

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).

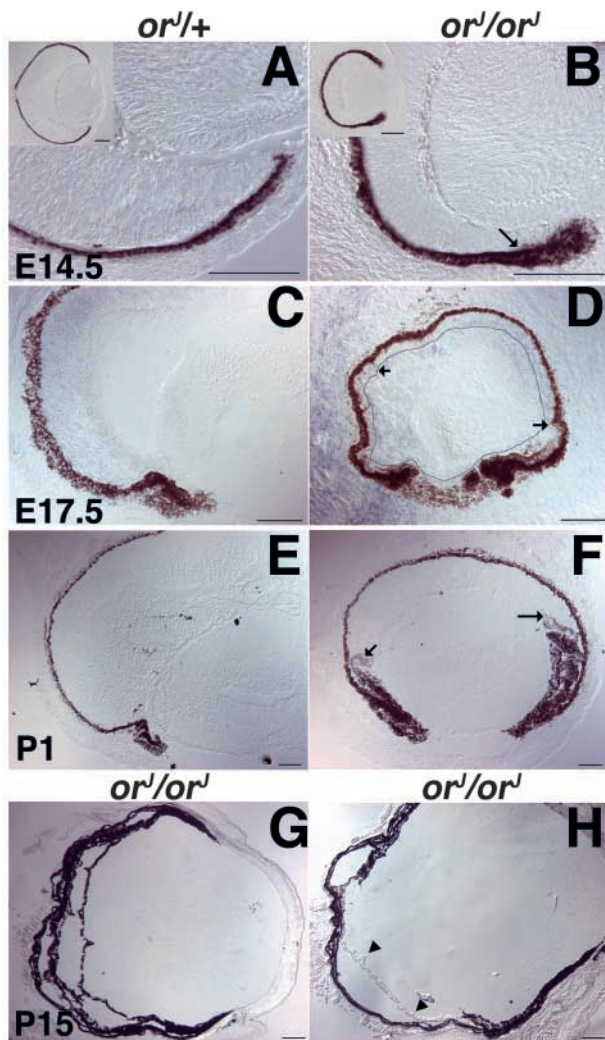


Fig. 1. Progressive pigmentation in *or^J* retinas. (A–H) Eyes were sectioned to analyze for pigmentation in *or^J/+* heterozygous (A, C, E) or *or^J/or^J* homozygous (B, D, F, G, H) littermates. Ages are indicated on the figure. (A, B) Insets show a full section of the eye (note scale bar differences). (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

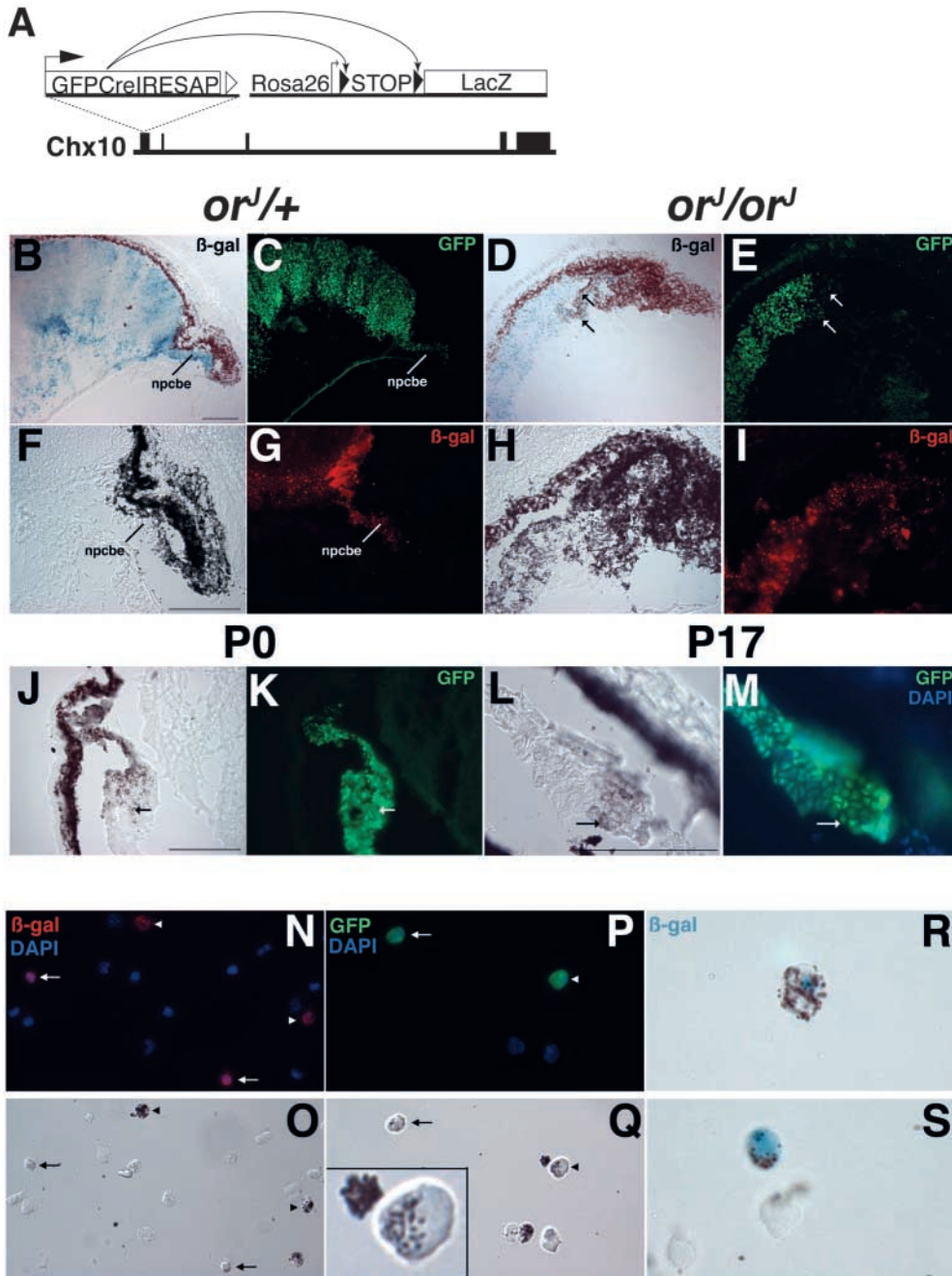


Fig. 2. Direct transdifferentiation of *or^J* retinal cells into pigmented cells. (A) Schematic of the transgenic mice used for fate mapping experiments. The *Chx10* BAC transgenic mouse directs expression of *GFP* and *AP* under control of *Chx10* regulatory sequences. This reporter is integrated in 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

