is capable of physical association with MITF, HeLa cells were transfected with
3xFLAG-PAX6, 3xFLAG-PAX6ΔPD, 3xFLAG-MITF or a combination of 3xFLAG-MITF with each PAX6 protein variant (Figure S6A). Cells were harvested and protein extracts were precipitated using MITF antibodies. Both PAX6 and PAX6ΔPD proteins were enriched in the immunoprecipitates when co-transfected with MITF (Figure S6A, right panel, lanes 9 and 10). These results suggest that the PAX6ΔPD variant is capable of associating with MITF as previously suggested [54]. We next conducted luciferase reporter assays using MITF and PAX6ΔPD. PAX6ΔPD had no transactivation effects on the transcriptional activity of MITF (Figure 5A,B) and did not show a dominant negative effect on the transactivation of the mTyrp1 promoter by MITF and PAX6 (Figure S6B). These results indicate that although the PAX6ΔPD variant is capable of association with MITF, the PD domain is necessary for the PAX6-MITF-mediated transcriptional activation of melanogenic genes.

Discussion

This study unravels the molecular mechanism through which a single transcription factor, which is expressed in multiple ocular and non-ocular cell types, controls a highly specialized differentiation program of the neuroepithelium-derived pigmented cells of the eye. We show that PAX6 regulates a gene regulatory network central to RPE differentiation. This activity is mediated by a coherent feed-forward loop, by which PAX6 controls the expression of Mitf and jointly with MITF triggers the expression of multiple downstream target genes that are required for the execution of distinct differentiation program of pigment formation (Figure 6). In this mode of action, MITF levels could serve as a sign-sensitive delay for the melanogenesis process in the RPE, as transactivation of pigmentation genes by PAX6 depends on Mitf transactivation by PAX6. This type of kinetic mechanism filters out fluctuations in input stimuli since it requires persistent co-expression of both transcription factors. Our data provide an explanation of how PAX6, which is expressed in most ocular lineages, can promote the highly specialized and distinct differentiation program of the RPE.

A role for Pax6 during specification of the OV to the PE lineage was deduced from the analyses of Pax6 mutants that also carry mutations in the transcription factors Pax2 or D-Mitf. The PE of the transgenic Pax2+/−;Pax6+/− and MitfΔD/ΔD;Pax6+/− mice develops into a second neuroretina [32,37]. In contrast, RPE cells that lost the expression of Pax6 after specification maintained their morphology of a single layer of polygonal epithelium (Figure 1). Accordingly, we did not detect changes in the expression of several epithelial markers (Figure 1O) or elevated expression of the neuronal gene CHX10 (Figure 3A-D) in the Pax6-mutant RPE. Although the mutant RPE transcriptome data did not reveal overt elevations in neuronal genes, we did detect alterations in the expression levels of MITF-regulators, both key RPE-specification factors such as Gli2 [55]; −1.1, p = 0.00003) and of the retinal promoting gene Msx2 [56]; +1.8, p = 0.016). These alterations in gene expression suggest partial changes in the differentiation program of the Pax6-deficient RPE and point to additional regulators of MITF that are controlled by PAX6. However, these changes were not sufficient to completely disrupt RPE differentiation, in contrast to the complete disruption observed following inhibition of the Wnt/β-catenin pathway in specified RPE [57]. A previous study showed that PAX6 together with Pax2 is required for expression of MITF during the specification stage, and that the former two proteins regulate the expression of the A-Mitf isoform in vivo [32]. In our analysis, the loss of Pax6 during RPE differentiation resulted in up-regulation of A-Mitf. These findings suggest stage-dependent roles for PAX6 during various stages of RPE development, from patterning to differentiation.

