

Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene *Chx10*

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Summary

The homeodomain transcription factor *Chx10* is one of the earliest markers of the developing retina. It is required for retinal progenitor cell proliferation as well as formation of bipolar cells, a type of retinal interneuron. *or^J* (ocular retardation) mice, which are *Chx10* null mutants, are microphthalmic and show expanded and abnormal peripheral structures, including the ciliary body. We show here, in a mixed genetic background, the progressive appearance of pigmented cells in the neural retina, concomitant with loss of expression of retinal markers. Fate mapping analysis using a multifunctional *Chx10* BAC reporter mouse revealed this process to be direct transdifferentiation of retinal cells into pigmented cells. Microarray and in situ hybridization analyses revealed a complex program underlying the transdifferentiation. This program involved the expansion of expression of genes

normally found only in the periphery into central regions of the eye. These genes included a transcription factor controlling pigmentation, *Mitf*, and the related factor *Tfec* (*Tfec* – Mouse Genome Informatics), which can activate a melanogenic gene expression program. Misexpression of *Chx10* in the developing retinal pigmented epithelium (RPE) caused downregulation of *Mitf*, *Tfec*, and associated pigment markers, leading to a nonpigmented RPE. These data link *Chx10* and *Mitf* to maintenance of the neural retina and RPE fates respectively. Further, they suggest a new role for *Chx10* in maintenance of compartment boundaries in the peripheral retina.

Key words: BAC transgenic, *Mitf*, *or^J*, Fate mapping, Microarray, Compartments

Introduction

The vertebrate retina and retinal pigmented epithelium (RPE) derive from the optic vesicle, an evagination of the developing forebrain. As the optic vesicle grows and encounters signals from the prospective lens ectoderm, the domains that form the neural retina and RPE become established (reviewed by Chow and Lang, 2001). The distal optic vesicle thickens and becomes the neural retina, while the immediate proximal optic vesicle gives rise to the RPE. The interface between these two domains forms the peripheral retina, a region that gives rise to several pigmented and nonpigmented structures, including the iris and ciliary body. Several transcription factors, many of them homeodomain-containing factors, have been shown to play important roles in the specification and development of the optic vesicle (Chow and Lang, 2001). Mutations in these genes in mice or humans frequently lead to anophthalmia or microphthalmia (Graw, 2003). Other transcription factors contribute to the definition of optic vesicle compartments. Two notable transcription factors, *Chx10* and *Mitf*, are expressed in the developing optic vesicle in patterns that are either temporally or spatially mutually exclusive (Bora et al., 1998;

Liu et al., 1994; Nguyen and Arnheiter, 2000). Mutations of both *Chx10* and *Mitf* are responsible for classically defined mouse ocular phenotypes affecting development of the retina or RPE, respectively.

Chx10, a paired-like homeodomain transcription factor, is expressed in the presumptive neural retina, probably in response to inductive signals from the prospective lens ectoderm (Liu et al., 1994; Nguyen and Arnheiter, 2000). In this respect, *Chx10* is the earliest characterized specific marker of retinal progenitor cells. Fibroblast growth factor (Fgf) signals from the surface ectoderm may play a role in patterning the domains of the optic vesicle and may be required for *Chx10* expression in the presumptive neural retina (Nguyen and Arnheiter, 2000). In mouse, as well as the chick, *Chx10* is expressed in what appears to be nearly all retinal progenitor cells, but is absent from all of the postmitotic cell types except for bipolar interneurons (Belecky-Adams et al., 1997; Burmeister et al., 1996; Chen and Cepko, 2000) and a subset of Müller glia (Rowan and Cepko, 2004). Mutations in *Chx10* cause the ocular retardation phenotype in mice, including the *or^J* mutant, which has a spontaneous mutation that leads to a premature stop codon and failure to produce *Chx10* protein

(Burmeister et al., 1996). This phenotype correlates with a reduction in proliferation of retinal progenitor cells, especially those in the periphery of the retina (Bone-Larson et al., 2000; Burmeister et al., 1996). The central retina does appear to undergo differentiation, but the retina differentiates into a poorly laminated structure (Bone-Larson et al., 2000; Burmeister et al., 1996). Molecular analysis has revealed the absence of bipolar cells in these retinas, although all other cell types could be found (Burmeister et al., 1996). Several peripheral structures of the eye are abnormal in *or^J* mice, including the ciliary body and iris, and the lens is cataractous (Bone-Larson et al., 2000; Tropepe et al., 2000). The ciliary body appears to be expanded at the periphery of the retina, but does not undergo proper morphogenesis. *Chx10* expression has not been analyzed in these peripheral structures in the mouse. In the chick, however, *Chx10* has proven to be a useful marker of cells in the peripheral retina and nonpigmented cells of the ciliary body (Fischer and Reh, 2003; Kubo et al., 2003).

It has been known for several decades that ocular retardation mutant mice can show a range of variability in severity of the phenotype, predicting the presence of genetic interactors (Osipov and Vakhrusheva, 1983). In fact, genetic interactions between ocular retardation mutants and a number of microphthalmia mutants, including the fidget mouse and two different naturally occurring *Mitf* mutant mice, microphthalmia (*Mitf^{Mi}*) and white (*Mitf^{Wh}*), have been observed (Koniukhov and Sazhina, 1985; Koniukhov and Ugol'kova, 1978; Koniukhov and Sazhina, 1966). In each case, the ocular retardation small eye and microphthalmia small eye phenotypes were ameliorated in the double mutant animals. The genetic interactions between *Mitf* and *Chx10* mutants indicate a functional antagonism between *Mitf* and *Chx10*, a supposition supported by their mutually exclusive expression patterns. Genetic modifiers for *or^J* mutants have been identified that affect the proliferation of progenitor cells, as well as the lamination of the differentiated retina. One set of modifiers was observed by crossing 129/Sv mice, the original background of *or^J* mice, to *Mus musculus castaneus*. These mice had intermediate-sized, well-laminated retinas, but the peripheral retina remained undifferentiated and the ciliary body did not form (Bone-Larson et al., 2000). A partially rescued *or^J* phenotype was also observed in crosses with *p27* nullizygous mice. These retinas also were intermediate in size and had lamination, although they did not generate bipolar cells (Green et al., 2003).

Mitf is a basic helix-loop-helix (bHLH)-Zip transcription factor that acts as a master regulator of pigment cell development, and is expressed throughout neural crest-derived melanocytes and the RPE (Hodgkinson et al., 1993). *Mitf* is initially expressed throughout the optic vesicle, but its expression is rapidly restricted to the proximal part of the optic vesicle, the presumptive RPE (Bora et al., 1998; Nguyen and Arnheiter, 2000). The restriction of *Mitf* expression both spatially and temporally correlates with induction of *Chx10* expression in the presumptive neural retina. While *Mitf* is not required for specification of the RPE, it is essential for its differentiation and maintenance (Bumsted and Barnstable, 2000; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). A regulatory pathway upstream of *Mitf* expression has been characterized involving redundant activities of *Pax6* and *Pax2* as well as *Otx1* and *Otx2* (Baumer et al., 2003; Martinez-

Morales et al., 2003; Martinez-Morales et al., 2001), but the transcriptional pathway leading to repression of *Mitf* within the distal optic vesicle has not been identified. It is possible that either signaling upstream of *Chx10* or *Chx10* itself is responsible for the downregulation of *Mitf* in this domain.

A fascinating feature of the developing RPE is its ability to transdifferentiate into retinal cells under the appropriate circumstances. This has been observed following the addition of *Fgf* to RPE in vivo or in culture (Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1991). Most studies have focused on chick RPE, which maintains the ability to transdifferentiate at later embryonic stages. Rodent RPE has the ability to transdifferentiate into retina in culture, but this capacity is lost relatively early in development (Nguyen and Arnheiter, 2000; Zhao et al., 1995). Transgenic mice have been generated that overexpress *Fgf9* in developing RPE, and these mice show dramatic RPE to retina transdifferentiation (Zhao et al., 2001). *Mitf* mutants show spontaneous transdifferentiation of dorsal RPE (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000), while mice lacking a cell cycle inhibitor, *Gas1*, have ventral RPE transdifferentiation (Lee et al., 2001). *Mitf* function appears to be a key target in transdifferentiation. In the chick, forced expression of *Mitf* prevents RPE transdifferentiation in the presence of *Fgf*, although on its own, *Mitf* does not cause retinal effects (Mochii et al., 1998). Mutation or overexpression of factors thought to be upstream of *Mitf*, such as *Pax2* and *Pax6*, have phenotypes consistent with their action being mediated by *Mitf*. Thus, *Mitf* appears to function in part as a safeguard against transdifferentiation of the RPE into the retina.

A full understanding of the role of *Chx10* in retinal development would require understanding its biochemical functions and the genes it regulates. To date this has been lacking. Two candidate *Chx10* target genes have been identified. One gene, *Foxn4*, is co-expressed in progenitor cells with *Chx10*, and is highly downregulated in the *or^J* mutant (Gouge et al., 2001). Another candidate gene is *cyclin D1*, whose RNA levels are significantly decreased in the *or^J* mice, possibly leading to deregulation of *p27* (Green et al., 2003). In neither case is it known if these effects reflect direct regulation by *Chx10*. To further our understanding of the function of *Chx10* and how it might act through downstream targets, we characterized the fate of cells within the *Chx10*-expressing domain in *or^J* mutant mice. In addition, we present a microarray analysis of *or^J* mutant versus wild-type retinas. We show that several genes are upregulated in the *or^J* mutant, including those associated with peripheral retina development and those controlling pigmentation. Examination of the developing periphery of *or^J* retinas revealed the progressive expansion of pigmented cells towards the center of the retina at the expense of retinal progenitor cells. Using a multifunctional *Chx10* BAC transgenic mouse reporter, we demonstrate that these pigmented cells transdifferentiated from retinal cells. Peripheral markers also expand centrally in the *or^J* retinas, suggesting a new function for *Chx10* in maintaining boundaries in the peripheral retina. Finally, we show that ectopic *Chx10* in the developing chick RPE causes lack of pigmentation of the RPE, perhaps through direct repression of *Mitf*. These studies link *Chx10* and *Mitf* together as critical determinants in maintenance of the retina and RPE respectively.

Materials and methods

Transgenic and mutant mice

or^J mice were obtained from Jackson Laboratories and were maintained on a 129/Sv background before being crossed to *Chx10* BAC transgenic mice. R26R mice (a gift from R. Awatramani and S. Dymecki, Harvard Medical School, Boston, MA, USA), created by P. Soriano (Soriano, 1999), were maintained on a C57BL/6 background before being crossed to *Chx10* BAC mice. *Mitf^{gag9}* mice (a gift from H. Arnheiter and L. Lamoreux, NIH, Bethesda, MD, USA) have been described previously (Hodgkinson et al., 1993), and were maintained on a C57BL/6 background. *Chx10* BAC transgenic mice are described elsewhere (Rowan and Cepko, 2004), and were maintained on a mixed background containing FVB, 129/Sv, SJL and C57BL/6.

