Chx10 repression of *Mitf* is required for the maintenance of mammalian neuroretinal identity

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Summary

During vertebrate eye development, the cells of the optic vesicle (OV) become either neuroretinal progenitors expressing the transcription factor Chx10, or retinal pigment epithelium (RPE) progenitors expressing the transcription factor Mitf. *Chx10* mutations lead to microphthalmia and impaired neuroretinal proliferation. *Mitf* mutants have a dorsal RPE-to-neuroretinal phenotypic transformation, indicating that *Mitf* is a determinant of RPE identity. We report here that *Mitf* is expressed ectopically in the *Chx10*$^{or\text{-}J/or\text{-}J}$ neuroretina (NR), demonstrating that *Chx10* normally represses the neuroretinal expression of *Mitf*. The ectopic expression of *Mitf* in the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR deflects it towards an RPE-like identity; this phenotype results not from a failure of neuroretinal specification, but from a partial loss of neuroretinal maintenance. Using *Chx10* and *Mitf* transgenic and mutant mice, we have identified an antagonistic interaction between *Chx10* and *Mitf* in regulating retinal cell identity. FGF (fibroblast growth factor) exposure in a developing OV has also been shown to repress *Mitf* expression. We demonstrate that the repression of *Mitf* by FGF is *Chx10* dependent, indicating that FGF, Chx10 and Mitf are components of a pathway that determines and maintains the identity of the NR.

Key words: Mouse, Mitf, Chx10, FGF, RPE, Neuroretina

Introduction

In vertebrate eye development, anterior ectodermal tissue undergoes specification to form the three major structures of the eye: the NR and the RPE, both of which originate from the neuroectodermal tissue of the OV; and the lens, which develops from the surface ectoderm (Chow and Lang, 2001). Recent evidence suggests that eye cell fate decisions begin early in embryogenesis and occur through a series of narrowing cell commitments (Baker and Bronner-Fraser, 2001; Zuber et al., 2003). However, the binary cell fate decision through which an OV neuroectodermal cell becomes either a neuroretinal or an RPE cell is poorly characterized, especially at the molecular level.

The majority of studies examining RPE versus neuroretinal cell identity decisions have focused on the ability of differentiated or developing RPE cells to 'transdifferentiate' into neuroretinal cells in response to external signals (Reh and Pittack, 1995). The neuroretinal-inducing signal appears to come from the surface ectoderm (Mikami, 1939), suggesting a model in which surface ectodermal signals induce the underlying OV neuroectoderm to become NR rather than RPE. Although FGF has long been considered the surface ectodermal signal that induces neuroretinal cell fate, recent evidence suggests that surface ectodermal FGFs (or factors mimicking FGF signaling) pattern and organize the RPE and NR in the OV, rather than inducing neuroretinal cells (Hyer et al., 1998; Nguyen and Arnheiter, 2000). This model is consistent with the step-wise specification of neuroretinal and RPE cells that begins early in embryogenesis, before the OV stage.

Of the regulatory molecules implicated in RPE cell specification, the basic helix-loop-helix leucine-zipper transcription factor Mitf is the best characterized. *Mitf* is considered to be crucial for the acquisition and maintenance of RPE cell identity. In the mouse, *Mitf* is expressed throughout the neuroectoderm of the E9.0 OV, and is subsequently downregulated in the presumptive NR at E9.5 (Nguyen and