We show that Pax6 is essential for the proper expression of Mitf and its melanogenic target genes. This activity requires both PAX6 and MITF to act synergistically, as shown by luciferase reporter

![Figure 6. Model of PAX6 control of melanogenesis in the RPE through a positive feed forward loop with MITF.](https://www.plosgenetics.org/journal.pgen.1004360.g006)
assays on the promoters of mD-Mitf, mTyrp1 and hTyr (Figure 3H and Figure 3A,B). Although PAX6 binding sites were identified in each of these three promoters (Tables S2–S4), the deletion of the PAX6 binding site in the Tyrp1 promoter did not reduce the transcriptional activity of PAX6 and MITF, while mutations in MITF binding sites in either mTyrp or hTyr promoters hampere their activity (Figure 5A,B). In contrast, the PAX6 binding site in the D-Mitf promoter (site 3, Figure 3F,G), but not the putative MITF binding sites, was essential for PAX6 and MITF transcriptional activity (Figure 3H). Together with the observation that PAX6 and MITF are capable of physical association (Figure 3D), these results suggest that the PAX6/MITF complex may trans-activate promoters through either PAX6 or MITF binding sites. This mode of action of PAX6 may account for the broad spectrum of PAX6 transcriptional targets. ChIP-Seq studies on embryonic RPE or hES-RPE cells are underway to determine the promoter occupancy of PAX6 and MITF on RPE genes.

The Mitf gene encodes at least 10 isoforms with alternative promoter or exon usage. The three RPE-specific isoforms (D-, A- and H-Mitf [18,19]) differ only in the N-terminal sequences [22]. The fact that all Mitf isoforms have different promoter sequences and predicted transcription factor binding sites suggests different regulation mechanisms [58]. Our data show that PAX6 is specifically required for the normal expression of D-Mitf. In both mouse mutant lines – Pax6loxP/loxP;DctCre and MitfAD/AD – we observed an up-regulation of A-Mitf and H-Mitf isoforms (Figure 3E and Figure 4E). However, while in the RPE of MitfAD/AD mice the total transcript level of Mitf was similar to that in the wild-type RPE, in the RPE of Pax6loxP/loxP;DctCre the level of pan-Mitf was 1.45-fold lower than the wild-type RPE. We therefore suggest that there is a feedback mechanism that balances the total level of MITF protein and that this mechanism requires full-length PAX6.

The observation that A- and H-Mitf are capable of compensating for the absence of D-Mitf activity in the MitfAD/AD transgenic mice, but not in the Pax6loxP/loxP;DctCre mutants, suggests that reduction in pigmentation in the RPE of Pax6loxP/loxP;DctCre transgenic mice might be caused by down-regulation of a MITF co-factor, either PAX6 itself or another protein. Other than MITF, the only transcription factor that has been demonstrated to have a role in regulation of RPE melanogenesis is OTX2. OTX2 plays an important role in RPE development [59,60] by trans-activating the melanogenic enzymes-encoding genes Tyr, Tryptp1 and Dct [61–63]. Since we did not detect a significant change in the expression pattern of Ot2 in the RPE of Pax6loxP/loxP;DctCre transgenic mice (Figure S3), it is unlikely that changes in its expression mediate the reduction in the expression of the melanogenic genes and pigmentation observed following Pax6 loss.

Another candidate that might be responsible for the reduced pigmentation in the Pax6loxP/loxP;DctCre is the bHLH leucine-zipper transcription factor TFE3. The amino acid sequences of TFE3 and MITF bHLH leucine-zipper show high similarity [64] and these two proteins bind to an E-box as heterodimer complex [17]. Bharti et al. [2012] showed that PAX6 trans-activates the expression of Tfe3 and that Tfe3 can rescue eye defects in mice with a mutation in the Mitf gene [37]. The transcript level of Tfe3 was indeed reduced in Pax6loxP/loxP;DctCre mutants (Figure S7A; p = 1.23 fold change, n = 6), and this minor down-regulation may have also contributed to the overall reduction in pigmentation, as TFE3 is capable of trans-activating mTyrp1 and hTyr promoters alone and synergistically with PAX6 (Figure S7B and data not shown). Thus, in addition to its known role during PE specification, TFE3 may also have a role in RPE differentiation where it acts like an additional isoform of MITF [17].