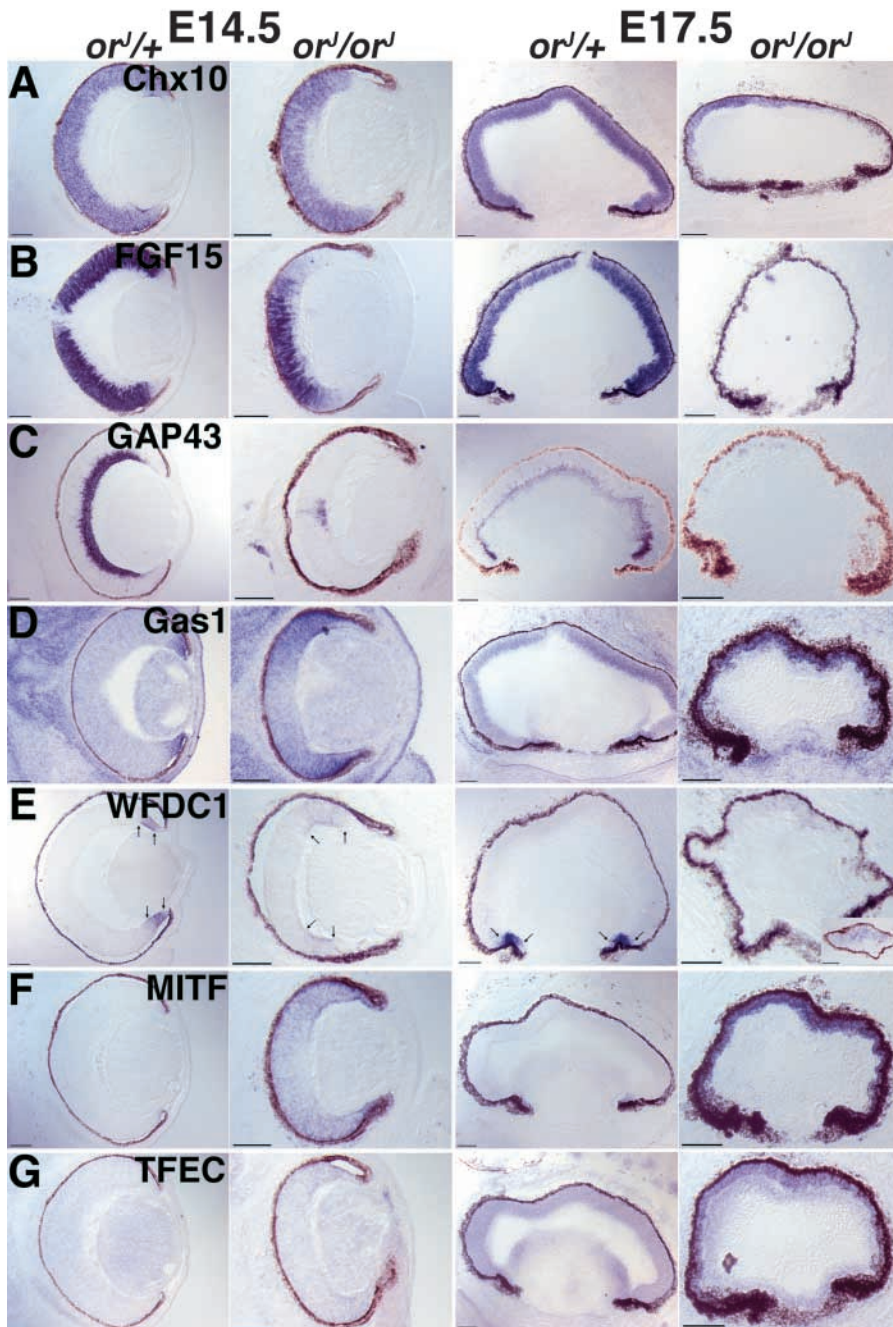


Fig. 3. In situ hybridization analysis of genes affected in *or^J* mutants. (A-G) Sections through E14.5 or E17.5 *or^{J/+}* heterozygous or *or^{J/or^J}* homozygous littermates, as indicated, were analyzed for the following RNAs: (A) *Chx10*, (B) *Fgf15*, (C) *Gap43*, (D) *Gas1*, (E) *Wfcd1*, (F) *Mitf*, (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

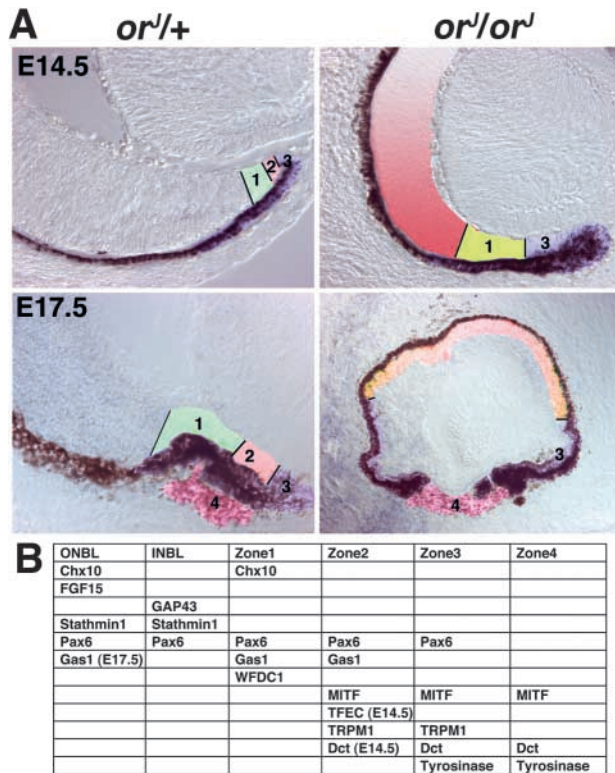


Fig. 4. Model of progressive transdifferentiation in the *or^J* mutant. (A) Representative sections through E14.5 or E17.5 embryos that were *or^J/+* heterozygotes or *or^J/or^J* homozygotes, as indicated, pseudocolored for expression of peripheral genes in zone 1 (green), zone 2 (red), zone 3 (blue), 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