Arnheiter, 2000). However, *Mitf* expression continues in the RPE and ciliary margin throughout early eye development. Mutations in mouse *Mitf* result in microphthalmia, a lack of RPE cell differentiation and an RPE-to-neuroretinal change in cell identity in the dorsal region of the eye (Bumstead and Barnstable, 2000; Hodgkinson et al., 1993; Hughes et al., 1993; Nguyen and Arnheiter, 2000). Other studies suggest that *Mitf* is involved in OV organization and pattern formation in response to external signals. *Mitf* expression is repressed by surface ectodermal FGF (Nguyen and Arnheiter, 2000), and upregulated by activin signals from the mesenchyme surrounding the RPE (Fuhrmann et al., 2000). In addition, *Pax2, Pax6* (Baumer et al., 2003), *Otx1*, *Otx2* (Martinez-Morales et al., 2003; Martinez-Morales et al., 2001), *Gas1* (Lee et al., 2001) and *Ap2a* (West-Mays et al., 1999) are necessary for RPE cell identity; *Pax2*, *Pax6* and the Otx genes are upstream of *Mitf*.

Apart from the role of FGF in regulating neuroretinal cell identity, little is known about the mechanisms of neuroretinal specification. In the mouse, ectopic expression of *Ras* (Zhao et al., 2001) or *Mek1* (Galy et al., 2002) results in RPE-to-NR changes in cell identity, presumably by mimicking the reception of an FGF signal from the surface ectoderm. In addition, misexpression of the early eye-specifying transcription factor Six6/Optx2 leads to an RPE-to-NR transformation in chick (Toy et al., 1998). In the mouse, however, no loss-of-function studies have identified a NR-specifying molecule.

We considered the Chx10 homeodomain transcription factor to be a strong candidate regulator of neuroretinal specification or maintenance. *Chx10* is the earliest known gene to be expressed specifically in the presumptive NR, beginning at E9.5 (Liu et al., 1994). Mutations in mouse *Chx10* result in microphthalmia and a pronounced defect in the proliferation of neuroretinal progenitor cells, but all the cell types of the mature retina are present, except bipolar cells (Burmeister et al., 1996). A comparable phenotype is seen in humans (Percin et al., 2000). Although *Chx10* does not appear to be necessary for neuroretinal specification, its early neuroretinal expression suggests that *Chx10* may contribute to the acquisition or maintenance of neuroretinal identity.

To investigate the role of *Chx10* in eye development, we examined the expression of candidate Chx10 target genes in the developing eye of the homozygous recessive *Chx10*$^{or\text{-}J/or\text{-}J}$ mouse (Burmeister et al., 1996). One potential target was the key eye development transcription factor gene *Mitf,* because mutations in this gene, like mutations in *Chx10*, are also associated with a small eye phenotype (Hodgkinson et al., 1993; Hughes et al., 1993). A possible regulatory relationship between *Chx10* and *Mitf* in early stages of mouse eye development is also suggested by the fact that *Mitf* expression in the presumptive NR (in the distal OV) is normally extinguished at E9.5 (Nguyen and Arnheiter, 2000), at the time that *Chx10* expression in the distal OV is first observed (Liu et al., 1994).

Materials and methods

Animals

Chx10or-J/+(*129/SvJ*), *Mitfmi/+* (*B6C3He)* and *C57BL/6* mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were placed together in the evening and females were considered to be at 0.5 days of gestation (E0.5) when a vaginal plug was detected the following morning. Mice were euthanised by cervical dislocation.

Chx10$^{or\text{-}J/+}$ mice were genotyped as previously described (Burmeister et al., 1996). *Mitf*$^{mi/+}$ mice were genotyped by PCR amplification with mouse *Mitf* intron 6 forward (5′-GGTGTG-CCTCAGTCACTAATG-3′) and exon 7 reverse (5′-CTGGATCA-TTTGACTTGGGG-3′) primers. Genotypes were determined by heteroduplex analysis on a 9% acrylamide gel. Heterozygous individuals yielded multiple bands instead of a single 200 bp band that arises from homozygous normal or mutant mice.

In situ hybridization and immunofluorescence

Embryos were processed and sectioned, and in situ hybridization and immunofluorescene were performed as previously described (Chow et al., 2001).