Association between PAX6 and MITF was previously shown in vitro by Planke et al. [2001]. However, in that study transfection of the two proteins caused a reduction in MITF transactivation of Tyr promoter [54]. The discrepancy with our results could be explained by the different ratio of Pax6/MITf levels used in the reporter assays, as Planke et al. used a ratio of 20:1, whereas in our study the ratio was 1:1. The importance of the ratio between PAX6 and MITF has also been demonstrated in vivo. The transcript levels of Tyr and Tryptp1 were lower in mice that over-express Pax6 (i.e. Pax6loxP/loxP mice) on the background of a MitfAD transgenic mice compared to either Pax6loxP/loxP or MitfAD/AD alone [37]. In these experiments, RPE pigmentation levels were consistent with the altered expression levels of Tyr and Tryptp1 [37] and Bharti, unpublished results). Interestingly, while during embryonic development PAX6 is eliminated from the RPE in a proximal to distal gradient, MITF and its downstream pigmentation genes are expressed along the entire length of the RPE. Thus, we infer that PAX6 is involved in the initiation of the pigmentation program but not in its maintenance.

The somatic mutation induced by the DetCre transgene deleted exons 4–6, which encode the initiation codon and the PD of Pax6. Interestingly, while the PD was eliminated from the Pax6loxP/loxP;DetCre embryos, a truncated transcript of Pax6 that gave rise to a Pax6APD variant was identified. The Pax6APD variant was not previously noted in somatic mutations of the Pax6 allele [40,65–69], probably because in some tissues, such as the lens placode and the peripheral optic cup, but not in the RPE, the expression of Pax6 depends on full-length Pax6 protein [70], which is absent due to the Cre-mediated deletion. In addition, it is possible that RPE-specific post-transcriptional mechanisms that alter splicing and RNA stability lead to more prominent accumulation of the Pax6APD transcript.

While the physiological activity of Pax6APD in the eye is still unknown, its over-expression results in microphthalmia due to aberrant lens and corneal development [71,72]. Thus, although we did not detect over-expression of the homeodomain of Pax6, we should consider the possible contribution of the over-expression of the Pax6APD isoform and the disruption of the Pax6/Pax6APD ratio to the pigment phenotype of the Pax6loxP/loxP;DetCre RPE. There are several lines of evidence that rule out a major effect of the Pax6APD isoform in the Pax6loxP/loxP;DetCre mutants: First, we detected a Pax6APD transcript in the control RPE, which was initiated from the P4 promoter (Figure S2A,C,E). Thus, the Pax6APD transcript is expressed during normal differentiation and onset of pigmentation in the RPE. Second, Pax6APD is expressed in the progenitors of the CB and is maintained there in the adult, both in the pigmented and non-pigmented epithelium [72]. Yet, mice carrying 10 copies of the Pax6 locus and over-expressing Pax6APD do not display any alteration in the pigmentation of the CB [72]. Therefore the Pax6APD isoform is unlikely to interfere with the pigmentation program. Third, we did not detect reduced pigmentation in the RPE of Pax6loxP/loxP;DetCre heterozygous mice, thus further arguing against a dominant-negative effect of this truncated product ([5] and data not shown). Finally, even though the Pax6APD isoform was able to associate with MITF in a co-IP assay (Figure S6A), it had no repressive or inductive effects on the promoters of mTyrp1 and hTyr either alone or when co-expressed with MITF or together with MITF and Pax6 (Figure 5A,B and Figure S6B).
development in different animal taxa [34,73–75]. According to this hypothesis an ancestor of a Pax6 gene was at the node of a gene regulatory network that controlled the morphogenesis of a primitive eye composed of a photoreceptor cell that contained pigment granules, as in Palamonomus pugio [76]. The evolutionarily earliest gene regulatory networks were likely to be hierarchically shallow and, as animal body parts gradually elaborated and gained more complex regional subdivision of the developing embryo, the underlying regulatory networks became hierarchically deeper and were terminally fixed into kernel genes, in which any minor change would lead to extremely harmful consequences [36,77]. In such a scenario, the development of the vertebrate eye into a complex structure that includes PE and multilayered neuroretina would require different cell specific transcription factors that in combination with PAX6 generate different cis-regulatory input functions that result in execution of distinct and highly specified differentiation programs. In this model, PAX6 acts as an accelerator directed by its tissue-specific partner to a specific transcriptional program.