Immunohistochemistry and X-gal histochemistry

For antibody staining, cryosections were prepared and stained as described previously (Chen and Cepko, 2002). Primary antibodies used were 1:1000 rabbit anti-GFP (Molecular Probes) and 1:500 rabbit anti- β -gal (5'3'). Secondary antibodies used were 1:250 Cy2- or Cy3-conjugated goat anti-rabbit (Jackson Immunologicals). Following antibody staining, 4',6-diamidino-2-phenylindole (DAPI) was applied to stain nuclei (Sigma), and the sections were coverslipped and mounted in Gel/Mount (Biomed). Dissociated cells were prepared by removing the lens and cornea from E17.5 *or^J* eyes and dissociating the remaining tissue using papain (Worthington) as described previously (Chen and Cepko, 2002). Tissue sections or dissociated cells were stained for β -galactosidase using standard methods (Kwan et al., 2001). Tissue was stained overnight at 37°C, washed in PBS, coverslipped, and mounted in Gelvatol (Air Products).

Microarray analysis

Retinal RNA samples were isolated from E12.5 or E13.5 *or^J* mutant embryos using the Trizol reagent (Gibco). Retinal RNA samples of E13.5 wild-type 129/Sv or E12.5 Swiss Webster were used as comparisons. Each RNA sample was pooled from an entire litter. Care was taken to avoid contamination from the RPE and lens. 0.3–0.7 μ g of total RNA was RT-PCR amplified for 14 to 18 cycles using the SMART amplification kit according to the manufacturer's instructions (Clontech). PCR-amplified cDNA from each experiment and control pair was Klenow labeled with Cy5 and Cy3, respectively, and color swapped with Cy3 and Cy5, respectively as described (Livesey et al., 2000). Labeled probe was then hybridized to microarray slides spotted with 11,500 cDNA clones from the brain molecular anatomy project (BMAP) library (kind gift of B. Soares, University of Iowa, see <http://trans.nih.gov/bmap/index.htm> for details) and 500 cDNA clones of known identity from our lab collection (list available by request). Slides were printed and hybridized as described (Dyer et al., 2003; Livesey et al., 2004). Slides were then scanned on an Axon Instruments GenePix 4000 scanner and images were analyzed using the GenePix Pro software package. Gene expression data were uploaded to the AMAD database for data management and filtering. Gene expression ratios were normalized after filtering the data to remove low-intensity and poor-quality spots.

In situ hybridization

Section in situ hybridization was performed as previously described (Murtaugh et al., 1999) using 20 μ m cryosections from OCT-embedded tissue. Whole-mount in situ hybridization was performed as previously described (Chen and Cepko, 2000). Riboprobes labeled with DIG were detected with NBT/BCIP (Sigma). Riboprobes, gene nomenclature, and relevant references are described in Table S1 (supplementary material).

Cloning of a partial chick *Tfec* cDNA

Total RNA from E4 chicks was isolated using the Trizol reagent

according to the manufacturer's instructions (Life Technologies) and cDNA was generated using superscript II reverse transcriptase (Life Technologies). cDNA was amplified using degenerate primers to the *Mitf* family bHLH domain as described elsewhere (Rehli et al., 1999). PCR products were subcloned and sequenced to determine their identity; 3/16 colonies coded for a bHLH domain that shared higher homology to *Tfec* orthologs than *Mitf*. The remaining clones encoded *Mitf*. 5' RACE was performed using the Marathon cDNA Amplification Kit (Clontech) with the following gene-specific primer: 5'-TCACAGCAGATACGCGGAGCAATGG-3' to obtain a partial chick *Tfec* cDNA, which was subcloned into pCR2.1 (Invitrogen) and sequenced. This sequence has been submitted to Genbank as accession number AY502941. Using available EST data, a full-length chick *Tfec* cDNA was compiled and the nucleotide sequence data are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK004078

In ovo electroporation

Electroporations were performed by injecting 0.5 μ g of pMiw-*Chx10* (1.3 Kb *EcoRI/KpnI* coding sequence fragment) along with 0.25 μ g pMiw-GFP expression vector (Schulte et al., 1999) into the right optic vesicle of HH stage 9–10 chick embryos as described previously (Chen and Cepko, 2002). Embryos were harvested at stage 15/16. Electroporated embryos with normal eye sizes and strong GFP fluorescence (visualized on a Leica MZFLIII dissecting microscope) were further analyzed by whole-mount in situ hybridization analysis.

Results

Progressive hyperpigmentation in the *or^J* eye

Changes in rates of proliferation and differentiation in the periphery of the *or^J* retina have been described in the 129/Sv genetic background (Burmeister et al., 1996). In the studies presented here, *or^J* animals on the 129/Sv background were crossed into a mixed genetic background that contained contributions from SJL, C57BL/6, FVB and 129/Sv strains. In contrast to crosses of *or^J* mutants with *Mus musculus castaneus* or *p27* nullizygous mice, which have ameliorated *or^J* phenotypes, a more severe phenotype was observed. Differences in eye size were apparent at E11.5 (data not shown), and by E14.5, the *or^J* eyes were significantly smaller than *or^J/+* littermates (Fig. 1A,B). The periphery of the retina was particularly thin, consistent with a decrease in the proliferation rate in the periphery. The peripheral RPE appeared to expand into the neural retina as pigment granules were detected (Fig. 1B, arrow). This expansion of pigmented cells continued more centrally as the eye continued to develop (Fig. 1D,F, arrows). In the periphery of the retina, extensive pigmentation was observed, resulting in what appeared to be a trilayered structure at P1 (Fig. 1F). More centrally, the pigmentation in the retina was lighter. The sections shown in Fig. 1 and those that were analyzed by in situ hybridization were taken from central parts of the *or^J* eye and showed a less dramatic extent of pigmentation than is typically observed in the rest of the eye. Attempts to dissect retinas and RPE in older *or^J* mice frequently proved unsuccessful. Some eyes had very thin retinas surrounded by masses of pigment cells. Sections through a P15 *or^J* mouse revealed that virtually the entire retina had become pigmented in a multilayered structure (Fig. 1G,H). In some central sections, a thin layer of nonpigmented cells could be observed (Fig. 1H, arrowheads).

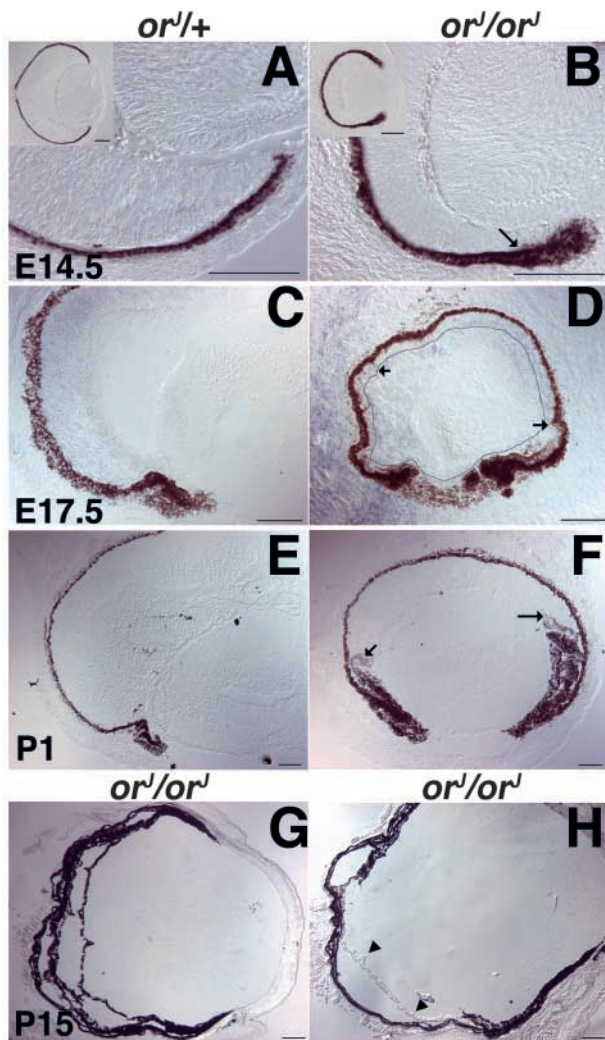


Fig. 1. Progressive pigmentation in *or^J* retinas. (A–H) Eyes were sectioned to analyze for pigmentation in *or^J/+* heterozygous (A, C, E) or *or^J/or^J* homozygous (B, D, F, G, H) littermates. Ages are indicated on the figure. (A, B) Insets show a full section of the eye (note scale bar differences). (B, D, F) Arrows indicate the central limit of ectopic pigmentation in the retinas of *or^J* mutants. (D) The lens is marked by a thin black line in the *or^J* eye at E17.5. (G, H) The peripheral retina in *or^J* mutants shows ectopic pigmentation, increasing over time until most of the retina appears pigmented at P15. (H) The presumptive retina is marked with arrowheads. Scale bar is 100 μ m.

Transdifferentiation of the retina into pigmented cells in *or^J* mutants

While expansion of the ciliary body has been characterized before in *or^J* mice, the source of the expanded population was not clear. We sought to determine whether the pigmented cells in the *or^J* retina arose via transdifferentiation of the retina, using a bacterial artificial chromosome (BAC) transgenic reporter mouse expressing both enhanced green fluorescent protein (GFP) fused to Cre recombinase, and alkaline phosphatase, under *Chx10* control (Fig. 2A). These mice were crossed with the R26R reporter mouse that expresses β -galactosidase (β -gal) following Cre-mediated excision of

a transcriptional stop cassette (Fig. 2A), and therefore permanently marks progeny of Cre-expressing cells. Thus, cells that expressed *Chx10* at the time of analysis could be detected using GFP (localized to the nucleus), while all cells that derived from *Chx10*-expressing cells could be analyzed for expression of β -gal using either histochemical X-gal staining or by anti- β -gal staining.

In *or^J/+* P1 mice, GFP was readily detectable throughout the outer neuroblastic layer (ONBL) in the retina, where progenitor cells are located, and in the non-pigmented ciliary body epithelium (npcbe) (Fig. 2C). Consistent with early expression of *Chx10* throughout progenitor cells, all of the different cell layers in the retina were β -gal-positive as determined by X-gal staining (Fig. 2B) or antibody staining for β -gal protein (Fig. 2G). The npcbe also stained positive for β -gal, with high levels transcribed from the *Rosa26* promoter in the more central part of the npcbe (Fig. 2B, G). *Chx10* protein was detected in the same pattern as GFP in *or^J/+* mice (data not shown). GFP and β -gal were not detected in pigmented cells in the developing ciliary body or in stromal cells outside the ciliary body. Analysis in *or^J/or^J* P1 eyes revealed the presence of GFP in retinal cells in the central part of the retina (Fig. 2E). The levels of GFP were markedly lower in *or^J/or^J* retinas than *or^J/+* retinas, and within *or^J/or^J* retinas, the levels were higher in the central retina than the periphery, where pigmentation was observed. However, GFP was detected in the lightly pigmented cells of *or^J/or^J* retinas near the periphery, showing that these cells were derived from *Chx10*-expressing cells (Fig. 2D, E arrows). Lack of GFP reporter activity in the heavily pigmented cells could indicate that GFP reporter activity was repressed in these tissues, that their origin was not a *Chx10*-expressing cell type, or that GFP fluorescence was obscured by the heavy pigmentation. Therefore, the R26R fate mapping cross was performed to determine what cells derived from *Chx10*-expressing cells. X-gal staining was found to be punctate and was detected in subsets of pigmented cells (Fig. 2D). To better visualize the extent of β -gal expression, immunofluorescence was performed using an anti- β -gal antibody. Analysis of the heavily pigmented peripheral region of the *or^J* eye, from the same section as Fig. 2D and at higher magnification, revealed that many pigmented cells did express β -gal protein, demonstrating their *Chx10*-expressing origin (Fig. 2I, also see below). Notably, the RPE and parts of the pigmented ciliary body did not express β -gal in these fate-mapping experiments. Overall, the presence of a largely β -gal-positive periphery showed the extent of retina to pigmented cell transdifferentiation in the *or^J* eye.