Mitf, *Tyr*, *Tyrp1*, *Dct*, *Math5* (*Atoh7* – Mouse Genome Informatics) and human *MITF* (see below) expression was identified using mRNA from full-length cDNAs; *Chx10* expression was examined using a 3′ UTR probe (Liu et al., 1994). The localization of Mitf protein was determined using rabbit anti-Mitf antibodies (Nguyen and Arnheiter, 2000) pre-adsorbed to mouse embryo powder (see below), at a dilution of 1:25. Chx10, Pax6 and NCAM proteins were identified with affinity-purified anti-Chx10 (Liu et al., 1994), anti-Pax6 (a gift from G. Mastick) and anti-NCAM (Santa Cruz Biotechnology, Santa Cruz CA, H-300) rabbit polyclonal antibodies. The final dilutions of these primary antibodies were 1:500, 1:500 and 1:100, respectively. Islet1 protein was identified with an anti-Islet1 (Developmental Studies Hybridoma Bank, University of Iowa; 39.4D5) mouse monoclonal antibody at a dilution of 1:100. Notch1 was identified using a goat anti-Notch1 (C-20; 1:25; Santa Cruz Biotechnology, Santa Cruz, CA; sc-6014) polyclonal antibody. Secondary AlexaFluor488-conjugated F(ab′)$_2$ fragment goat anti-mouse IgG (Molecular Probes, Eugene, OR) and secondary Cy3-conjugated goat anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and donkey anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) were used at a dilution of 1:100. Stained sections were examined on a LSM 510 Zeiss confocal microscope and on a Nikon Eclipse E-1000 conventional upright fluorescence microscope.

Anti-Mitf antibody was adsorbed on acetone-extracted E15.5 *Mitf*^{mi}/+ embryo powder in phosphate-buffered saline (PBS) and stored in 10% goat serum, 50% glycerol, 0.05% sodium azide/PBS.

Hematoxylin and Eosin staining

To perform Hematoxylin and Eosin staining, paraffin wax-embedded sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Sections were then immersed in Hematoxylin for 10 minutes, in tap water for 30 seconds and acid alcohol (0.5% HCl/70% ethanol) for 10 seconds. The slides were then incubated in 0.5% Eosin/70% ethanol for 3 minutes, dehydrated in a graded ethanol series, immersed in xylene and mounted in Permount (Fisher Scientific).

NR-MITF transgenic mice

The *NR-MITF* construct consisted of the human *PAX6* neuroretinal enhancer and minimal *β-globin* promoter driving the expression of the human *M-MITF* cDNA (without exon 6) followed by an SV40 polyadenylation signal (see Fig. S1 in the supplementary material).

A human M isoform *MITF* (*M-MITF*) cDNA was obtained by generating a hybrid EST clone (due to an EST mutation: 5′ EST Accession Number N36632, 3′ EST Accession Number N34462). The 5′ and 3′ sequences were linked using a *Cla*I site at nucleotide 478 of the *M-MITF* cDNA. The sequence starting at nucleotide 68 of the *M-MITF* cDNA and extending to the poly-A tail (nucleotide ~1900) was cloned into *pT3T7D* (Pharmacia), thus creating the plasmid *M-MITF-pT3T7D*. This *M-MITF* cDNA does not contain the alternatively

spliced exon 6. This plasmid was digested with *Acc*I, blunted and digested with *Hin*dIII.