UNIQUID	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

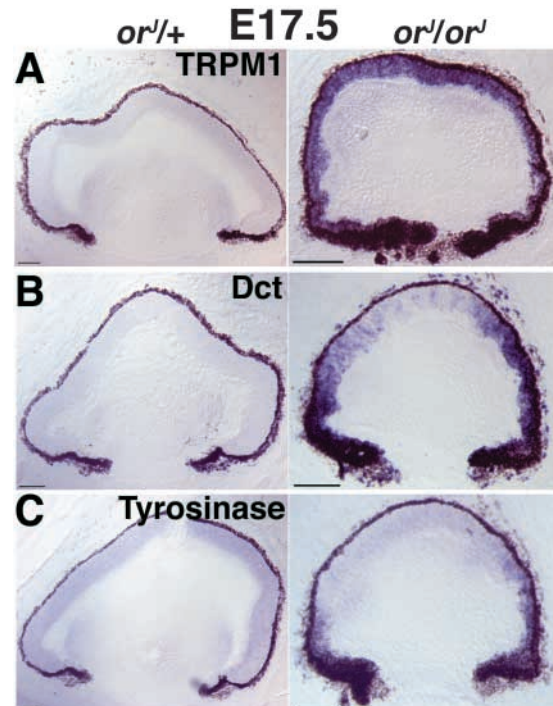


Fig. 5. In situ hybridization analysis of *Mitf* target genes in *or^J* mutants. (A–C) Sections through E17.5 *or^J/+* heterozygous or *or^J/or^J* homozygous littermates, as indicated, were analyzed for the following RNAs: (A) *Trpm1*, (B) *Dct*, (C) *Tyrosinase*. Full expression patterns are discussed 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,

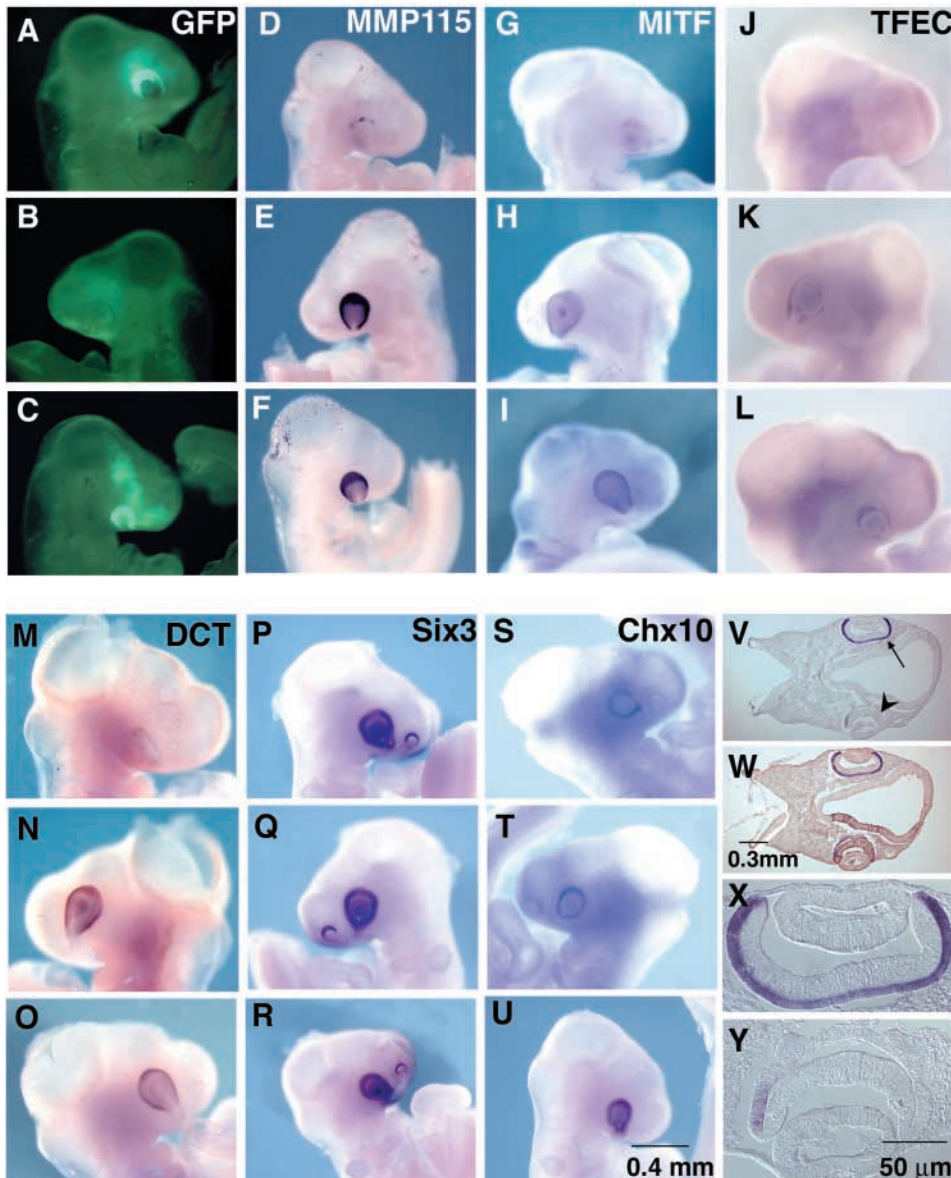


Fig. 6. Misexpression of *Chx10* by in ovo electroporations. (A-U) HH Stage 10 embryos were electroporated with GFP and *Chx10* (A,D,G,J,M,P,S) or GFP alone (C,F,I,L,O,R,U) and allowed to develop an additional day. (B,E,H,K,N,Q,T) Images are from the left non-electroporated eye of the same embryo shown 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>