Material and Methods

Mouse lines

The mouse lines employed in this study, Mtagfp [37], Pax6loxP [40] and DctCre [5] have been previously described. The latter two were used to establish Pax6loxP/DctCre somatic mutants. Pax6loxP/DctCre littermates were used as controls. The genetic background of all mice used in this study was C57BL/6J, except for in situ staining, for which mice of the outbred ICR genetic background were used. All animal work was conducted according to national and international guidelines and approved by the Tel Aviv University review board.

Statistical analysis

All data were examined using two-tailed Student’s t-test.

Immunofluorescence, ISH and flat-mount

Immunofluorescence analysis was performed on 10 μm paraffin sections as previously described [40], using the following primary antibodies: rabbit anti-PAX6 (1:400, Covance, prb-278b), mouse anti-PAX6 (1:25, Santa Cruz, sc-32766), rabbit anti-SOX9 (1:200, Chemicon, ab5535), sheep anti-CHX10 (1:1000, Exalpha, X11805), rabbit anti-OTX2 (1:50, Millipore, AB9566), rabbit anti-MIF1 [18], rabbit anti-Tyr [1:1,000, a gift from the Vincent Haring lab, NCI], rabbit anti-TYRP1 [1:1,000, a gift from the Vincent Haring lab, NCI]. Secondary antibodies were donkey anti-rabbit conjugated to alexa594 (1:1000, Invitrogen, A21207) and alexa488 donkey anti mouse/sheep (1:1000, Invitrogen, A21202/A11015).

In situ hybridization (ISH) was performed on 14 μm cryosections using DIG-labeled RNA probes as previously described [78]. The Pax6 intron 7 probe was generated from a 849bp PCR fragment (forward, 5’TGGAGGCCCTCAGACCTTCT-3; reverse, 5’TGCACAGTGTGGCCAGG-3). Plasmid for antisense transcription of silver was kindly provided by the laboratory of Dr. William Pavan (NIH) [79].

Flat-mount samples were prepared as follows: Eyes at E19.5 were enucleated and immediately fixed in 4% paraformaldehyde for 30 minutes. The RPE was carefully dissected from the rest of eye structures, sliced radially to four pieces and flattened on membrane filters (Schleicher & Schull, 0.45 μm D-37582). Samples were blocked and stained with phalloidin (1:100, Invitrogen, A12379). Thereafter, RPE was flattened on its basal side on a slide and sealed for observation.

Transmission electron microscopy

The heads of E15.5 embryos and perforated eyes of P1 neonatal mice were fixed in 0.1 M cacodylate-buffered fixative containing 2.5% paraformaldehyde and 2% glutaraldehyde and further processed as described previously [80]. Ultrathin sections were cut with a Leica Ultramicrotome UCT (Leica Microsystems), stained with uranyl acetate and lead citrate and analyzed with a H7600 transmission electron microscope (Hitachi).

RNA isolation and microarray analysis

Exact timed matings were performed by overnight cohabitation of an inbred Pax6loxP/DctCre male with Pax6loxP/loxP females. Pregnant females were harvested on day E15.5 and the RPE of the embryos was dissected as previously described [18]. RPEs were pooled into two separate tubes according to their pigmentation intensity, and tubes were stored at −80°C. Tail cuts of the embryos were collected for genotyping verification. Each tube was considered as one biological repeat. RNA was extracted using the QiAashereder and the RNeasy kits (QIA-GEN). RNA isolated from three control and three mutant samples was processed for microarray analysis using the Affymetrix GeneChip 1.0ST as described previously [81]. Differentially expressed genes with p-values lower than 0.05 and with a fold-change cutoff of 1.5 are listed in Table S1. The expression data were submitted to the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under series accession no. GSE56548.

Reverse transcription and quantitative real-time and end-point PCR

Reverse transcription of 1 μg of RNA from each sample was performed using the SuperScript III First Strand kit (Invitrogen). cDNA was amplified using the Power SYBR Green Mix (Applied Biosystems) in a 384-well optical reaction plate using ABI Prism 7000 Sequence Detection System (Applied Biosystems). All primer pairs were first tested for specificity and amplicon size using end-point PCR. Formation of a dimer structure was ruled out by analyzing the dissociation curve at the end of each amplification reaction. Results were calibrated in relation to an average of two house-keeping genes, Ppia and Tbp, after verifying that their levels were consistent in normal and mutant RPE. Raw data was processed using the comparative Ct method by the formula 2−ΔΔCt. Each amplification reaction was performed in triplicate using 20 ng of cDNA for each sample. Primers used to amplify and sequence the two Pax6APD transcript variants are listed in Table S6.