As the putative neural retinal enhancer (NRE) of the human *PAX6* gene is located in intron 4 (Kammandel et al., 1999), a 6 kb *Eco*RI fragment encompassing exons 3, 4 and part of exon 5 of the *PAX6* gene was identified and subcloned into the *pBluescript-SK* (*pBS-SK)* cloning vector (Stratagene). This fragment was then excised as a *Not*I fragment for subsequent ligation into the *Not*I site of the *p1229* reporter plasmid that contains the minimal *β-globin* promoter, the *lacZ* gene and the polyadenylation sequence from SV40. Transgenic studies demonstrated that this *PAX6* enhancer is sufficient to direct expression in the developing mouse neuroretinal epithelium as detected by staining for *lacZ* expression (G.C.S., unpublished). The *β-globin* promoter from *PAX6-NRE-βglobin-lacZ-SV40* was amplified from this plasmid with the primers T7 (5′-GTAATACGACTCACTATAGGGC-3′) and *lacZ*5′Rev (5′-AAGTTGGGTAACGCCAGGG-3′). This PCR product was digested with *Nco*I, blunted and digested with *Sac*II. The *M-MITF* cDNA and *β-globin* fragments were ligated into *pBS-SK* digested with *Sac*II and *Hin*dIII, to generate the plasmid *BMpBS-SK*. The SV40 polyadenylation sequence was amplified from *PAX6-NRE-βglobin-lacZ-SV40* with the primers SV40Fwd (5′-CCATCGATCCGGGCAGGCCATGTCTGC-3′) and T3 (5′-AATTAACCCTCACTAAAGGG-3′). This PCR product was digested with *Cla*I and *Hin*dIII and subcloned into *pBS-SK* to generate the plasmid *SV40pBS-SK*. *SV40pBS-SK* was digested with *Hin*dIII and the liberated fragment was ligated into the *Hin*dIII site of *BMpBS-SK* to generate *BMSpBS-SK*. The *PAX6-NRE-βglobin-lacZ-SV40* construct was digested with *Not*I and *Sal*I to remove all inserted sequences, which were replaced with the *Not*I-*Sal*I fragment from *BMSpBS-SK*, thus generating the plasmid *BMS*. The 6 kb *PAX6-NRE* was ligated into the *Not*I site of *BMS* to generate the *NR-MITF* construct (see Fig. S1 in the supplementary material). The *NR-MITF* insert was released with *Sal*I prior to microinjection.

The purified *NR-MITF* insert was injected into both *C57BL/6;SJL* (Hospital for Sick Children Transgenic Facility) and *C57BL/6;C3H* (Ottawa Health Research Institute) donors; germline-transmitting *NR-MITF* mice were identified using Southern blot analysis. *Eco*RI-digested genomic DNA was probed with radiolabeled full-length *M-MITF* cDNA; *NR-MITF*-positive individuals yielded 1 kb and 5 kb bands. Transgenic mice were also identified by PCR using primers in exon 3 (5′-CCCAGGCATGAACACACATTCAC-3′) and exon 9 (5′-GTGCTCCGTCTCTTCCATGC-3′), resulting in a 1 kb product.

RPE-CHX10 transgenic mice

The *RPE-CHX10* construct consisted of the mouse *Dct* enhancer/promoter driving the expression of the human *CHX10* cDNA (see Fig. S1 in the supplementary material).

The *Dct* enhancer/promoter construct (*pPdct*) was a generous gift from T. Hornyak. This construct contains ~3.3 kb of sequence upstream of the open reading frame of the mouse *Dct* gene in a *pcDNA* vector backbone (Hornyak et al., 2001). To generate the human *CHX10* cDNA construct, a second *Xho*I site was inserted 3′ to a 3.1 kb human *CHX10* cDNA in *pBS-KS* to generate *CHX10-2Xho*. *CHX10-2Xho* was digested with *Xho*I to release the *CHX10* cDNA, which was then ligated to *Xho*I-digested *pPdct* to generate *RPE-CHX10* (see Fig. S1 in the supplementary material). The *RPE-CHX10* insert was released with *Sal*I prior to microinjection.

The purified *RPE-CHX10* insert was injected into *C57BL/6;C3H* donors (Ottawa Health Research Institute) and germline-transmitting mice were identified. *RPE-CHX10* mice were genotyped using *Eco*RI-digested genomic Southern blots probed with a radiolabeled full-length human *CHX10* insert. *RPE-CHX10*-positive individuals yielded a major 3 kb band, and minor undigested bands that varied with the individual transgenic mouse lines. *RPE-CHX10* mice were also identified by PCR using primers located in the homeodomain (5′-GAAGAAGCGGCGACACAGG-3′) and CVC domain (5′-CCTCCAGCGACTTTTTGTG-3′), resulting in a 350 bp product.