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Table S1. Riboprobe templates used in this study

Name	Unigene name	Probe	References
Mouse genes:			
Chx10	C. elegans ceh-10 homeodomain containing homolog	3'UTR MscI/XhoI fragment from full-length mouse Chx10	Liu et al., 1994
Notch1	Notch gene homolog 1 (Drosophila)	EST – AW047868	Bao and Cepko, 1999
NeuroD	Neurogenic differentiation 1	Full length cDNA	Morrow et al., 1999
FGF15	Fibroblast growth factor 15	EST – BE952015	Thut et al., 2001
BTG2	B-cell translocation gene 2, anti-proliferative	EST – AI851775	Iacopetti et al., 1999
E2F1	E2F transcription factor 1	EST – BE953801	Dagnino et al., 1997
GAP43	Growth associated protein 43	EST – BE949941	Reh et al., 1993
Stathmin 1	Stathmin 1	EST – AI850105	Koppel et al., 1990
Gas1	Growth arrest specific 1	EST – AI846734	Lee et al., 2001
WFDC1	WAP four-disulfide core domain 1	EST – BF465460	Larsen et al., 2000
Pax6	Paired box gene 6	Full length cDNA, gift from H. Matsunami	Walther and Gruss, 1991
MITF	Microphthalmia-associated transcription factor	Full length M-form probe	Hodgkinson et al., 1993
TFEC	Transcription factor EC	Full coding sequence RT-PCR product: 5'-ATGACCTTTGACTGTCGGGTATGC-3', 5'-AGCCTGTTGCGGCTTACAACCTC-3'	
TRPM1	Transient receptor potential cation channel, subfamily M, member 1	Partial cDNA RT-PCR product: 5'-TGGCTGACAACGGCACC-3', 5'-GCTGATACGACTGGGACTTGCT-3'	Miller et al., 2004
DCT	Dopachrome tautomerase	1200 bp cDNA gift from H. Arnheiter	Steel et al., 1992; Nakayama et al., 1998
Tyrosinase	Tyrosinase	1978 bp cDNA of coding sequence gift from H. Arnheiter	Beerman et al., 1992; Nakayama et al., 1998
Cyclin E1	Cyclin E1	EST – BF462594	
Dll1	Delta-like 1 (Drosophila)	EST – AW047187	Bao and Cepko, 1999; Henrique et al., 1997
Math5	Atonal homolog 7 (Drosophila)	EST – BE951657	Yang et al., 1993
Brn3b	POU domain, class 4, transcription factor 2	EST – BE951391	Gan et al., 1996
Hes1	Hairy and enhancer of split 1 (Drosophila)	EST – BI557608	Tomita et al., 1996
Cyclin D1	Cyclin D1	EST – AI850048	Sicinski et al., 1995
SNAP25	Synaptosomal-associated protein 25	EST – AI843805	Catsicas et al., 1992
CRHBP	Corticoliberin-binding protein	EST – AI854101	Blackshaw et al., 2004
p57	Cyclin-dependent kinase inhibitor 1C	Full length cDNA	Dyer and Cepko, 2000
Otx1	Orthodenticle homolog 1 (Drosophila)	EST – BE988140	Acampora et al., 1996
CD63	Cd63 antigen	EST – AI838302	
Otx2	Orthodenticle homolog 2 (Drosophila)	3' UTR clone pOtd9	Ang et al., 1994; Bovolenta et al., 1997
IGF2	Insulin-like growth factor 2	EST – BF466901	Ohlsson et al., 1994
Chick genes:			
MMP115	115-kDa melanosomal matrix protein	Full length cDNA, gift from G. Eguchi	Mochii et al., 1998
MITF	Microphthalmia-associated transcription factor	Full length cDNA clone cmi9, gift from G. Eguchi	Mochii et al., 1998
TFEC	Transcription factor EC	5' partial cDNA described in materials and methods	
DCT	Dopachrome tautomerase	3' coding sequence, RT-PCR product: 5'-GGCCAAGAGACTCGGGTGAACAG-3', 5'-AAGGGAACAAAGCCAAGGCACTA-3'	April et al., 1998
Six3	Sine oculis-related homeobox 3 homolog (Drosophila)	3'UTR 0.2Kb fragment from full length cSix3 (accession number AY373324) amplified using 5'-CCAAGCTTAGAGACAGAGCGGCGGCT-3' and T3 primer	
Chx10	C. elegans ceh-10 homeodomain containing homolog	full length cDNA	Chen and Cepko, 2000
Chx10 3'UTR	homolog	3'UTR 0.85Kb fragment from full length cChx10 amplified using 5'-ATCTCGAGCCAGGCTACCCAGCCTG-3' and T3 primer	

All genes studied by in situ hybridization in Figs 3, S1 and S2 are shown. The common name referred throughout the text is listed, as is the associated Unigene name. Probes include EST or PCR templates. References refer to the origin of the riboprobe template as well as published expression patterns (referred to the Results section).

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Table S2. Genes upregulated in *or^J* mutants by microarray analysis