Luciferase reporter assay

Reporter assays were performed in HeLa cells using the Dual-Luciferase Reporter Assay System (Promega). Cells were seeded in a 24-well plate and 24 hours later were transfected using jetPEI DNA transfection reagent (Polyplus-transfection). Each well was co-transfected with three types of vectors in a total amount of 1210 ng of DNA: 1) 400 ng of a luciferase reporter vector (pGL3 basic) under the regulation of the examined promoter; 2) A total of 800 ng of expression vector (p3XFlag-CMV-10), either carrying no insert or containing an insert encoding the ORF of Pax6, Pax6APD or A-Miff, 400 ng of each; 3) 10 ng normalizing vector (pRL-TK). Cells were harvested 48 hours after transfection and luminescence was evaluated. Each treatment was carried out in duplicate, and each assay was repeated at least three times.
Site-directed mutagenesis

End-point PCR of 17 cycles was performed using oligonucleotides containing the desired mutated sites (Table S6, mutated nucleotides are in lower case) and the wild-type promoter reporter plasmid (pGL3 basic) as template. The PCR products were treated with 12U DdeI restriction enzyme (Fermentas) for 1 hour at 37°C, and 5 µl of the DNA was transformed into E. coli XL-1Blue strain, followed by colony-picking mini-prep and midi-prep plasmid purification (Qiagen). All plasmids were verified by sequencing.

Immunoprecipitation and immunoblotting

Transfection into HeLa cells was performed as described in the reporter assay section. Cells were seeded in 90-mm dishes and transfected with total of 10 µg of DNA. Cells were washed with phosphate buffered saline (PBS), scraped in 1 ml lysis buffer (10 mM HEPES pH 8.0, 100 mM NaCl, 1 mM MgCl2, 0.5% NP-40) containing protease inhibitor (Roche, complete Mini EDTA-free) and incubated on ice for 30 minutes. Extracts were subjected to pre-clearing using 150 µl of protein A agarose beads (Millipore, 16-157) for 2 hours at 4°C, followed by centrifugation at 10,000 g for 1 minute at 4°C. To avoid nonspecific binding of proteins to the beads, extracts were pre-incubated with 5 µl of protein A beads for 2 hours at 4°C. The beads were then washed four times with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) and the complexes were eluted in SDS sample buffer by boiling for 5 minutes. Samples were subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membrane and reacted with mouse anti-FLAG antibody (1:10,000, Sigma F3165) followed by anti-mouse horseradish peroxidase-conjugated secondary antibody. The reaction was examined by enhanced chemiluminescence detection kit (Biological Industries).

Co-immunoprecipitation from ARPE19 cells was performed essentially as described above, except cells were scraped in RIPA buffer containing protease inhibitor (Roche, complete Mini EDTA-free). Antibodies used for IP were either rabbit anti-PAX6 (Millipore, AB2237) or rabbit anti-MITF (kindly provided by David E. Fisher, MGH [82]) with rotation overnight at 4°C. The resulting immuno-complexes were incubated with 30 µl of protein A beads for 2 hours at 4°C. The beads were then washed three times with RIPA buffer (50 µM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM MgCl2, 0.5% NP-40) containing protease inhibitor (Roche, complete Mini EDTA-free) and incubated on ice for 30 minutes. Extracts were clarified by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was kept at 4°C. Input samples (50 µl of the supernatant) were kept at −20°C for input analysis and the cleared extracts were incubated with 5 µl of rabbit anti-MITF (kindly provided by David E. Fisher, MGH [82]) with rotation overnight at 4°C. The resulting immuno-complexes were incubated with 30 µl of protein A beads for 2 hours at 4°C. Samples were subjected to SDS–polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membrane and reacted with mouse anti-FLAG antibody (1:10,000, Sigma F3165) followed by anti-mouse horseradish peroxidase-conjugated secondary antibody. The reaction was examined by enhanced chemiluminescence detection kit (Biological Industries).