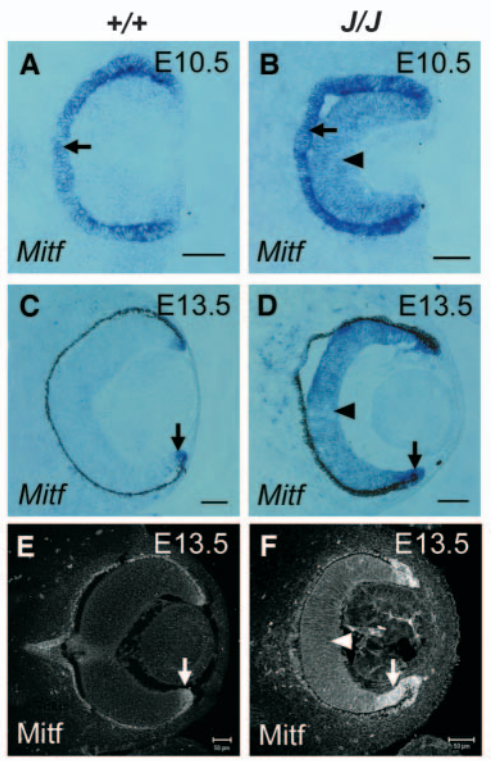

Fig. 1. Mitf is ectopically expressed in the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR. (A) In the eye of an E10.5 wild-type (+/+) embryo, *Mitf* mRNA is present only in the presumptive RPE (arrow). (B) In an E10.5 *Chx10*$^{or\text{-}J/or\text{-}J}$ (*J/J*) mutant eye, *Mitf* is expressed normally in the presumptive RPE (arrow), and ectopically in the NR (arrowhead). (C) By E13.5, *Mitf* is localized to the RPE and presumptive ciliary margin (arrow) in a wild-type embryo. (D) E13.5 *Chx10*$^{or\text{-}J/or\text{-}J}$ animals continue to misexpress *Mitf* in the NR (arrowhead). In addition, the presumptive ciliary margin domain (arrow) is expanded, as evidenced by a larger area of increased *Mitf* expression. (E) Mitf protein levels mirror the RPE and presumptive ciliary margin (arrow) *Mitf* mRNA expression domains in a wild-type E13.5 embryo. (F) Mitf protein is synthesized in the E13.5 *Chx10*$^{or\text{-}J/or\text{-}J}$ NR (arrowhead) and the enlarged presumptive ciliary margin region can also be clearly identified (arrow). Scale bars: 25 μm in A,B,D; 32 μm in C; 50 μm in E,F.

OV cultures

OV cultures and associated immunofluorescence were performed as previously described (Nguyen and Arnheiter, 2000).

Results

Chx10 represses *Mitf* in eye development

We examined the expression of *Mitf* in wild-type and *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant eyes, and confirmed that *Mitf* is normally expressed in the RPE and the ciliary margin (Nguyen and Arnheiter, 2000) (Fig. 1A,C,E). In the *Chx10*$^{or\text{-}J/or\text{-}J}$ embryo, however, we unexpectedly found that both the *Mitf* mRNA and protein are ectopically expressed in the NR, from at least E10.5 until P0 (Fig. 1B,D,F; data not shown), whereas the expression of *Mitf* in the RPE appears unchanged. The ectopic expression of *Mitf* in the developing *Chx10*$^{or\text{-}J/or\text{-}J}$ NR establishes that one function of *Chx10* in eye formation is to repress, directly or indirectly, the expression of *Mitf* in neuroretinal progenitor cells. This finding is consistent with a report that the CHX10