To better illustrate the pattern of neopigmentation, *or^J* mutant *Chx10* BAC transgenic animals were immunofluorescently stained for GFP at P0 (Fig. 2K) or P17 (Fig. 2M). In both cases, regions of mostly non-pigmented neuroepithelium included some lightly pigmented cells (Fig. 2J, L arrows). These regions expressed high amounts of GFP (Fig. 2K, M), demonstrating that they expressed *Chx10*. Furthermore, GFP-positive nuclei could be observed surrounded by pigment granules. At P1, the newly transdifferentiating region was still peripherally located within the eye, whereas by P17 it was more central, illustrating the progressive feature of transdifferentiation in the *or^J* eye.

In order to unambiguously determine whether β -gal- or GFP-positive cells were pigmented, as well as quantify the extent of transdifferentiation, E17.5 *Chx10* BAC/R26R double

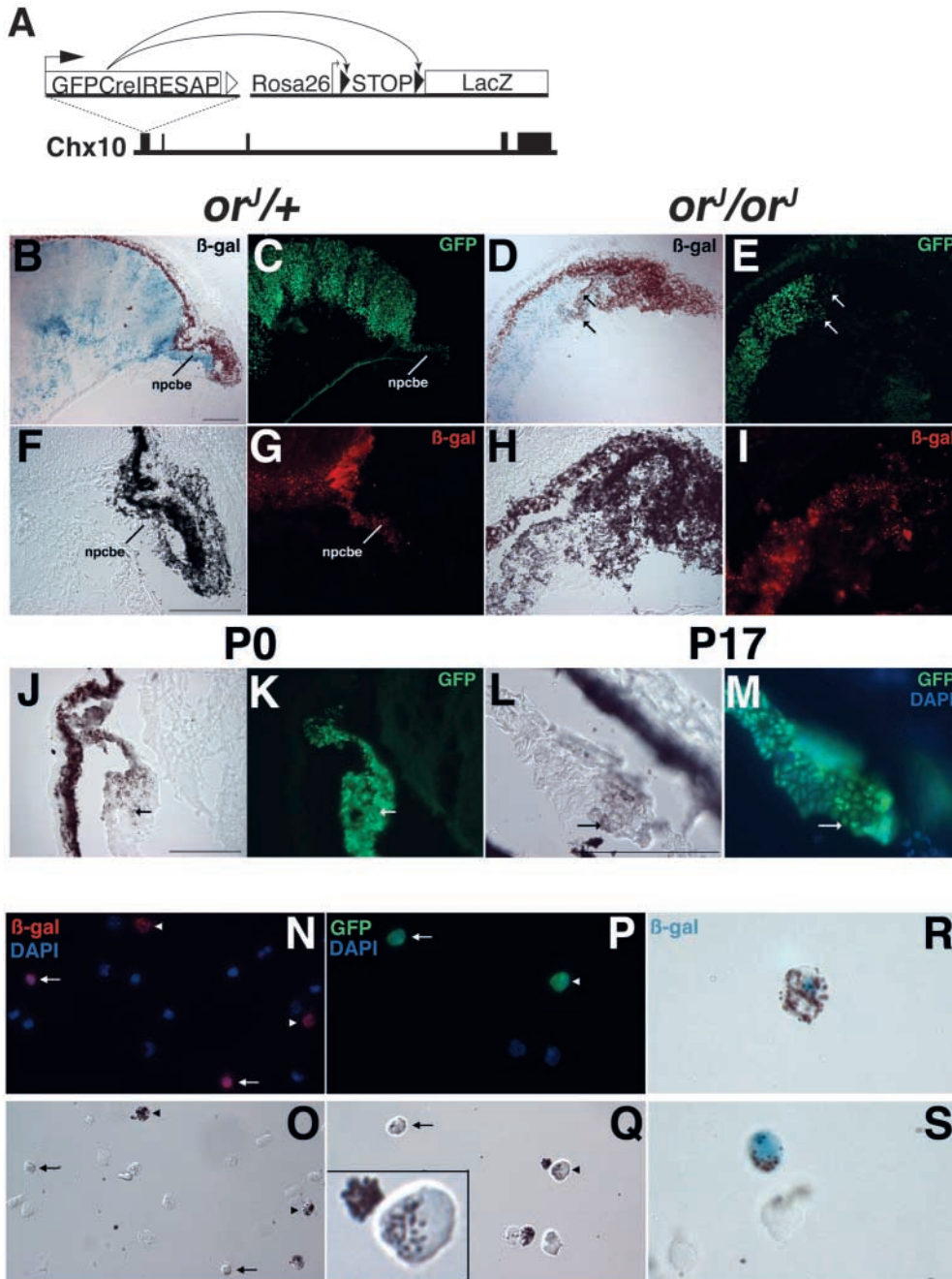


Fig. 2. Direct transdifferentiation of *or^J* retinal cells into pigmented cells. (A) Schematic of the transgenic mice used for fate mapping experiments. The *Chx10* BAC transgenic mouse directs expression of *GFP* and *AP* under control of *Chx10* regulatory sequences. This reporter is integrated in the first exon of *Chx10* (exons are indicated by solid vertical bars) within the *Chx10* RNA (black horizontal line) such that the 5' most reporter utilizes the *Chx10* start methionine (large arrow). Cre recombinase mediates recombination (curved arrows) at loxP sites (triangles) in the *R26R* allele. This removes a transcriptional stop cassette allowing *lacZ* expression from the *Rosa26* promoter (small arrow). (B-I) Sections through *Chx10* BAC/R26R transgenic eyes from *or^J/+* (B,C,F,G) or *or^J/or^J* (D,E,H,I) P0 littermates. (B,G) β -gal expression is observed throughout the retina as well as in the nonpigmented ciliary body epithelium (npcbe), but not in pigmented cells, in *or^J/+* eyes as detected histochemically (B) or with anti- β -gal antibody (G). (C) GFP reporter activity is detected in the outer neuroblastic layer (ONBL) as well as npcbe. (D,I) β -gal expression is observed throughout the retina as well as in many pigmented cells in the peripheral pigmented regions. (E) GFP reporter activity is observed throughout the retina as well as in lightly pigmented regions (arrows in D,E)). (J-L) Sections through *Chx10* BAC transgenic *or^J* mutant eyes at P0 (J,K) and P17 (L,M). (K,M) GFP reporter activity is detected throughout the peripheral retina including regions of the retina that are lightly pigmented (J,L) and in pigmented cells themselves (arrows). (M) DAPI nuclear counterstaining confirms that the GFP-expressing nucleus is within a

pigmented cell. (N-S) Dissociated cells from an E17.5 *Chx10* BAC/R26R transgenic *or^J* mutant eye antibody stained for β -gal (N) or GFP (P) with DAPI nuclear counterstaining. Arrows indicate cells that express β -gal (N) or GFP (P) in unpigmented cells (O,Q), while arrowheads indicate pigmented cells. (Q) Inset shows higher magnification image of cell marked with arrowhead. (R,S) Histochemical β -gal staining showing pigmented cells also expressing β -gal. All scale bars are 100 μ m.

transgenic *or^J* eyes were dissociated and immunofluorescently or histochemically stained for β -gal (Fig. 2N,R,S) or GFP (Fig. 2P). Examination of β -gal-positive cells under Nomarski illumination revealed that many of these cells (59/100) were pigmented, with some of them heavily pigmented (Fig. 2N, arrowheads). Similar analysis of GFP-positive cells also indicated that a significant, but smaller number of cells (18/100), contained pigment granules. These GFP-positive pigmented cells were more lightly pigmented than typical

pigmented cells (Fig. 2Q arrowhead and insert) and were probably cells in the process of transdifferentiation. Histochemical staining of dissociated cells for β -gal also revealed cells that were clearly pigmented and expressing β -gal (Fig. 2R,S).

Identification of genes affected in the *or^J* mutant

To identify genes affected in the *or^J* mutant, a microarray analysis was performed comparing wild type to *or^J* retinas.

Table 1. Genes upregulated in *or^J* mutants by microarray analysis

UNIQUID	Name	E12.5 Average	E13.5 Average	Average	s.d.
	Transcription factor EC (Tfec)	8.88	4.073	5.67	2.86
AI852420	Leucine-rich repeat protein 3, neuronal	2.69	3.555	3.27	0.62
AI851397	Gap-junction membrane channel protein α 1	3.06	2.563	2.73	0.54
AI843797	Hemoglobin α , adult chain 1	2.04	2.678	2.47	0.88
AI845279	RIKEN cDNA 2700084L06 gene	2.005	2.585	2.39	0.93
AI854349	Serum/glucocorticoid regulated kinase	2.41	2.345	2.37	0.37
AI845477	I- κ B α chain mRNA	1.915	2.558	2.34	0.95
AI847573	Protease, serine, 25	1.455	2.688	2.28	1.29
AI838302	CD63 antigen	1.575	2.58	2.25	0.58
	Fibroblast growth factor receptor 2	2.78	1.45	2.11	0.65
AI845640	RIKEN cDNA D430039N05 gene	2.805	1.685	2.06	0.61
AI838762	Hemoglobin, β adult major chain	1.4	2.293	1.99	0.96
AI845491	Microsomal glutathione S-transferase 1	2.21	1.785	1.93	0.36
AI835174	Carbonic anhydrase 14	2.145	1.785	1.91	0.26
AI851848	Aldehyde dehydrogenase family 1, subfamily A1	1.225	2.228	1.89	0.74
AI846734	Growth arrest specific 1 (Gas1)	2.28	1.598	1.83	0.41
AI838156	Carbonic anhydrase 8	1.195	2.133	1.82	0.99
AI848419	H3 histone, family 3A	1.58	1.903	1.79	0.34
AI852943	Chondroitin sulfate proteoglycan 5	1.585	1.82	1.74	0.32
AI854206	Hexosaminidase B	1.655	1.785	1.74	0.23
AI834768	RIKEN cDNA 1810027I20 gene	1.835	1.66	1.72	0.29
	Cyclin-dependent kinase inhibitor 2A (p16 INK4a)	1.525	1.825	1.72	0.34
AI842572	Rho, GDP dissociation inhibitor (GDI) beta	1.72	1.705	1.71	0.25
AI845761	RIKEN cDNA 2010319C14 gene	1.27	1.933	1.71	0.46
AI853186	Fatty acid binding protein 3, muscle and heart	1.775	1.658	1.7	0.48
AI850915	UDP-glucose ceramide glucosyltransferase	2.2	1.448	1.7	0.41
	Paired box gene 6 (Pax6)	1.13	1.613	1.45	0.29
	Cyclin-dependent kinase inhibitor 1C (p57)	1.03	1.628	1.43	0.33
AI854101	Corticoliberin-binding protein (CRHBP)	1.155	1.36	1.29	0.16

Genes listed are those upregulated at least 1.7-fold. The last three genes in the table were confirmed to be upregulated by in situ hybridization analysis, although they were below the 1.7-fold cutoff. Genes shown in bold have been analyzed by in situ hybridization. Genes listed without unique ID are full-length clones from within our laboratory. The average of E12.5 was determined from one hybridization and its color swap. The average from E13.5 was determined from two hybridizations and their color swaps. The total average was calculated from all six data points and is shown with its standard deviation (s.d.). Individual data points and Unigene numbers are included in Table S2.