UNIQID	Unigene	Name	E12.5	E12.5 swap	E13.5-1	E13.5-1 swap	E13.5-2	E13.5-2 swap
	Mm.36217	Transcripton Factor EC (TFEC)	6.65	11.11	4.23	4.35	3.86	3.85
AI852420	Mm.39913	Leucine rich repeat protein 3, neuronal	3	2.38	3.93	3.03	4.03	3.23
AI851397	Mm.4504	Gap junction membrane channel protein alpha 1	3.34	2.78	2.46	1.89	3.27	2.63
AI843797	Mm.196110	Hemoglobin alpha, adult chain 1	2.23	1.85	2.02	1.59	3.77	3.33
AI845279	Mm.200924	RIKEN cDNA 2700084L06 gene	2.32	1.69	2.45	1.39	4.06	2.44
AI854349	Mm.28405	serum/glucocorticoid regulated kinase	2.6	2.22	2.28	1.75	2.57	2.78
AI845477	Mm.170515	I-kappa B alpha chain mRNA	2.24	1.59	2.13	1.49	4.11	2.5
AI847573	Mm.288626	Protease, serine 25	1.73	1.18	2.07	1.23	4.59	2.86
AI838302	Mm.4426	Cd63 antigen	1.4	1.75	2.36	2.27	2.83	2.86
	Mm.16340	Fibroblast growth factor receptor 2	2.53	3.03	1.41	1.49		
AI845640	Mm.26101	RIKEN cDNA D430039N05 gene	2.58	3.03	1.93	1.75	1.59	1.47
AI838762	Mm.276220	hemoglobin, beta adult major chain	1.62	1.18	1.93	1.22	3.75	2.27
AI845491	Mm.14796	microsomal glutathione S-transferase 1	2.5	1.92	2.15	1.79	1.75	1.45
AI835174	Mm.224836	carbonic anhydrase 14	2.37	1.92	1.85	1.85	1.88	1.56
AI851848	Mm.4514	aldehyde dehydrogenase family 1, subfamily A1	1.36	1.09	2.09	1.59	3.19	2.04
AI846734	Mm.22701	Growth arrest specific 1	2.43	2.13	1.67	1.49	1.9	1.33
AI838156	Mm.156583	carbonic anhydrase 8	1.21	1.18	1.67	1.22	3.75	1.89
AI848419	Mm.89136	H3 histone, family 3A	1.71	1.45	2.32	1.61	2.09	1.59
AI852943	Mm.38496	chondroitin sulfate proteoglycan 5	1.76	1.41	1.67	1.49	2.3	1.82
AI854206	Mm.27816	Hexosaminidase B	1.67	1.64	1.85	1.47	2.13	1.69
AI834768	Mm.274892	RIKEN cDNA 1810027I20 gene	2.06	1.61	1.54	1.28	1.97	1.85
	Mm.4733	Cyclin-dependent kinase inhibitor 2A (p16 INK4a)	1.72	1.33	2.16	1.52	2.1	1.52
AI842572	Mm.2241	Rho, GDP dissociation inhibitor (GDI) beta	1.44	2	1.5	2	1.57	1.75
AI845761	Mm.37613	RIKEN cDNA 2010319C14 gene	1.19	1.35	1.94	1.45	2.42	1.92
AI853186	Mm.22220	fatty acid binding protein 3, muscle and heart	1.51	2.04	1.14	2.38	1.26	1.85
AI850915	Mm.198803	UDP-glucose ceramide glucosyltransferase	2.07	2.33	1.26	1.43	1.49	1.61
AI836483	Mm.22699	selenoprotein P, plasma, 1	1.72	1.35	1.9	1.45	2.04	1.69
AI849606	Mm.2135	folate receptor 1 (adult)	1.64	1.67	1.14	2	1.54	2.08
AI853837		EST	2.73	2.5	1.36	1.16	1.28	1.04
AI851910	Mm.100923	dystrobrevin alpha	2.11	2.63	1.35	1.61	1.07	1.33
	Mm.1571	Cadherin 11	2.1	2.04	1.67	1.35	1.44	1.35
AI845827	Mm.29391	RIKEN cDNA 1110007F23 gene	1.57	1.43	1.53	1.54	1.91	1.85
AI853444	Mm.204	RIKEN cDNA 2610042L04 gene	2.98	2.38	1.28	1.12	1.18	0.91
AI851001	Mm.578	Insulin-like growth factor binding protein 5	2.48	2.04	1.2	1.32	1.36	1.41
AI850263	Mm.486	Lysosomal membrane glycoprotein 2	1.52	1.49	1.62	1.39	2.05	1.67
AI836560	Mm.20948	transcobalmin II (Tcn2)	1.51	1.27	1.51	1.41	2.07	1.89
AI842286	Mm.27856	protein tyrosine phosphatase, receptor type, K	1.74	1.82	1.38	1.72	1.38	1.56
AI851745	Mm.29027	SPARC-like 1 (mast9, hevin)	1.23	1.96		1.67	1.51	0
AI853697	Mm.182434	Follistatin-like	1.4	1.54	1.1	1.23	2.05	2.13
AI851210	Mm.34108	RIKEN cDNA C030003H22 gene	1.88	2	1.39	1.45	1.41	1.28
AI854781	Mm.44814	Transcription factor XLMO1 homolog	1.69	1.39		1.43	1.53	1.75
AI848129	Mm.154529	RIKEN cDNA 6430520M22 gene	2.15	2.08	1.24	1.39	1.27	1.1
AI840291	Mm.260244	D430021N15 product:unknown EST	1.66	1.59	1.14	1.69	1.25	1.92
AI854164	Mm.40243	EST	1.18	1.47	1.86	1.75	1.48	1.52
	Mm.2423	Procollagen, type II, alpha 1	1.42	1.32	1.91	1.64	1.52	1.27
AI838713	Mm.12715	RIKEN cDNA 2310016C16 gene	1.55	1.1	1.74	1.16	2.11	1.32
AI845303	Mm.24873	nucleolar protein family A, member 1	1.35	1.61	1.24	1.45	2.13	1.2
AI843783	Mm.658	solute carrier family 25, member 5	1.85	1.12	1.54	1.06	2.09	1.33
AI846715	Mm.37938	spondin 1, (f-spondin) extracellular matrix protein	1.75	1.23		1.09	1.94	1.43
AI841883		EST	1.41	1.04		1	2.49	1.45
	Mm.3918	ATP-binding cassette, sub-family A (ABC1), member 4 (ABCR)	1.22	1.19	0.77	0.83	2.28	2.56
AI851312	Mm.27230	RNA binding motif, single stranded interacting protein 1	1.36	1.75	1.43	1.49	1.37	1.39
AI849044	Mm.28144	apoptosis related protein APR-3	1.32	1.28	1.61	1.82	1.55	1.22
AI834785	Mm.142760	RIKEN cDNA E130016I23 gene	1.28	1.27	1.65	1.37	1.67	1.45
	Mm.3608	Paired box gene 6 (Pax6)	1.04	1.22	1.69	1.39	1.83	1.54
AI845199	Mm.22699	selenoprotein P, plasma, 1	1.45	1.15	1.28	1.56	1.67	1.52
AI848342	Mm.30064	gamma-aminobutyric acid receptor associated protein	1.46	1.19	1.61	1.54	1.55	1.28
	Mm.168789	Cyclin-dependent kinase inhibitor 1C (p57)	1	1.06	1.69	1.41	1.77	1.64
AI854333	Mm.121878	tropomyosin 1, alpha	1.37	1.43	1.45	1.72	1.18	1.43
AI836902	Mm.24118	Glutathione S-transferase, theta 2	1.43	1.16	1.7	1.23	1.86	1.22
AI841689	Mm.29658	chemokine-like factor super family 3	1.26	1.22	1.24	2.04	1.2	1.54
AI846379	Mm.213114	RIKEN cDNA 9130423L19 gene	1.48	1.2	1.52	1.15	1.74	1.41
AI853807	Mm.22271	smt3-specific isopeptidase 1	1.51	1.56	1.27	1.45	1.25	1.41
	Mm.201322	Activity-dependent neuroprotective protein (ADNP)	1.66	1.49	1.17	1.75	1.24	1.14
		Paired box gene 2 (Pax2)	1.29	1.06	1.58	1.33	1.83	1.39
AI845968	Mm.7046	Protective protein for beta-galactosidase	1.56	1.28	1.18	1.35	1.31	1.69
AI850677	Mm.34977	neuron navigator 1	1.84	1.41	1.51	1.12	1.35	1.11
AI849964	Mm.40272	RIKEN cDNA 9930033H14 gene	1.3	1.22	1.45	1.59	1.38	1.33
AI850370	Mm.119265	RIKEN cDNA 9430020K16 gene	1.65	1.67	1.27	1.32	1.17	1.2
AI850161	Mm.28771	RIKEN cDNA 1110035H23 gene	1.19	1.3	1.24	1.45	1.41	1.61
AI835916	Mm.158897	RIKEN cDNA 2900027G03 gene	1.64	1.25	1.44	1.28	1.43	1.16
AI847949	Mm.248937	RIKEN cDNA 0610007H07 gene	1.3	1.23	1.35	1.25	1.59	1.49
AI844522	Mm.1945	phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha	1.5	1.3	1.51	1.22	1.41	1.28
AI842777	Mm.65357	leucine-rich repeat LGI family, member 1	1.33	1.35	1.54	1.37	1.18	1.45