ChIP was performed as previously described [83,84]. Briefly, hES-RPE cells were grown as described [52]. Fixed chromatin was extracted from 2x10^7 cells and immunoprecipitated using rabbit anti-PAX6 (Millipore, AB2237) or non-immune rabbit IgG (Rockland) as a negative control. The primers used for ChIP analysis are listed in Table S6.

Electrophoretic mobility shift assay (EMSA)

HEK-293T cells were transfected with p3XFlag-CMV-10 encoding the ORF of full-length Pax6. Nuclear extracts were obtained as previously described [85]. Nuclear extract [1 ml] or 1:10 diluted nuclear extract was incubated for 10 minutes on ice in 8.5 mM HEPES pH 7.9, 30 mM KC1, 1.5 mM MgCl2, 0.4 mM DTT, 2 mg polydI/dC (Sigma). Binding with 1 ml double-stranded 59-c-ATP-labeled probe (30,000 cpm) was performed at room temperature for 20 minutes and 200 ng of “cold” PAX6 consensus site (PAX6CON) was used for competition [86].

Supporting Information

Figure S1 A Pax6ApD protein is expressed in the RPE of Pax6loxP/loxP;DctCre mutant mice. Paraffin sections of (A-D) control, Pax6loxP/loxP;DctCre, and (E-H) mutant, Pax6loxP/loxP;DctCre, eyes stained for the N-terminus (red, amino acids 1-206) and C-terminus (green, last 18 amino acids) of Pax6. (A’-H’ insets) Higher magnifications of indicated regions and nuclear staining with DAPI. (E-H’) Pax6ApD is not detected in the RPE of Pax6loxP/loxP;DctCre mutants at E12.5, E15.5 or E19.5 (red). Nevertheless, a Pax6ApD polypeptide is detected in the Pax6loxP/loxP;DctCre RPE (green). The spatiotemporal expression pattern of Pax6 isoforms is similar in wild-type and Pax6loxP/loxP;DctCre RPE: (A,E) At E12.5, Pax6 isoforms are expressed in the entire RPE; (B,F) At E15.5, Pax6 isoforms are highly expressed in the distal RPE; (D,H) and at E19.5 Pax6 isoforms are expressed only in the distal most cells of the RPE. (C,D,G,H’ insets) The boundary region along the RPE, where the expression of Pax6 isoform is gradually reduced, is shown in higher magnifications. Scale bar is 50 µm. (TIF)

Figure S2 Pax6 gene structure and transcripts expressed in the RPE of Pax6loxP/loxP;DctCre mutant and control mice. (A) A scheme of Pax6ApD transcript expressed under the regulation of the P4 promoter. Striped rectangle indicates the location of the ISH probe used to identify the expression pattern of the Pax6ApD transcribed from promoter P4. (B) A scheme of the abnormal Pax6ApD transcript that is expressed in the RPE of Pax6loxP/loxP;DctCre mice. Coding exons are marked with large rectangles and non-coding exons are marked with small rectangles. The PD and HD coding exons are marked in light blue and blue, respectively. Locations of primers that were used to sequence the two Pax6ApD variants are indicated with red arrows. Locations of possible start codons for the Pax6ApD transcript variants are indicated by ATG codons. (C) PCR products generated using primers designed to amplify a Pax6 exon 7 to exon 9 fragment, suggestive of the product shown in panel A. (D) PCR products generated using primers designed to amplify the a Pax6 exon 3 to exon 8 fragment, suggestive of the product shown in panel B. (E) A view of the distal OC of cryo-section subjected to in situ hybridization with a probe corresponding to Pax6 exon 7. Scale bar is 50 µm. (F) Transcript levels of Pax6 exons 7-8 in the RPE of control Pax6loxP/loxP and mutant Pax6loxP/loxP;DctCre mice at E15.5. (TIF)

Figure S3 Expression of the RPE transcription factors Otx2 and Sox9 was similar in wild-type and Pax6loxP/loxP;DctCre mice. (A) Relative transcript levels of Otx2 and Sox9 in RPE fractions from wild-type and mutant mice determined using QRT-PCR (n = 5). (B) Distal OC view of paraffin sections labeled with antibodies against (B,D) OTX2 and (C,E) SOX9. Scale bar is 25 µm. Only few cells in the distal most region of the RPE of Pax6loxP/loxP;DctCre do not express OTX2 (B,D’ insets). The expression of SOX9 in a proximal to distal gradient is maintained in the RPE of Pax6loxP/loxP;DctCre mutants. (C,E’ insets) The boundary region along the RPE, where the expression of SOX9 gradually increases. (TIF)