protein acts as a transcriptional repressor in vitro (Bremner et al., 1997). We investigated the ability of CHX10 to represses human *MITF* directly using *MITF* promoter-driven (Fuse et al., 1996; Takeda et al., 2002; Udono et al., 2000) luciferase reporter gene expression in cultured RPE (D407, ARPE19), melanocyte (melan-a), neural (NG108) or fibroblast (COS7) cells. We examined the *M*-, *D*- and *H-MITF* promoters because each possesses a CHX10 consensus DNA-binding site (TAATTAGG/C/T) (Percin et al., 2000). However, baseline promoter activity was detected only with the *H-MITF* construct. The co-transfection of CHX10 and the *H-MITF* construct did not result in significant repression of the *H-MITF* promoter (data not shown).

The Chx10-mediated repression of *Mitf* expression may normally prevent *Mitf* from implementing an RPE differentiation program in the developing NR. The failure to repress *Mitf* expression in *Chx10*$^{or\text{-}J/or\text{-}J}$ neuroretinal progenitors might therefore be associated with additional evidence of differentiation of the mutant NR towards an RPE cell identity, particularly as high levels of *Mitf* expression can initiate a program leading to partial pigment cell identity (Lister et al., 1999; Planque et al., 1999; Tachibana et al., 1996). Consequently, we examined the expression in the *Chx10*$^{or\text{-}J/or\text{-}J}$ optic cup of five other RPE genes whose transcripts are not normally detected in the early developing NR: the pigment cell-specific Mitf-activated melanogenesis genes *Tyr*, *Tyrp1* and *Dct,* as well as *Otx1* and *Otx2*, which together act to regulate RPE cell identity (Martinez-Morales et al., 2001). The expression of all five genes was normal in the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR (Fig. 2B,D,F; data not shown). However, the domain of expression of *Mitf* (Nguyen and Arnheiter, 2000), *Tyr*, *Tyrp1* and *Dct* (Zhao and Overbeek, 1999) that defines the presumptive ciliary margin was greatly expanded in the *Chx10* mutant (Fig. 1D,F; Fig. 2B,D,F), consistent with the enlarged ciliary margin seen in the adult *Chx10*$^{or\text{-}J/or\text{-}J}$ eye (Tropepe et al., 2000).