AI845880	Mm.260164	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	1.15	1.11	1.64	1.19	1.68	1.45
AI844804	Mm.197518	lysosomal-associated protein transmembrane 4B	1.42	1.52	1.31	1.27	1.31	1.41
AI853088	Mm.182434	Follistatin-like	1.29	1.52	1.19	1.3	1.36	1.59
AI854552	Mm.951	interferon-induced protein with tetratricopeptide repeats 3	1.02	1.75	1.35	1.32	1.35	1.35
AI851746	Mm.30155	ATPase, H+ transporting, V0 subunit C	1.24	1.37	1.34	1.35	1.44	1.43
AI854095	Mm.139166	IMAGE:5006616, mRNA	1.63	2.63	1.01	1.2	0.63	1.06
AI848876	Mm.22673	Fc receptor, IgE, high affinity I, gamma	1.27	1.3	1.38	1.2	1.57	1.45
AI846973		EST	1.39	1.18	1.39	1.19	1.66	1.37
AI839644	Mm.7500	Ferritin light chain 1	1.35	1.16	1.74	1.27	1.59	1.08
AI854232	Mm.8154	Imprinted and ancient	1.56	1.3	1.33	1.12	1.51	1.25
AI842171	Mm.230635	Heme oxygenase (decycling) 1	0.89	1.43	1.32	1.49	1.46	1.49
AI847838	Mm.11218	Ercc3	1.08	1.35	1.54	1.54	1.13	1.45
AI854187	Mm.16340	Fibroblast growth factor receptor 2	1.34	1.72	1.07	1.37	1.08	1.52
AI842667	Mm.684	Cathepsin C	1.25	1.35	1.25	1.54	1.15	1.56
AI842767	Mm.28099	Sterol O-acyltransferase 1	1.42	1.3	1.18	1.47	1.36	1.39
AI839581	Mm.259670	Porcupine homolog (Drosophila)	1.23	1.18	1.52	1.33	1.38	1.39
AI839138	Mm.77432	thioredoxin interacting protein	1.14	1.3	1.6	1.54	1.13	1.33
AI850126	Mm.43660	Palmitoyl-protein thioesterase	1.35	1.28	1.18	1.32	1.38	1.54
AI846363	Mm.260164	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	1.13	1.18	1.42	1.43	1.36	1.54
AI846398	Mm.141021	RIKEN cDNA 1110004C05 gene	1.47	1.23	1.44	1.3	1.39	1.23
AI850638	Mm.270278	thyrotroph embryonic factor	1.11	1.47	1.11	1.59	1.18	1.28
AI838693	Mm.22699	selenoprotein P, plasma, 1	1.4	1.27	1.42	1.27	1.37	1.28
AI850670	Mm.206505	Tissue inhibitor of metalloproteinase 2	1.45	1.32	1.37	1.59	1.05	1.23
AI851537	Mm.20829	Epithelial membrane protein 3	0.97	1.22	1.41	1.59	1.3	1.43
AI849556	Mm.25248	expressed sequence AA960558	1.34	1.61	1.18	1.45	1.11	1.25
AI849135	Mm.4948	RIKEN cDNA 1110048B16 gene	1.49	1.56	1.22	1.35	1.07	1.14
AI848880	Mm.381	Adipose differentiation related protein	1.34	1.28	1.36	1.27	1.32	1.27
AI852792	Mm.29648	Degenerative spermatocyte homolog (Drosophila)	1.15	1.32	1.35	1.09	1.53	1.41
AI838435	Mm.34087	RIKEN cDNA A730024A03 gene	1.37	1.39	1.36	1.41	1.32	1.01
AI850788	Mm.140496	RIKEN cDNA 1110001M20 gene	1.24	1.14	1.45	1.27	1.55	1.23
AI847599	Mm.28362	Immunoglobulin heavy chain 6	1.18	1.35	1.31	1.41	1.38	1.25
AI844356	Mm.38055	esterase 10	1.15	1.45	1.3	1.45	1.36	1.18
AI850349	Mm.14530	RAB1, member RAS oncogene family	1.42	1.28	1.08	1.52	1.12	1.47
AI843750	Mm.7821	Tumor protein D52-like 1	1.17	1.43	1.32	1.52	1.16	1.18
AI847007	Mm.25203	NCK-associated protein 1	1.45	1.39	1.45	1.25	1.19	1.04
AI847927		EST	1.34	1.49	1.07	1.52	1.04	1.32
AI852636	Mm.18494	MAP kinase kinase 3	1.21	1.35	1.27	1.04	1.5	1.41
AI852449	Mm.12559	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	1.24	1.06	1.4	1.14	1.65	1.3
AI844604	Mm.3126	Four and a half LIM domains 1	1.48	1.15	1.26	1.45	1.27	1.19
AI843396		EST	1.4	1.2	1.37	1.02	1.63	1.19
AI851074	Mm.17958	recombination activating gene 1 gene activation	1.14	1.1	1.56	1.28	1.37	1.37
AI845824	Mm.104920	Sorbitol dehydrogenase 1	1.09	1.1	1.59	1.35	1.62	1.08
AI844081	Mm.49689	sarcospan	1.17	1.67	1.35	1.14	1.19	1.32