RNA samples were compared at E12.5 or E13.5 in development, very early time points following the onset of *Chx10* expression. At this time, the difference in eye size is small and transdifferentiation is not observed (data not shown). Table 1 shows the genes that were upregulated greater than 1.7-fold in the *or^J* versus wild-type retinas and Table 2 shows the genes that were downregulated 0.5-fold compared to wild-type retinas. These cutoffs were arbitrarily determined, and a number of genes with ratios below the cutoffs showed expression differences as analyzed by in situ hybridization (see below). A more complete list of putatively affected genes is shown in Tables S2 and S3 (supplementary material).

Examination of genes upregulated in the *or^J* mutant revealed that *Tfec*, a bHLH leucine-zipper transcription factor in the *Mitf* family, was the most upregulated gene in this analysis. This was a striking result, as *Tfec* is normally only expressed in the early developing RPE and in the peripheral retina (see Fig. S3B,D in the supplementary material, data not shown). This expression change, at a time prior to pigmentation in the *or^J* retina, suggested a link between the transdifferentiation observed in the *or^J* retina and gene expression changes. Other transcription factors and enzymes involved in pigmentation were not represented on the microarray, but were evaluated by in situ hybridization analysis (see below). Also upregulated in the *or^J* mutant were two well-characterized cell cycle inhibitors, *p57* and *Gas1*.

Table 1 also shows a number of genes evaluated by in situ

hybridization (in bold). The genes that were tested by in situ hybridization from the array list were verified to be differentially expressed in the *or^J* mutant (see below). One particular gene, *Crhbp*, evaluated because of a known expression pattern in the peripheral retina (Blackshaw et al., 2004), was verified to be upregulated in the *or^J* mutant, even though the fold upregulation by microarray analysis was 1.29. This suggested that a high percentage of the genes identified by microarray analysis might be significantly differentially expressed in the *or^J* mutant.

Downregulated genes (Table 2) with known functions fitted into a more definite pattern than upregulated genes. The bHLH transcription factors, *Neurod1* and *Math3*, are required for neuronal differentiation and were highly downregulated. Several other genes found to be downregulated corresponded to structural genes and known markers (e.g. *Gap43* and *Snap25*) of neurons. In situ hybridization analysis of a number of genes identified in Table 2 showed significant downregulation of these genes (see below). Two markers of cell cycle progression, *cyclin D1* and *E2f1*, were downregulated according to the microarray analysis, although less so than the cutoff assigned to Table 2. Cyclin D1 has previously been shown to be downregulated in *or^J* retinas (Green et al., 2003).

In situ hybridization analysis of affected genes

In order to verify that some genes identified as differentially regulated by microarray analysis were changed in expression

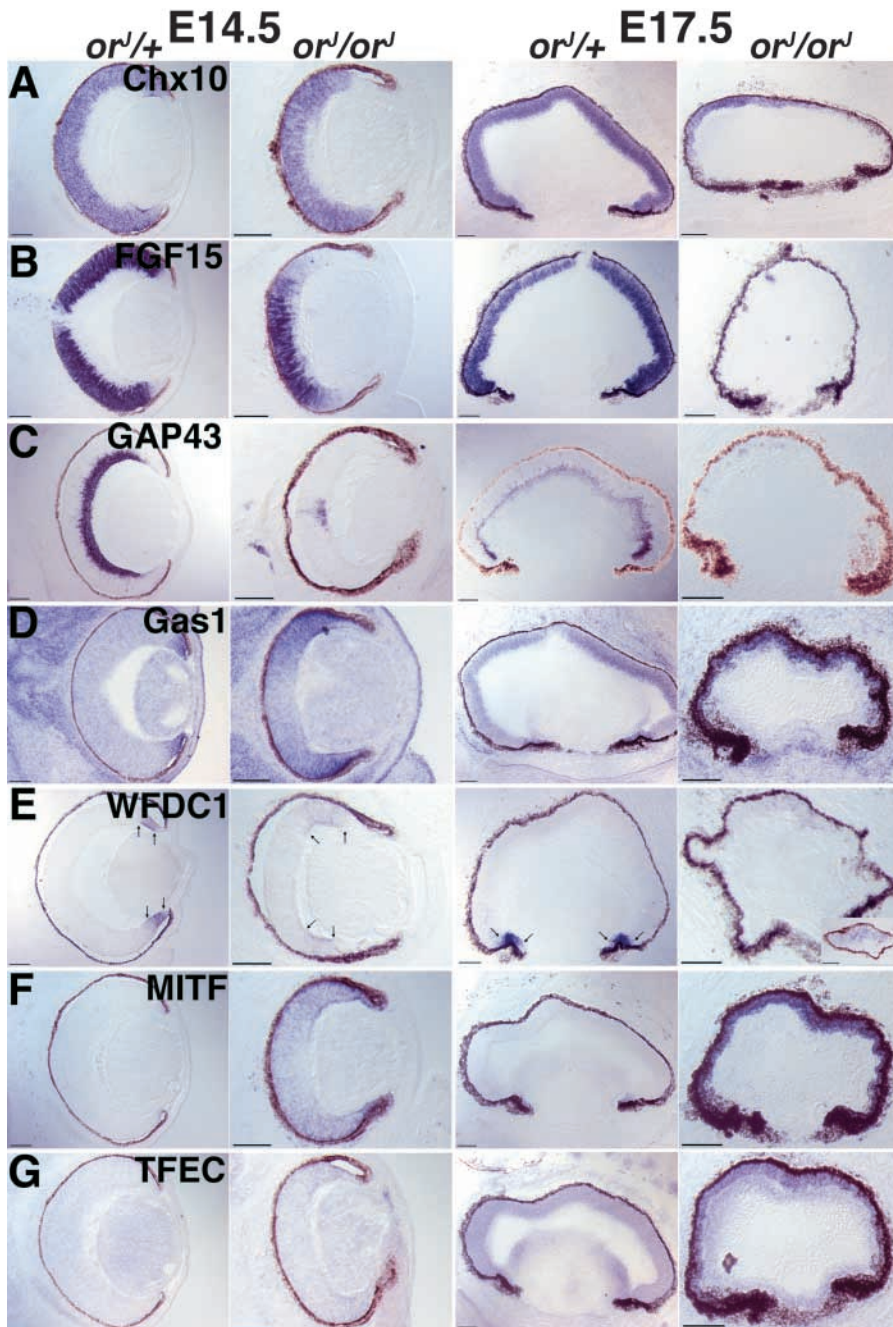


Fig. 3. In situ hybridization analysis of genes affected in *or^J* mutants. (A-G) Sections through E14.5 or E17.5 *or^{J/+}* heterozygous or *or^{J/or^J}* homozygous littermates, as indicated, were analyzed for the following RNAs: (A) *Chx10*, (B) *Fgf15*, (C) *Gap43*, (D) *Gas1*, (E) *Wfcd1*, (F) *Mitf*, (G) *Tfec*. Full expression patterns are discussed in Results and summarized in Fig. 4. Note scale bar differences between *or^{J/+}* and *or^{J/or^J}* sections. (E) Arrows indicate boundaries of *Wfcd1* expression. (E) Inset shows peripheral section from the same eye. All scale bars are 100 μ m.

RPE, where the tissue is thickened. Zone 4 contains loosely packed pigment cells, presumably neural crest-derived melanocytes. Within the neural retina (i.e. the tissue that abuts zone 1 and extends more centrally), specific expression patterns were observed in the outer neuroblastic layer (ONBL), containing a mixture of progenitor cells, newly postmitotic cells, and differentiating photoreceptors, and the inner neuroblastic layer (INBL), where postmitotic amacrine and ganglion cells reside. Summaries of the expression patterns in Fig. 3 are listed in Fig. 4B. References of known expression patterns or functions are included in Table S1 (supplementary material).

Class I: ONBL-expressed genes

In *or^{J/+}* retinas, *Chx10* was expressed at E14.5 and E17.5 throughout the ONBL in progenitor cells, and extended peripherally into zone 1, but was not expressed in zone 2 (Fig. 3A). In *or^J* eyes, *Chx10* was expressed in the ONBL at comparable levels, but the expression ended slightly more centrally than in *or^{J/+}* mice, in a region that was becoming pigmented. At E17.5, *Chx10* was still expressed in the center of the eye, but was not detected more peripherally in the

transdifferentiating region. To further analyze the expression of genes specific to retinal progenitor cells, *Fgf15* was examined (Fig. 3B). *Fgf15* was expressed throughout the ONBL at E14.5 and E17.5, but was not expressed in any of the zones of the peripheral eye in *or^{J/+}* mice. In the *or^J* mutant, its expression was restricted centrally at E14.5, but its expression levels appeared normal. At E17.5, *Fgf15* was only detected in a few cells in the center of the *or^J* mutant retina. A large number of genes expressed in the ONBL exhibited similar patterns to that of *Fgf15* in the *or^J* mutant retina. These included genes controlling retinal progenitor cell function or proliferation (*Notch1*, *E2f1*, *cyclin E1*, *cyclin D1*), bHLH transcription factors (*Neurod1* and *Math5*), and genes associated with cell cycle exit (*Btg2* and *Dll1*) (see Fig. S1A-H in the

level, and to better identify genes that may play a role in transdifferentiation, in situ hybridization analysis was performed (Fig. 3; see Fig. S1 in the supplementary material). Several additional genes not present on the microarray were analyzed because of their previously characterized expression patterns. In situ hybridizations were performed at E14.5, when early phenotypes were apparent, and at E17.5, when transdifferentiation was at an intermediate stage (see Fig. 1).

To better describe the patterns that were observed, the peripheral eye was subdivided into four separate zones (see Fig. 4A). Zone 1 is in the peripheral retina and normally does not express pigment markers. Zone 2 is peripheral to zone 1 and contains non-pigmented cells that directly abut pigmented cells. Zone 3 consists of pigmented cells at the periphery of the

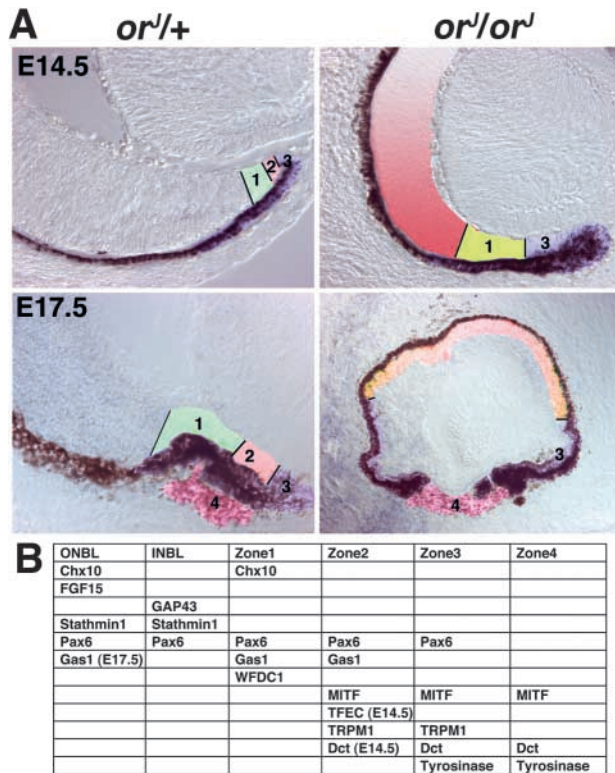


Fig. 4. Model of progressive transdifferentiation in the *or^J* mutant. (A) Representative sections through E14.5 or E17.5 embryos that were *or^J/+* heterozygotes or *or^J/or^J* homozygotes, as indicated, pseudocolored for expression of peripheral genes in zone 1 (green), zone 2 (red), zone 3 (blue), or zone 4 (magenta). Regions of tissue co-expressing zone 1 and zone 2 markers are pseudocolored as yellow/orange. (B) Tabular summary of some expression patterns shown in Figs 3, 4, S1 and S2 (supplementary material).

supplementary material). A number of these genes (*Neurod1*, *Btg2*, *cyclin D1* and *E2f1*) were identified as dependent on *Chx10*, by the microarray analysis as described above. Based on in situ hybridization analysis, RNA levels did not appear significantly altered within expressing cells, suggesting that the decrease of RNA levels was due to a decreased number of cells expressing these genes. Together, these data indicate a progressive loss of retinal progenitor cell identity and neurogenesis in the *or^J* mutant.