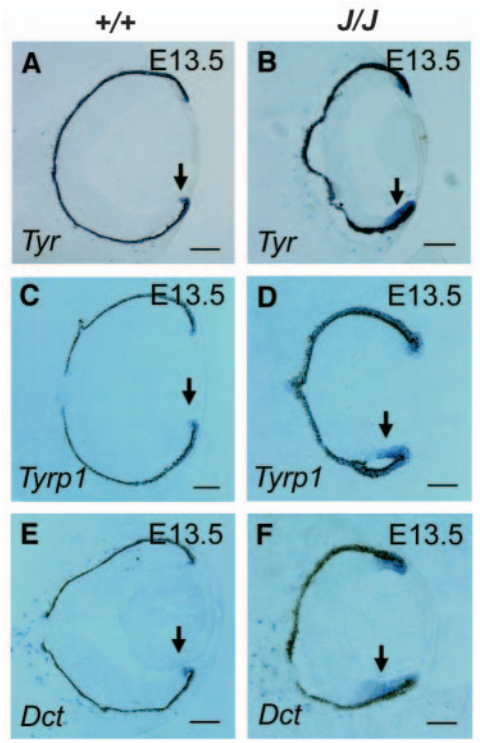

Fig. 2. *Tyr*, *Tyrp1* and *Dct* expression in the cilary margin is expanded in the *Chx10*$^{or\text{-}J/or\text{-}J}$ E13.5 NR. (A) *Tyr* is expressed in a wild-type E13.5 RPE and ciliary margin (arrow). (B) By contrast, in the *Chx10*$^{or\text{-}J/or\text{-}J}$ E13.5 ciliary margin, the *Tyr* expression domain is expanded (arrow). (C) *Tyrp1* is expressed in the wild-type RPE and ciliary margin (arrow). (D) *Tyrp1* expression in the *Chx10*$^{or\text{-}J/or\text{-}J}$ ciliary margin is expanded (arrow). (E) A wild-type section showing expression of *Dct* in the RPE and ciliary margin (arrow). (F) In the *Chx10*$^{or\text{-}J/or\text{-}J}$ ciliary margin, the *Dct* expression domain is enlarged (arrow). Scale bars: 40 μm in A; 32 μm in B,C,E; 25 μm in D,F.

Chx10 and *Mitf* are necessary for their reciprocal mutant eye phenotypes

The ectopic expression of *Mitf* in the *Chx10*$^{or\text{-}J/or\text{-}J}$ mutant retina suggested that some of the phenotypic features of the *Chx10*$^{or\text{-}J/or\text{-}J}$ eye phenotype might be the result of *Mitf* neuroretinal expression. For example, the decreased proliferation of *Chx10*$^{or\text{-}J/or\text{-}J}$ neuroretinal progenitors may reflect a *Mitf*-dependent constraint on proliferation, as *Mitf*-expressing RPE progenitors normally proliferate less than neuroretinal progenitors during optic cup development (Packer, 1967). Conversely, some of the phenotypes observed in the *Mitf*$^{mi/mi}$ mouse eye may require the ectopic *Chx10* expression observed in the RPE. These considerations predicted that the eyes of double *Mitf*$^{mi/mi}$;*Chx10*$^{or\text{-}J/or\text{-}J}$ mutants may have at least partial correction of the phenotypes seen in both *Chx10*$^{or\text{-}J/or\text{-}J}$ and *Mitf*$^{mi/mi}$ mutant eyes. In fact, double *Mitf*$^{mi/mi}$;*Chx10*$^{or/or}$ mutants were reported previously to have increased retinal progenitor cell proliferation and normalization of the size of the NR and RPE (Konyukhov and Sazhina, 1966). To corroborate those findings using mice with a well-defined *Chx10* allele (*or*^{J}), we generated *Mitf*$^{mi/mi}$;*Chx10*$^{or\text{-}J/or\text{-}J}$ mice and confirmed that the eyes of these double mutant animals are larger than those of *Chx10*$^{or\text{-}J/or\text{-}J}$ mice (Fig. 3A-D), that the NR displays near-normal lamination (Fig. 3C,D,D′), and that the dorsal RPE is a normal-appearing monolayer (Fig. 3E-H). The requirement for *Mitf* function in the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR phenotype was also evident from the significant improvement of neuroretinal lamination in *Chx10*$^{or\text{-}J/or\text{-}J}$ mice heterozygous for the *Mitf*^{mi} allele (Fig. 3C). In addition, as expected (Bone-Larson et al., 2000; Green et al., 2003) (e.g. see Fig. 3E-H), inspection of the *Mitf*$^{mi/mi}$;*Chx10*$^{or\text{-}J/or\text{-}J}$ phenotype in two different backgrounds (50% *129/SvJ*; 50% *B6C3He* and >90% *129/SvJ*) confirmed that *Mitf* and *Chx10* interact genetically, that *Mitf* is at least partially necessary for the *Chx10*$^{or\text{-}J/or\text{-}J}$ phenotype, and that *Chx10* is necessary for the *Mitf*$^{mi/mi}$ phenotype.

Chx10 and *Mitf* function antagonistically to regulate retinal cell identity

The repression both of the size of the presumptive ciliary margin and of neuroretinal *Mitf* expression by Chx10 suggested that *Chx10* and *Mitf* may act in opposition during early eye development, a model supported by the rescue in double *Mitf;Chx10* mutant animals of both the RPE and neuroretinal phenotypes. We therefore hypothesized that the role of *Chx10* may be to define or maintain neuroretinal identity either by repressing *Mitf*, or by other mechanisms, and that the role of *Mitf* is to define or maintain an RPE identity.