Genes listed are those upregulated at least 1.3-fold. Genes listed without unique IDs are full-length clones from within our laboratory. Unigene numbers and full names are indicated. Data points include one E12.5 sample and two E13.5 samples with associated color swaps.

Table S3. Genes downregulated in *or^l* mutants by microarray analysis

UNIQUID	Unigene	Name	E12.5	E12.5 swap	E13.5-1	E13.5-1 swap	E13.5-2	E13.5-2 swap
AI852799	Mm.22231	synuclein, gamma	0.33	0.25	0.31	0.21	0.21	0.17
	Mm.4636	Neurogenic differentiation 1 (NeuroD)	0.24	0.21	0.31	0.27	0.23	0.23
AI841966	Mm.6670	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	0.34	0.31	0.28	0.21	0.24	0.18
AI840972	Mm.29580	stathmin-like 2	0.28	0.3	0.32	0.24	0.26	0.26
AI836519	Mm.25165	ALL1-fused gene from chromosome 1q	0.34	0.28	0.32	0.22	0.36	0.27
AI841303	Mm.1222	growth associated protein 43	0.35	0.31	0.46	0.43	0.35	0.3
AI835930		Ribosomal RNA	0.25	0.31	0.44	0.32	0.53	0.54
AI848068	Mm.4323	microtubule-associated protein 1 B	0.48	0.4	0.43	0.44	0.36	0.3
AI851775	Mm.903	B-cell translocation gene 2, anti-proliferative	0.19	0.15	0.43	0.57	0.56	0.57
AI841267	Mm.2319	stathmin-like 3	0.49	0.65	0.35	0.43	0.33	0.25
	Mm.10695	Neurogenic differentiation 4 (Math3)	0.33	0.52	0.44	0.46	0.37	0.47
AI848445	Mm.27578	melanoma antigen, family D, 1	0.48	0.4	0.56	0.43	0.43	0.31
AI843116	Mm.61526	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	0.5	0.37	0.47	0.53	0.5	0.41
AI849592	Mm.3970	ELAV (Drosophila)-like 4 (Hu antigen D)	0.42	0.43	0.54	0.56	0.44	0.46
AI848983	Mm.32884	tubulin, alpha 8	0.52	0.42	0.54	0.37	0.6	0.47
AI848368	Mm.4435	synaptosomal-associated protein 25	0.42	0.45	0.7	0.54	0.38	0.44
AI850105	Mm.28479	stathmin 1	0.67	0.45	0.63	0.37	0.52	0.32
AI841233	Mm.101976	potassium voltage gated channel, Shaw-related subfamily, member 4	0.5	0.52	0.58	0.46	0.51	0.41
AI846286	Mm.218788	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	0.66	0.47	0.64	0.4	0.51	0.35
	Mm.22288	Cyclin D1	0.33	0.36	0.74	0.68	0.47	0.47
AI845079		EST	0.54	0.52	0.54	0.41	0.58	0.47
AI851861	Mm.156164	RIKEN cDNA 6430547I21 gene	0.63	0.55	0.89	0.36	0.37	0.31
AI840604	Mm.1155	tubulin, alpha 4	0.6	0.43	0.58	0.41	0.68	0.42
AI854858	Mm.256966	microtubule-associated protein 2	0.52	0.56	0.61	0.58	0.43	0.44
AI850627		EST	0.63	0.42	0.61	0.39	0.68	0.42
AI838066	Mm.182912	growth hormone inducible transmembrane protein	0.57	0.52	0.61	0.42	0.53	0.51
AI835385	Mm.29324	lactate dehydrogenase 1, A chain	0.55	0.4	0.58	0.39	0.72	0.54
AI846851	Mm.39472	farnesyl diphosphate synthetase	0.62	0.5	0.58	0.43	0.6	0.46
AI837019	Mm.40068	tubulin, beta 3	0.8	0.61	0.57	0.39	0.51	0.35
AI837745	Mm.22421	telomerase binding protein, p23	0.66	0.4	0.66	0.4	0.73	0.41
AI848759	Mm.3204	farnesyl diphosphate farnesyl transferase 1	0.59	0.48	0.57	0.43	0.71	0.53
AI849399	Mm.45759	Musculus, clone IMAGE:5325914, mRNA	0.65	0.42	0.7	0.41	0.72	0.42
AI839893	Mm.231463	tubulin, alpha 2	0.64	0.44	0.54	0.44	0.76	0.51
AI839009	Mm.29902	phosphoserine aminotransferase 1	0.58	0.5	0.5	0.44	0.71	0.62
AI837103	Mm.40673	expressed sequence AI316785	0.68	0.52	0.62	0.4	0.67	0.46
AI850603	Mm.133615	RIKEN cDNA 2810457I06 gene	0.62	0.43	0.61	0.47	0.74	0.54
AI849291	Mm.3111	ribosomal protein L28	0.81	0.58	0.62	0.4	0.58	0.41
AI850549	Mm.37819	DNA segment, Chr 5, Brigham & Women's Genetics 0860 expressed	0.58	0.57	0.51	0.55	0.62	0.58
AI845046	Mm.4142	trans-golgi network protein 1	0.63	0.55	0.62	0.55	0.61	0.5
AI844837		EST	0.75	0.43	0.61	0.47	0.67	0.53