Figure S4 Schemes of the mouse Mitf gene locus, the deleted DNA segment in the MitfΔ/Δ transgenic mice and the different MITF isoforms expressed in the RPE and choroid. (A) Scheme of Mitf gene structure presenting only the exons that constitute the four main isoforms expressed in the RPE (A-, D- and H-Mitf) and choroid (M-Mitf). The alternative transcription (TSS) and translation (ATG) start sites are indicated. White rectangles mark...
untranslated regions and colored rectangles mark coding sequences. A graphic scheme of all known Mitf isoforms and their unique alternative first exons is presented in Bharti et al. (2008) [18]. (C) An enlargement of the rectangular inset in (A). In the MitfD transgenic allele, a 5.0 kb DNA fragment that includes exon D (253bp) and covers a region of ~200bp downstream and ~5.6 kb upstream to the D-Mitf TSS was replaced by a neomycin cassette [37].

Figure S5  PAX6 trans-activates the promoters of mMlana in the presence of MITF. Activity of luciferase under the regulation of the mMlana promoter co-transfected into HeLa cells along with different combinations of expression vectors and/or their backbones lacking the ORF, as indicated (n = 3). The positions of binding sites for MITF (E-box, green rectangles) and potential binding sites for PAX6 (light blue rectangles) are indicated relative to the TSS.

Figure S6  The PAX6ΔPD variant is capable of association with MITF but probably does not take part in the MITF-mediated transcriptional activation of melanogenic genes. (A) co-IP of MITF and PAX6 or PAX6ΔPD. HeLa cells were transfected with vectors expressing 3xFlagPax6, 3xFlagMitf, 3xFlagPax6ΔPD or their combinations, as indicated. Cell lysates were prepared (input) and immunoprecipitated with anti-MITF antibodies. Samples were subjected to SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody (input: lanes 1-5; IP: lanes 6-10). Both full-length PAX6 and PAX6ΔPD were found to be in association with MITF (lanes 9-10). (B) Activity of luciferase under the regulation of wild-type mTyrp1 promoter co-transfected into HeLa cells along with different combinations and amounts of expression vectors and/or their backbones lacking the ORF, as indicated. PAX6ΔPD had no effect on PAX6-MITF mediated transactivation of the mTyrp1 promoter.

Figure S7  The slight reduction in Tfe transcript level in the RPE of Pax6loxP/loxP;DctCre mice may contribute to the reduction in pigmentation. (A) Activity of luciferase under the regulation of wild-type mTyrp1 promoter (promoter scheme shown in Figure 5A) co-transfected into HeLa cells along with different combinations of expression vectors and/or their backbones lacking the ORF, as indicated. (B) Relative transcript levels of Tfe in wild-type and Pax6loxP/loxP;DctCre RPE fractions using QRT-PCR (n = 6).

Table S1  Genes differentially expressed in the Pax6-deficient RPE relative to wild-type RPE according to the microarray results.

Table S2  Putative MITF and PAX6 binding sites in mD-Mitf promoter (from +6 to −1153 relative to the TSS).

Table S3  Putative MITF and PAX6 binding sites in mTyrp1 promoter (from +5 to −252 relative to the TSS).

Table S4  Putative MITF and PAX6 binding sites in hTyr promoter (from +80 to −115 relative to the TSS).

Table S5  Putative MITF and PAX6 binding sites in promoter of mMlana (from +6 to −1153 relative to the TSS).

Table S6  Primers used in this study.

Text S1  Supporting references.

Author Contributions
Conceived and designed the experiments: SR SRL RAP. Performed the experiments: SR KB SRL RG RRA GL HA RAP. Analyzed the data: SR KB SRL RG YCT RS NE EM. Contributed reagents/materials/analysis tools: JL AZ MI BR. Wrote the paper: SR SRL RAP.

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