Most genes analyzed with ONBL expression patterns were not expressed in the zones of the peripheral eye in *or^J/+* mice, or were only expressed transiently in the peripheral retina (*E2f1*, *cyclin D1* and *cyclin E1*) with the exception of *Chx10*. To determine whether *Chx10* was differentially regulated in *or^J* mutants from other ONBL-expressed genes because of its peripheral expression, *Hes1*, a gene expressed similarly to *Chx10* was analyzed (see Fig. S1I in the supplementary material). *Hes1* expression in the *or^J* mutant behaved like that of *Chx10* rather than other ONBL-expressed genes (see Fig. S1I in the supplementary material), suggesting that peripheral gene expression was differentially affected in the *or^J* mutant.

Class II: INBL- and INBL+ONBL-expressed genes

The loss of expression of genes enriched in retinal progenitor

cells, as well as genes that play a role in neurogenesis, suggested that the genesis of retinal neurons might be affected in *or^J* mutant retinas. Since a number of genes found by microarray analysis to be downregulated in *or^J* mutants were markers of early-born neurons, we used in situ hybridization to examine genes expressed in the INBL. *Gap43*, *Brn3b* (*Pou4f2* – Mouse Genome Informatics) and *Snap25* were expressed in the INBL at E14.5 and E17.5 in *or^J/+* heterozygotes (Fig. 3C and Fig. S1J,K in the supplementary material). *Gap43* was restricted to only a few cells in the INBL in the central retina in *or^J* mutant retinas (Fig. 3C). Similar results were obtained for *Brn3b* and *Snap25* (see Fig. S1J,K in the supplementary material). This decrease in the number of cells expressing markers of retinal ganglion cells and amacrine cells correlated with the restricted location and reduced number of cells expressing ONBL markers, and was further indicated by the finding of a hypocellular INBL in *or^J* mutant retinas.

Stathmin 1, one of three stathmin family member genes observed by microarray analysis to be downregulated, was evaluated by in situ hybridization. In *or^J/+* eyes, *stathmin 1* was expressed throughout the INBL and in many ONBL cells at E14.5 and E17.5 (see Fig. S1L in the supplementary material). In *or^J* mutant retinas, *stathmin 1* was expressed in a similar fashion to the combination of *Gap43/Brn3b* and *Neurod1*, and in a much-reduced number of cells, which was especially apparent at E17.5 (see Fig. S1L in the supplementary material). The paired box transcription factor *Pax6* was expressed throughout the ONBL and INBL, and at high levels in zones 1, 2 and 3 (see Fig. S1M in the supplementary material). Unlike *stathmin 1*, *Pax6* appeared slightly upregulated in *or^J* mutant retinas and persisted even in pigmented regions. These data, like the comparison of *Chx10* and *Hes1* with other ONBL-expressed genes, suggest that the peripheral component of *Pax6* may have contributed to the high expression level of *Pax6* in the *or^J* mutant retina.

Class III: Peripherally-expressed genes

Gas1 and *p57* were both found to be significantly upregulated in the *or^J* mutant microarray analysis. In situ detection of *Gas1* at E14.5 in *or^J/+* eyes showed expression exclusively in the peripheral retina in zones 1 and 2, but not in pigmented cells (Fig. 3D). At E17.5, it remained highly expressed in these zones and was also expressed at low levels in the ONBL in *or^J/+* eyes. In *or^J* eyes, *Gas1* was highly upregulated in the peripheral retina and expressed at lower levels in more central parts (Fig. 3D). At E17.5, *Gas1* was highly upregulated throughout the entire *or^J* retina. Another gene also functioning as a cell cycle inhibitor, *p57*, was highly expressed in the peripheral retina and showed a similar expression pattern to *Gas1* in *or^J/+* and *or^J* eyes (see Fig. S1N in the supplementary material). A gene not represented on the microarrays, but with known important roles in ciliary body development, *Otx1*, was analyzed by in situ hybridization, and it too showed an expression pattern in *or^J/+* and *or^J* eyes highly similar to *Gas1* and *p57* (see Fig. S1O in the supplementary material).

To address the role of gene expression specific to zone 1, *Wfdc1*, a potential tumor suppressor gene and a peripheral retinal marker (J. Trimarchi and C.L.C., unpublished), was analyzed by in situ hybridization (Fig. 3E). The limits of *Wfdc1* expression defined zone 1 (Fig. 3E, arrows). In the *or^J* retina

Table 2. Genes downregulated in *or^J* mutants by microarray analysis

UNIQID	Name	E12.5 Average	E13.5 Average	Average	SD
AI852799	Synuclein γ	0.29	0.23	0.25	0.06
	Neurogenic differentiation 1 (Neurod1)	0.23	0.26	0.25	0.04
AI841966	Protein kinase, AMP-activated, γ 1 chain	0.33	0.23	0.26	0.06
AI840972	Stathmin-like 2	0.29	0.27	0.28	0.03
AI836519	ALL1-fused gene from chromosome 1q	0.31	0.29	0.3	0.05
AI841303	Growth associated protein 43 (Gap43)	0.33	0.39	0.37	0.06
AI835930	EST	0.28	0.46	0.4	0.12
AI848068	Microtubule-associated protein 1 B	0.44	0.38	0.4	0.06
AI851775	B-cell translocation gene 2, anti-proliferative (Btg2)	0.17	0.53	0.41	0.19
AI841267	Stathmin-like 3	0.57	0.34	0.42	0.14
	Neurogenic differentiation 4 (Math3)	0.43	0.44	0.43	0.07
AI848445	Melanoma antigen, family D, 1	0.44	0.43	0.44	0.08
AI843116	HMG-Coenzyme A synthase 1	0.44	0.48	0.46	0.06
AI849592	ELAV (Drosophila)-like 4 (Hu antigen D)	0.43	0.5	0.47	0.06
AI848983	Tubulin, α 8	0.47	0.5	0.49	0.08
AI848368	Synaptosomal-associated protein 25 (Snap25)	0.44	0.52	0.49	0.12
AI850105	Stathmin 1	0.56	0.46	0.49	0.14
AI841233	Potassium voltage-gated channel, Shaw-related, 4	0.51	0.49	0.5	0.06
AI846286	β -1,3-glucuronyltransferase 1	0.57	0.48	0.5	0.12
	Cyclin D1	0.35	0.59	0.51	0.17
	E2F1 (human)	0.78	0.88	0.84	0.09

Genes listed are those downregulated at least 0.5-fold, an arbitrary cut-off point. The last two genes are significantly downregulated, as determined by in situ hybridization analysis. Genes shown in bold were analyzed by in situ hybridization. Genes listed without unique IDs are full-length clones from within our laboratory clones. Note that the E2F-1 clone on the array was a full-length human cDNA. The average from E12.5 was determined from one hybridization and its color swap. The average from E13.5 was determined from two hybridizations and their color swaps. The total average was calculated from all six data points and is shown with its standard deviation (s.d.). Individual data points and Unigene numbers are included in Table S3.

at E14.5, *Wfdc1* expression levels were not significantly altered, but its expression was expanded centrally. At E17.5, *Wfdc1* expression was observed in some cells in the center of the *or^J* retina and throughout peripheral parts of the retina that had not yet undergone transdifferentiation (Fig. 3E, inset). Another peripherally-expressed gene, *Crhbp*, showed essentially the same expression pattern (see Fig. S1P in the supplementary material). *Crhbp* showed a small but significant upregulation by microarray analysis. This upregulation was prominent at E13.5 but not at E12.5, and like the in situ hybridization analysis, indicated a slow expansion of zone 1. A third marker of zone 1, *Igf2* (J. Trimarchi and C.L.C., unpublished), was altered in the *or^J* retina like *Wfdc1* and *Crhbp* (see Fig. S1Q in the supplementary material).

Class IV: RPE transcription factors

To examine changes in genes expressed in zones 2 and 3 and evaluate factors involved in controlling pigmentation, *Mitf*, *Tfec* and *Otx2* were examined by in situ hybridization. In *or^{J/+}* eyes, *Mitf* was expressed throughout the RPE at E14.5 and at higher levels in peripheral RPE in zone 3 and zone 2 as well (Fig. 3F). *Mitf* was also expressed in migrating melanoblasts and in zone 4 at E17.5. *Mitf* expression abutted *Chx10* in the peripheral retina at E14.5 and E17.5, and was never detected in wild-type retinas. In the *or^J* retina, *Mitf* was ectopically expressed at high levels in the retina, in a manner similar to *Gas1*, while there was a lessened expression in the center. However, by E17.5, *Mitf* was highly upregulated throughout the entire *or^J* retina (Fig. 3F). *Tfec* was the most upregulated gene as evaluated by microarray analysis. At E14.5, in *or^{J/+}* eyes, *Tfec* was barely detectable in zone 2 and was not detected at E17.5 (apparent staining is likely to be background) (Fig. 3G). In the *or^J* retina, *Tfec* was ectopically expressed in the retina, but more peripherally than *Mitf* and at lower levels. At

E17.5, *Tfec* was moderately expressed throughout the entire retina in *or^J* eyes. In *or^{J/+}* eyes (Fig. 3G), *Otx2* was expressed in both the ONBL and RPE, including zone 3, at E14.5, but not the peripheral retina (see Fig. S1R in the supplementary material). The *Otx2* expression pattern mimicked that of ONBL-specific genes in the *or^J* retina, except that *Otx2* was detected in the periphery in lightly pigmented cells in the peripheral retina only at E14.5.