	Mm.8008	Cone-rod homeobox containing gene (Crx)	0.63	0.78	0.45	0.53	0.51	0.56
AI845893	Mm.42242	ISL1 transcription factor, LIM/homeodomain (islet 1)	0.76	0.6	0.65	0.55	0.48	0.44
AI836851	Mm.27307	guanine nucleotide binding protein (G protein), gamma 3 subunit	0.64	0.68	0.62	0.55	0.5	0.5
AI839739		EST	0.7	0.45	0.66	0.43	0.78	0.47
AI842035		EST	0.83	0.79	0.63	0.47	0.46	0.35
AI849910		EST	0.81	0.51	0.59	0.39	0.85	0.45
AI840446	Mm.24059	schwannomin interacting protein 1	0.62	0.56	0.68	0.65	0.59	0.49
AI840227	Mm.18923	minichromosome maintenance deficient 7 (S. cerevisiae)	0.74	0.61	0.52	0.5	0.67	0.57
AI849333	Mm.218657	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	0.7	0.67	0.51	0.44	0.67	0.63
AI843193	Mm.138083	RIKEN cDNA E130114P18 gene	0.57	0.64	0.63	0.7	0.56	0.55
AI838225	Mm.41484	RIKEN cDNA 1500015O20 gene	0.54	0.61	0.67	0.69	0.69	0.5
AI845505	Mm.246377	tubulin, beta 2	0.81	0.68	0.62	0.48	0.64	0.48
AI843805	Mm.4435	synaptosomal-associated protein 25	0.45	0.66	0.66	0.85	0.51	0.61
AI853846	Mm.741	fatty acid binding protein 5, epidermal	0.79	0.75	0.71	0.5	0.56	0.44
	Mm.20355	Wingless-related MMTV integration site 4 (Wnt4)	0.69	0.5	0.76	0.46	0.79	0.54
	Mm.1956	Neurofilament, light polypeptide (NF68)	0.8	0.85	0.64	0.51	0.49	0.47
AI845354	Mm.232930	neuritin 1	0.74	0.8	0.68	0.71	0.42	0.43
AI853301	Mm.16533	polymerase (RNA) II (DNA directed) polypeptide A	0.77	0.71	0.75	0.54	0.54	0.49
AI838520	Mm.41803	developmentally regulated GTP binding protein 2	0.82	0.59	0.72	0.49	0.72	0.47
AI844406	Mm.137278	RIKEN cDNA C030046I01 gene	0.67	0.57	0.8	0.59	0.64	0.55
AI843545	Mm.155896	heterogeneous nuclear ribonucleoprotein A2/B1	0.72	0.56	0.67	0.46	0.85	0.6
AI836520	Mm.268166	histone 3, H2bb	0.6	0.51	0.97	0.67	0.68	0.47
AI854838	Mm.42972	Ywhae	0.75	0.6	0.74	0.54		0.65
AI848998	Mm.29075	reticulon 1	0.67	0.7	0.8	0.78	0.52	0.49
AI849379	Mm.1147	Calmodulin 2	0.59	0.57	0.76	0.59	0.77	0.69
AI845040	Mm.254	tumor protein, translationally-controlled 1	0.86	0.66	0.64	0.48	0.75	0.57
AI852915	Mm.5101	synaptotagmin 1	0.67	0.52	0.86	0.75	0.74	0.43
AI845300	Mm.2223	t-complex protein 1	0.64	0.51	0.79	0.59	0.81	0.65
AI851946	Mm.140700	RIKEN cDNA 2610316D01 gene	0.69	0.85	0.44	0.67	0.62	0.74
AI845631	Mm.235585	dihydropyrimidinase-like 2	0.64	0.44	0.83	0.66	0.73	0.72
AI852614	Mm.28323	RIKEN cDNA G630055P03 gene	0.63	0.39	0.72	0.61	1.06	0.63
AI843214	Mm.36683	eukaryotic translation elongation factor 1 epsilon 1	0.55	0.59	0.65	0.79	0.7	0.77
AI841020	Mm.188	phosphoglycerate kinase 1	0.7	0.53	0.73	0.51	0.94	0.68
AI840089	Mm.6787	splicing factor, arginine/serine-rich 3 (SRp20)	0.75	0.57	0.74	0.54	0.83	0.68

AI841335		EST	0.91	0.67	0.73	0.51	0.75	0.56
AI835506		EST	0.66	0.65	0.67	0.61	0.79	0.75
AI852638	Mm.26381	spastic paraplegia 3A homolog (human)	0.86	0.76	0.74	0.71	0.59	0.47
AI841389	Mm.90587	enolase 1, alpha non-neuron	0.74	0.52	0.76	0.47	1	0.68
AI836137	Mm.216135	pyruvate kinase, muscle	0.57	0.61	0.62	0.51	1.04	0.84
AI849319	Mm.140672	Smarcd3	0.69	0.6	0.68	0.58	0.88	0.75
AI847555	Mm.4762	kinesin family member 1A	0.76	0.67	0.65	0.53	0.79	0.8
AI846050	Mm.196219	RIKEN cDNA 1110005A05 gene	0.82	0.6	0.82	0.64	0.75	0.6

Genes listed are those downregulated at least 0.7-fold, as represented by the ratio of signal in *or^l* mutants relative to wild-type controls. Genes listed without unique IDs are full-length clones from within our laboratory. Unigene numbers and full names are indicated. Data points include one E12.5 sample and two E13.5 samples with associated color swaps.