Activation of *Mitf* target genes in transdifferentiation

To address whether ectopic *Mitf* and *Tfec* in the *or^J* retina was sufficient to activate their cognate target genes, *Mitf* target genes were analyzed by in situ hybridization at E17.5, during an intermediate stage of transdifferentiation (Fig. 5). *Trpm1* is a direct target of *Mitf* and depends on *Mitf* for expression in the developing RPE. Like *Mitf*, *Trpm1* was expressed in zone 2 in *or^{J/+}* heterozygotes and was ectopically expressed throughout the retina in *or^J* mutants, including unpigmented cells (Fig. 5A). In contrast, *Dct* was highly expressed in pigmented cells in *or^J* retinas as well as in regions of non-pigmented cells in the central retina. Its expression correlated with regions that had lost neural retinal identity, but had not yet undergone pigmentation, and complemented the expression of ONBL and INBL markers (Fig. 5B). *Tyrosinase*, the rate-limiting enzyme in melanin synthesis, was only expressed in pigmented cells in both *or^{J/+}* heterozygotes and *or^J* mutant retinas (Fig. 5C). Therefore, different classes of *Mitf* target genes define a progressive program of transdifferentiation beginning with direct *Mitf* target genes like *Trpm1*, then proceeding to express *Dct*, and finally *tyrosinase*, coinciding with the appearance of pigment. This ordered gene expression progression was also observed at E14.5 in *or^J* mutant retinas (see Fig. S2A-C in the supplementary material).

While RPE genes, including *Mitf* and melanin synthesis

enzymes, were generally not represented on the microarray, one that was on the array and showed *or^J* upregulation was *Cd63* (a melanoma antigen in humans). By in situ hybridization, *Cd63* was upregulated in the *or^J* retinas, similarly to *Trpm1* (see Fig. S2D,E in the supplementary material), and was dramatically downregulated in *Mitf*-deficient RPE, especially at E14.5 when *Tfec* was only weakly expressed (see Fig. S3A,C in the supplementary material), suggesting it is a direct target of *Mitf* and possibly *Tfec*. In contrast, *Tfec* expression was not dependent on *Mitf* (see Fig. S3B in the supplementary material), and *Otx2* was neither upregulated in the *or^J* retina, nor was it altered in *Mitf*-deficient RPE (see Fig. S3E in the supplementary material). Other genes expressed in zone 1 and zone 2, including *p57*, *Pax6* and *Crhbp* were also unaffected in the peripheral retina in *Mitf* nullizygous mice (see Fig. S3F-H in the supplementary material), suggesting their upregulation in the *or^J* retina was a consequence of their peripheral expression and not indirectly through *Mitf*.

Chx10 is sufficient to repress the expression of RPE genes

Microarray and in situ hybridization analysis pointed to *Mitf* family members as possible key target genes for *Chx10* regulation. To determine if the expression of *Chx10* could lead to repression of these genes, *Chx10* was misexpressed in the developing RPE in chick by in ovo electroporation. A plasmid encoding chick *Chx10*, along with another plasmid encoding GFP as a cotransfection reporter, were transduced into early chick optic vesicles by electroporation, and embryos were allowed to develop for 1 day in ovo (Fig. 6A). Non-transfected optic vesicles from the same embryo (Fig. 6B) or electroporations with GFP alone (Fig. 6C) were used as controls and showed identical marker expression (compare Fig. 6F,I,L,O,R,U to Fig. 6E,H,K,N,Q,T).

Electroporation of *Chx10* resulted in a strong downregulation of *Mitf* (Fig. 6G) and *Tfec* (Fig. 6J). Additionally, a direct target gene of *Mitf* in chick RPE, *Mmp115*, and an additional RPE marker *Dct*, were also strongly downregulated upon *Chx10* misexpression (Fig. 6D,M). *Mmp115* was analyzed in sections and showed strong downregulation only in the transfected eye (Fig. 6W,X) and within regions of the RPE that were electroporated, as judged by GFP antibody staining. The retinal progenitor cell markers *Six3* (Fig. 5P) and *Chx10* (Fig. 5S) were unaffected by *Chx10* misexpression. The restriction of *Six3* and *Chx10* to the neural retina also confirmed that transdifferentiation was not occurring upon *Chx10* misexpression, in contrast to misexpression of other retinal homeobox transcription factors in the RPE (C.-M.A.C. and C.L.C., unpublished) (Toy et al., 1998). These data place *Mitf* and *Tfec* as potentially direct target genes for *Chx10* repression in the absence of a direct cell fate change.

Discussion

Transdifferentiation of retina to pigment cells in the *or^J* mutant

The data presented here demonstrate that the retina in the *or^J* mutant mouse directly transdifferentiates into pigmented cells. This is the first description of retinal tissue becoming pigmented in vivo, whereas a number of studies have shown

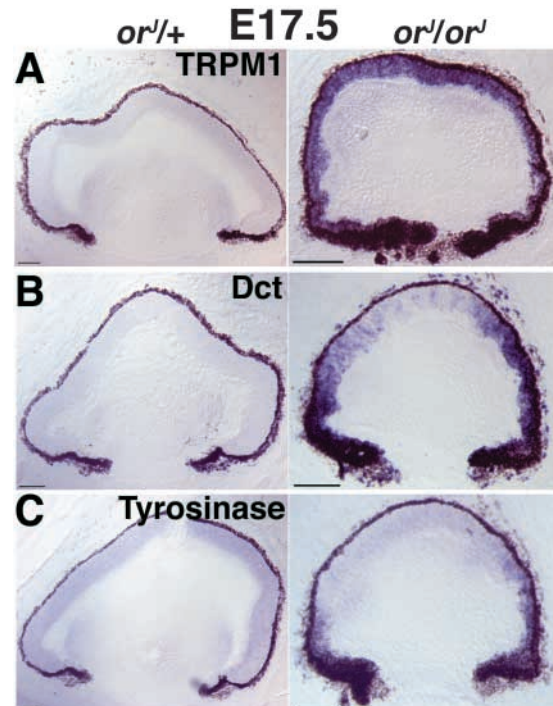


Fig. 5. In situ hybridization analysis of *Mitf* target genes in *or^J* mutants. (A–C) Sections through E17.5 *or^J/+* heterozygous or *or^J/or^J* homozygous littermates, as indicated, were analyzed for the following RNAs: (A) *Trpm1*, (B) *Dct*, (C) *Tyrosinase*. Full expression patterns are discussed in Results and summarized in Fig. 4. Note scale bar differences between *or^J/+* and *or^J/or^J* sections. All scale bars are 100 μ m.

the opposite transition. It is difficult to know whether transdifferentiation in either direction is a physiologically relevant process, or simply the result of aberrant development. There is some suggestion that transdifferentiation of RPE could serve as a source of retinal cells in regeneration (Fischer and Reh, 2001), but there is no evidence to suggest that retinal cells ever change their identity to pigmented cells. Rather, this transdifferentiation seems to be specific to loss of *Chx10*. Nonetheless, individuals with diseases of the RPE might benefit from this observation when engraftment of RPE cells is being considered.

Several lines of evidence support the notion that the neural retina directly transdifferentiates into pigmented cells. However, the additional expression domain of *Chx10* in the nonpigmented ciliary body epithelium raises the possibility that the transdifferentiation observed is one of nonpigmented ciliary body cells into pigmented cells. In this model, the neural retina would die and be replaced with an expanding population of pigment-fated cells. The fate mapping analysis and extensive expression studies presented here do not support this alternative model. Firstly, in situ hybridizations and microarray data do not indicate that pigment-fated cells undergo the rapid proliferation required to populate the *or^J* retina, nor do they indicate abnormally high rates of cell death in the neural retina. Specifically, markers of proliferation, including *E2f1*, *cyclin E1*, and *cyclin D1*, were downregulated in regions of the *or^J* retina undergoing pigmentation, while cell cycle inhibitors,

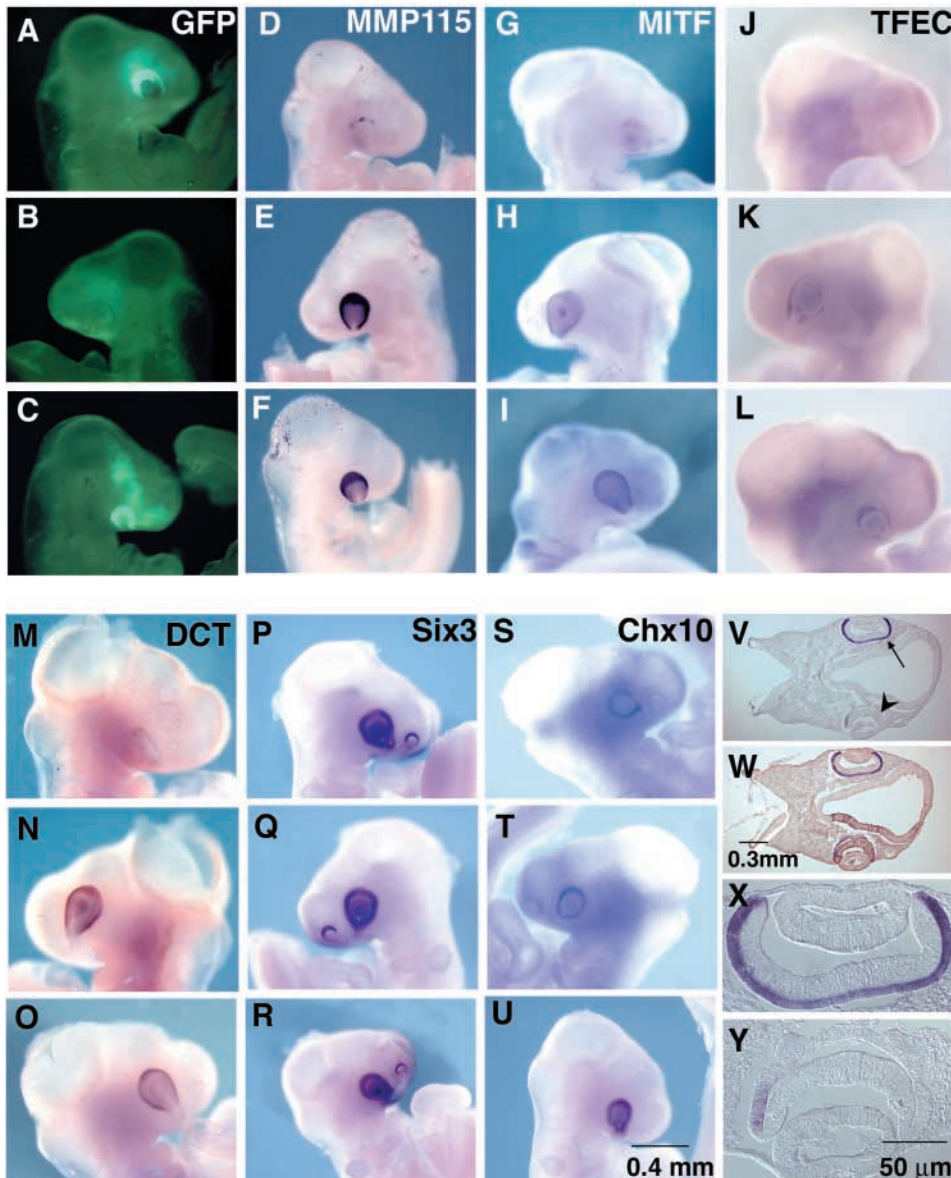


Fig. 6. Misexpression of *Chx10* by in ovo electroporations. (A-U) HH Stage 10 embryos were electroporated with GFP and *Chx10* (A,D,G,J,M,P,S) or GFP alone (C,F,I,L,O,R,U) and allowed to develop an additional day. (B,E,H,K,N,Q,T) Images are from the left non-electroporated eye of the same embryo shown in (A,D,G,J,M,P,S) respectively. (A-C) HH16-18 embryos were harvested and photographed for GFP to observe transfected regions. (D-U) HH16-18 embryos were harvested and whole-mount in situ hybridization was performed to detect the following RNAs: (D-F) *Mmp115*, (G-I) *Mitf*, (J-L) *Tfec*, (M-O) *Dct*, (P-R) *Six3*, (S-T) *Chx10* 3'UTR, (U) *Chx10*. (V-Y) Sections through the *Chx10* and GFP electroporated embryo detected for *Mmp115* (D,E). (V) *Mmp115* RNA was detected in the RPE of the eye that was not electroporated (arrow, high magnification image shown in (X)), but was highly reduced in the eye electroporated with GFP and *Chx10* (arrowhead, high magnification image shown in (Y)). (W) Section from (V) further stained with an antibody against GFP to show the region of the embryo that was electroporated (brown stain), including the retina and RPE in the bottom eye.

including *p57* and *Gas1*, were concomitantly upregulated in these regions. Secondly, analysis of markers from the peripheral retina, particularly in zone 2, showed their expansion into parts of the retina that also expressed bona fide neural retina markers well before transdifferentiation was observed. The overall temporal pattern of gene expression and high number of fate-mapped pigmented cells even at E17.5 support the direct transdifferentiation of neural retinal cell into pigmented cells.

A molecular program of transdifferentiation

Genes expressed in either the RPE or different peripheral regions of the retina were upregulated in the *or^J* mutant retina and were identifiable by microarray analysis. By analyzing candidate genes and other genes with known expression patterns by in situ hybridization, we have formed a model for transdifferentiation that also possibly explains why it occurs progressively (Fig. 4A). The model designates zones of gene expression for purposes of description,

although as discussed below, these zones may represent functional compartments (except zone 4, which is not part of the neuroepithelium). Fig. 4B is a tabular summary of some of the genes presented in this study (Fig. 3, Fig. 5 and Fig. S1,S2 in the supplementary material) and their inclusion in the different zones. The expansion and ectopic expression of genes normally restricted to zone 2 of the peripheral retina appears to be an early event in the *or^J* retina. The upregulation, however, was not uniform and was stronger in the periphery than the center. While *Mitf*, and some of its target genes, showed broad upregulation, other melanogenic genes were restricted to peripheral regions, and thus pigmentation did not occur in all *Mitf*-expressing cells. Where zone 1 genes and zone 2 genes then began to overlap (shown as yellow in Fig. 4A), *Dct* expression became pronounced. Shortly thereafter, *tyrosinase* was expressed and the tissue became pigmented and adopted a zone 3-like appearance. Concurrent with pigmentation was a downregulation of genes expressed in zone 1, as well as *Gas1* and *p57*. The next progression occurred within zone 1 which began to expand centrally. As new parts of the retina began coexpressing zone 1 and zone 2 genes, *Dct* expression expanded centrally and marked non-pigmented cells programmed to initiate pigmentation. The cells did not initiate pigmentation until tyrosinase, the rate-limiting enzyme in melanin production, became expressed.

It is striking how the progression of transdifferentiation occurred so slowly. Even as late as P17, there were still regions of the retina just beginning to undergo pigmentation (see Fig. 2L). The rate-limiting step appears related to the expansion of zone 1, as zone 2 completely expanded throughout the retina well before pigmentation reached this area. The central boundary of zone 1 was defined by the expression of progenitor cell genes in the ONBL. The downregulation of ONBL-expressed genes was observed in the peripheral retina prior to those regions acquiring pigmentation, and by E17.5, the entire retina appeared to lose neural identity. These events may precipitate loss of the central boundary of zone 1 and lead to the expansion of zone 1 into central parts of the retina. Determination of the ONBL-expressed genes that are responsible for defining the boundary with zone 1 will yield interesting insights into the mechanism of compartmentalization of the retina.

Genetic modifiers of the *or^J* phenotype

Modifiers of the *or^J* phenotype have been described that partially rescue eye size and lamination. In neither case was the peripheral *or^J* phenotype rescued (Bone-Larson et al., 2000; Green et al., 2003). This raised the possibility that another factor could compensate for some Chx10 functions in the central but not peripheral retina. One would then predict that in the genetic background employed here, the compensating factor was detrimentally affected, leading to a more severe phenotype. A candidate gene for this function is the *Chx10* homolog, *Vsx-1*. *Vsx-1* in other organisms including chick and zebrafish is co-expressed with *Chx10* in retinal progenitor cells, but has not been detected in mammalian progenitor cells (Chen and Cepko, 2000; Chow et al., 2001; Passini et al., 1997). We performed in situ analysis for *Vsx-1* and did not see it expressed in either *or^J/+* mice or *or^J* mice on any background, probably ruling out *Vsx-1* as a modifying allele (data not shown).

An alternative possibility for a genetic modifier relates to the extent of upregulation of *Mitf* and other zone 2 genes. *Mitf* is normally not expressed at any level in the retina, thus *Mitf* upregulation in the *or^J* mutant may be influenced by genetic modifiers in terms of the spatial limits of *Mitf* expression or the absolute quantity of RNA. One might then predict that *Mitf* in rescued *or^J* mice might only be ectopically expressed in the periphery and/or may be expressed at lower levels. A further prediction is that broad and strong overexpression of *Mitf* in a mildly affected *or^J* strain would lead to a more dramatic phenotype. Interestingly, *Mitf* was one of the candidate genes preliminarily identified by Bone-Larson and colleagues as a modifier of the *or^J* phenotype (Bone-Larson et al., 2000).

Another candidate gene that might control the degrees of severity of the *or^J* transdifferentiation phenotype is the *Mitf*-related transcription factor *Tfec*. Normally, *Tfec* is only transiently expressed in the RPE and zone 2, and its ectopic expression in the retina along with *Mitf* might have distinct consequences. It is also notable that *Tfec* appeared to be upregulated less centrally than *Mitf* at E14.5, possibly marking the region of the retina actively undergoing transdifferentiation. *Gas1* is also an interesting candidate, not only because of its characterized role as a cell cycle inhibitor, but also its functions in the RPE. Mice lacking *Gas1* show transdifferentiation of the ventral RPE to neural retina,

suggesting a role for *Gas1* in ventral RPE identity (Lee et al., 2001). *Mitf* mutant mice also show transdifferentiation of RPE to neural retina, but this occurs only dorsally (Bumsted and Barnstable, 2000). Thus, the concurrent expression of *Gas1* and *Mitf* in the retina of *or^J* mice may have a distinct effect on cell fate, functioning in an opposite direction to their loss-of-function phenotypes. Finally, there remains the possibility that mesenchymal cells play a role in controlling the spatial and temporal features of transdifferentiation, and these functions could be altered by genetic background.

Transcriptional targets of Chx10

One of the goals of *or^J* microarray analysis was to identify genes that may be directly controlled by Chx10. If Chx10 functioned as a transcriptional activator, one would predict that direct target genes would be downregulated in the *or^J* retina. A large number of downregulated transcripts were observed by microarray analysis, but the ones studied in greater detail by in situ hybridization were not necessarily downregulated on a per cell transcript level, but rather through a reduction in the number of cells expressing them. Many of these downregulated genes were markers of neurogenesis and early differentiation. Further characterization of genes downregulated in the *or^J* retina may give new insights into this molecular program.

Genes upregulated in the *or^J* retina were the focus of this study and are candidate targets for repression by Chx10, e.g. *Mitf*, *Tfec*, *Gas1*, *p57* and *Otx1*. However, some upregulated genes are likely targets of *Mitf*-dependent activation rather than repression by Chx10. Genes like *Trpm1* and *Cd63* are probably upregulated through *Mitf* transcription. *Trpm1*, in particular, is transcriptionally activated in a concentration-dependent fashion by *Mitf* and is not expressed in *Mitf* mutant RPE (Miller et al., 2004). In this respect, analysis of *or^J* mice by microarray analysis would be an ideal way to identify new target genes of *Mitf* and *Tfec*, provided the array had a large set of RPE genes. Neither *Gas1*, *p57* nor *Tfec* require *Mitf* for their expression and therefore their misexpression in *or^J* mice is probably secondary to, or independent of, *Mitf* expression (see Fig. S3 in the supplementary material). However, *p57* is expressed elsewhere in the retina in subsets of progenitor cells, and *Gas1* is expressed in the ONBL at E17.5 in cells that potentially express *Chx10*, so *Gas1* and *p57* may not be ideal candidate target genes for repression by Chx10.

The finding that misexpression of *Chx10* in developing chick RPE led to a depigmentation and downregulation of *Mitf*, *Tfec* and other pigment markers further supports the notion that *Mitf* and *Tfec* are direct targets of Chx10. Overexpression of a number of transcription factors in the RPE (e.g. *Six3*, *Six6* and *Rax/Rx*) can lead to depigmentation, but this usually occurs via transdifferentiation of the RPE to retina (C-M.A.C. and C.L.C., unpublished) (Toy et al., 1998). RPE made to express Chx10 seemed to maintain proper RPE identity, at least as judged by the lack of conversion to a retinal identity as viewed through expression of *Six3* and *Chx10*. These data further suggest that the aberrant expression of *Mitf*, *Tfec* and their target genes was not secondary to alterations in the center-periphery pattern also observed in the *or^J* mutant. *Mitf* has been implicated as a Chx10 target gene previously, based on its temporal and spatial expression in early eye development. The boundary between *Chx10* and *Mitf* persists throughout eye development (discussed further below). An attractive model then is that

Chx10 functions in the optic vesicle to repress *Mitf* and permanently keep it repressed in the retina. This repression does not require continuous *Chx10* expression, as mature retinal cell types that lose *Chx10* expression do not ever express *Mitf*. Possible derepression of *Mitf* in the optic vesicle in *or^J* mice may help explain the broad ectopic expression of *Mitf* in the retina of these mutants. Identification of a physiologically meaningful Chx10 binding site in *Mitf* regulatory regions is a necessary next step in testing this hypothesis of direct regulation by Chx10.

Insights into other *Chx10* mutant phenotypes

The results of this study provide new insights into a number of phenotypes observed in mice and humans lacking Chx10 function. Human patients with mutations in *Chx10* present with microphthalmia, cataracts and iris abnormalities (Percin et al., 2000). While microphthalmia is expected, based upon the earlier characterization of the *or^J* phenotype, the anterior eye phenotypes were unexplained. Conversion of nonpigmented ciliary body epithelium into pigmented tissue might disrupt formation and function of the ciliary body and iris. Even in the *or^J* animals rescued by a *Mus musculus castaneus* genetic background, the ciliary body did not form properly, although the iris partially developed (Bone-Larson et al., 2000). Overt hyperpigmentation was not observed in these eyes, suggesting that Chx10 may play a direct role in differentiation of the ciliary body where it is expressed in the presumptive nonpigmented ciliary body epithelium.

An intriguing observation made in *or^J* mice was the presence of a five-fold increase in the number of retinal stem cells compared to wild-type counterparts (Tropepe et al., 2000). Retinal stem cells derive from cells in the pigmented ciliary body in mammals, which may be a homolog of the ciliary marginal zone of fish and amphibians (Perron and Harris, 2000). It was proposed that retinal progenitor cells might negatively regulate the number of stem cells in the retina, and as a consequence of fewer progenitor cells in the *or^J* retina, more stem cells would be produced. Another possibility might be that the retinal cells that transdifferentiated into pigment cells form a larger niche for retinal stem cells, or themselves become retinal stem cells. The possibility that retinal progenitor cells could become retinal stem cells via transdifferentiation opens up new experimental possibilities in studying retinal stem cells.

Compartment boundaries in the peripheral retina

The study of compartment boundaries has been elegantly conducted in *Drosophila* using classical genetics and molecular markers (see Dahmann and Basler, 1999; Wolpert, 2003). Much less is known in vertebrate systems about where compartments are found and how they are regulated. Several aspects of the data presented here provide evidence for compartment boundaries in the peripheral retina. First, early gene expression patterns in the peripheral retina were largely maintained. For example, *Notch1*, *Fgf15*, and several bHLH factors examined were never expressed in the peripheral zones in the retina. *Wfdc1* also was never expressed outside of zone 1, although *Crhbp* and *Igf2* showed some zone 2 expression. Not only was the spatial organization maintained, but most markers of the periphery remained expressed in the mature ciliary body. Second, a combination of expression and fate-

mapping analysis of *Chx10* at the periphery revealed it to be expressed in zone 1 throughout development. Fate mapping analysis using R26R in wild type or *or^{J/+}* heterozygotes showed that labeled cells stayed within their boundaries and labeled cells were never observed to be pigment cells. This finding strongly implicates a boundary between future pigmented and nonpigmented cells within an initially nonpigmented population, and suggests that even if some cell mixing occurs, the eventual fate of a cell is still lineage-fixed relative to its initial specification. Third, loss of function of Chx10 in the *or^J* mutant caused normally peripherally restricted genes to become expanded to the center of the retina. Therefore, Chx10 function appears linked to maintaining at least one important boundary in the peripheral retina. Strikingly, the most peripherally expressed genes, in zone 2, expanded more dramatically and temporally before zone 1 cells in the *or^J* mutant. This finding implies that formation and/or maintenance of the boundary between zone 1 and zone 2 requires Chx10.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/131/20/5139/DC1>

References

- Baumer, N., Marquardt, T., Stoykova, A., Spieler, D., Treichel, D., Ashery-Padan, R. and Gruss, P. (2003). Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6. *Development* **130**, 2903-2915.
- Belecky-Adams, T., Tomarev, S., Li, H. S., Ploder, L., McInnes, R. R., Sundin, O. and Adler, R. (1997). Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest. Ophthalmol. Vis. Sci.* **38**, 1293-1303.
- Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W. P., Fraioli, R. E., Choi, S.-H., Yung, R., Asch, E. et al. (2004). Genomic analysis of mouse retinal development. *PLoS Biol.* **2**, e247.
- Bone-Larson, C., Basu, S., Radcliff, J. D., Liang, M., Perozek, T., Kapousta-Bruneau, N., Green, D. G., Burmeister, M. and Hankin, M. H. (2000). Partial rescue of the ocular retardation phenotype by genetic modifiers. *J. Neurobiol.* **42**, 232-247.
- Bora, N., Conway, S. J., Liang, H. and Smith, S. B. (1998). Transient overexpression of the Microphthalmia gene in the eyes of Microphthalmia vitiligo mutant mice. *Dev. Dyn.* **213**, 283-292.
- Bumsted, K. M. and Barnstable, C. J. (2000). Dorsal retinal pigment epithelium differentiates as neural retina in the microphthalmia (mi/mi) mouse. *Invest. Ophthalmol. Vis. Sci.* **41**, 903-908.
- Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. I. et al. (1996). Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat. Genet.* **12**, 376-384.
- Chen, C. M. and Cepko, C. L. (2000). Expression of Chx10 and Chx10-1 in the developing chicken retina. *Mech. Dev.* **90**, 293-297.

- Chen, C. M. and Cepko, C. L. (2002). The chicken RaxL gene plays a role in the initiation of photoreceptor differentiation. *Development* **129**, 5363-5375.
- Chow, R. L. and Lang, R. A. (2001). Early eye development in vertebrates. *Annu. Rev. Cell Dev. Biol.* **17**, 255-296.
- Chow, R. L., Snow, B., Novak, J., Looser, J., Freund, C., Vidgen, D., Ploder, L. and McInnes, R. R. (2001). Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. *Mech. Dev.* **109**, 315-322.
- Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet.* **15**, 320-326.
- Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat. Genet.* **34**, 53-58.
- Fischer, A. J. and Reh, T. A. (2001). Transdifferentiation of pigmented epithelial cells: a source of retinal stem cells? *Dev. Neurosci.* **23**, 268-276.
- Fischer, A. J. and Reh, T. A. (2003). Growth factors induce neurogenesis in the ciliary body. *Dev. Biol.* **259**, 225-240.
- Gouge, A., Holt, J., Hardy, A. P., Sowden, J. C. and Smith, H. K. (2001). Foxn4 – a new member of the forhead gene family is expressed in the retina. *Mech. Dev.* **107**, 203-206.
- Graw, J. (2003). The genetic and molecular basis of congenital eye defects. *Nat. Rev. Genet.* **4**, 876-888.
- Green, E. S., Stubbs, J. L. and Levine, E. M. (2003). Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. *Development* **130**, 539-552.
- Guillemot, F. and Cepko, C. L. (1992). Retinal fate and ganglion cell differentiation are potentiated by acidic FGF in an in vitro assay of early retinal development. *Development* **114**, 743-754.
- Hodgkinson, C. A., Moore, K. J., Nakayama, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A. and Arnheiter, H. (1993). Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* **74**, 395-404.
- Koniukhov, B. V. and Sazhina, M. V. (1966). Interaction of the genes of ocular retardation and microphthalmia in mice. *Folia Biol. (Praha)* **12**, 116-123.
- Koniukhov, B. V. and Sazhina, M. V. (1985). Interaction of mutant white, aphakia and ocular retardation genes in the mouse. *Izv. Akad. Nauk. SSSR Biol.* **722-730**.
- Koniukhov, B. V. and Ugol'kova, T. S. (1978). Site of fidget gene action and its interaction with the ocular retardation gene in cultured mouse embryo retinas. *Ontogenes* **9**, 475-480.
- Kubo, F., Takeichi, M. and Nakagawa, S. (2003). Wnt2b controls retinal cell differentiation at the ciliary marginal zone. *Development* **130**, 587-598.
- Kwan, C. T., Tsang, S. L., Krumlauf, R. and Sham, M. H. (2001). Regulatory analysis of the mouse Hoxb3 gene: multiple elements work in concert to direct temporal and spatial patterns of expression. *Dev. Biol.* **232**, 176-190.
- Lee, C. S., May, N. R. and Fan, C. M. (2001). Transdifferentiation of the ventral retinal pigmented epithelium to neural retina in the growth arrest specific gene 1 mutant. *Dev. Biol.* **236**, 17-29.
- Liu, I. S., Chen, J. D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V. I. and McInnes, R. R. (1994). Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. *Neuron* **13**, 377-393.
- Livesey, F. J., Furukawa, T., Steffen, M. A., Church, G. M. and Cepko, C. L. (2000). Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene Crx. *Curr. Biol.* **10**, 301-310.
- Livesey, F. J., Young, T. L. and Cepko, C. L. (2004). An analysis of the gene expression program of mammalian neural progenitor cells. *Proc. Natl. Acad. Sci. USA* **101**, 1374-1379.
- Martinez-Morales, J. R., Dolez, V., Rodrigo, I., Zaccarini, R., Lecante, L., Bovolenta, P. and Saule, S. (2003). OTX2 Activates the Molecular Network Underlying Retina Pigment Epithelium Differentiation. *J. Biol. Chem.* **278**, 21721-21731.
- Martinez-Morales, J. R., Signore, M., Acampora, D., Simeone, A. and Bovolenta, P. (2001). Otx genes are required for tissue specification in the developing eye. *Development* **128**, 2019-2030.
- Miller, A. J., Du, J., Rowan, S., Hershey, C. L., Widlund, H. R. and Fisher, D. E. (2004). Transcriptional regulation of the melanoma prognostic marker melastatin (TRPM1) by MITF in melanocytes and melanoma. *Cancer Res.* **64**, 509-516.
- Mochii, M., Mazaki, Y., Mizuno, N., Hayashi, H. and Eguchi, G. (1998). Role of Mitf in differentiation and transdifferentiation of chicken pigmented epithelial cell. *Dev. Biol.* **193**, 47-62.
- Murtaugh, L. C., Chyung, J. H. and Lassar, A. B. (1999). Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* **13**, 225-237.
- Nakayama, A., Nguyen, M. T., Chen, C. C., Opdecamp, K., Hodgkinson, C. A. and Arnheiter, H. (1998). Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. *Mech. Dev.* **70**, 155-166.
- Nguyen, M. and Arnheiter, H. (2000). Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development* **127**, 3581-3591.
- Osipov, V. V. and Vakhrusheva, M. P. (1983). Variation in the expressivity of the ocular retardation gene in mice. *Tsitol. Genet.* **17**, 39-43.
- Park, C. M. and Hollenberg, M. J. (1989). Basic fibroblast growth factor induces retinal regeneration in vivo. *Dev. Biol.* **134**, 201-205.
- Passini, M. A., Levine, E. M., Canger, A. K., Raymond, P. A. and Schechter, N. (1997). Vsx-1 and Vsx-2: differential expression of two paired-like homeobox genes during zebrafish and goldfish retinogenesis. *J. Comp. Neurol.* **388**, 495-505.
- Percin, E. F., Ploder, L. A., Yu, J. J., Arici, K., Horsford, D. J., Rutherford, A., Bapat, B., Cox, D. W., Duncan, A. M., Kalnins, V. I. et al. (2000). Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. *Nat. Genet.* **25**, 397-401.
- Perron, M. and Harris, W. A. (2000). Retinal stem cells in vertebrates. *Bioessays* **22**, 685-688.
- Pittack, C., Jones, M. and Reh, T. A. (1991). Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina in vitro. *Development* **113**, 577-588.
- Rehli, M., Lichanska, A., Cassidy, A. I., Ostrowski, M. C. and Hume, D. A. (1999). TFEC is a macrophage-restricted member of the microphthalmia-TFE subfamily of basic helix-loop-helix leucine zipper transcription factors. *J. Immunol.* **162**, 1559-1565.
- Rowan, S. and Cepko, C. L. (2004). Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. *Dev. Biol.* **271**, 388-402.
- Schulte, D., Furukawa, T., Peters, M. A., Kozak, C. A. and Cepko, C. L. (1999). Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. *Neuron* **24**, 541-553.
- Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
- Toy, J., Yang, J. M., Leppert, G. S. and Sundin, O. H. (1998). The optx2 homeobox gene is expressed in early precursors of the eye and activates retina-specific genes. *Proc. Natl. Acad. Sci. USA* **95**, 10643-10648.
- Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R. and van der Kooy, D. (2000). Retinal stem cells in the adult mammalian eye. *Science* **287**, 2032-2036.
- Wolpert, L. (2003). Cell boundaries: knowing who to mix with and what to shout or whisper. *Development* **130**, 4497-4500.
- Zhao, S., Hung, F. C., Colvin, J. S., White, A., Dai, W., Lovicu, F. J., Ornitz, D. M. and Overbeek, P. A. (2001). Patterning the optic neuroepithelium by FGF signaling and Ras activation. *Development* **128**, 5051-5060.
- Zhao, S., Thornquist, S. C. and Barnstable, C. J. (1995). In vitro transdifferentiation of embryonic rat retinal pigment epithelium to neural retina. *Brain Res.* **677**, 300-310.