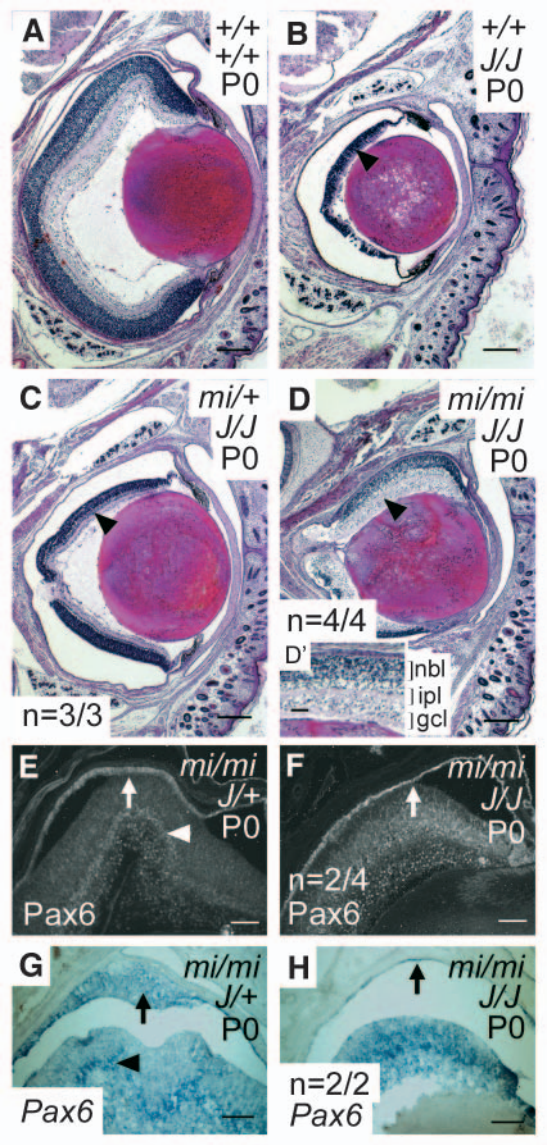

Fig. 3. *Chx10* and *Mitf* are necessary for their reciprocal *Mitf* and *Chx10* mutant retinal phenotypes. (A) A Hematoxylin and Eosin-stained coronal section through a wild-type (+/+; +/+) P0 eye showing normal morphology. (B) An identical Hematoxylin and Eosin-stained section through an *Mitf* wild-type (+/+);*Chx10*$^{or\text{-}J/or\text{-}J}$ (*J/J*) eye showing the thin, hypocellular, non-laminated NR (arrowhead). (C) The loss of one copy of *Mitf* (*mi/+*) on the *Chx10* mutant background (*J/J*) results in a thicker, multicellular, laminated NR (arrowhead). Neuroretinal rescue in the *mi/+;J/J* eye (*n*=3/3). (D) Double *Mitf;Chx10* mutants (*mi/mi;J/J*) have a dramatic normalization of the NR (arrowhead) when compared with the *Chx10*$^{or\text{-}J/or\text{-}J}$ mouse (B). Neuroretinal rescue in the *mi/mi;J/J* eye (*n*=4/4). (D′) An enlargement of the NR (shown in the region of the arrowhead in D) illustrating the lamination of the double mutant NR. nbl, neuroblastic layer; ipl, inner plexiform layer; gcl, ganglion cell layer. (E) *Mitf*$^{mi/mi}$ (*mi/mi*) mice lacking one copy of *Chx10* (*J/+*) express the neuroretinal marker protein Pax6 in a thickened neuroretinal-like layer (NRLL) in the dorsal part of the RPE (arrow) and in the NR (arrowhead). (F) The loss of both copies of *Chx10* (*J/J*) in the *Mitf* mutant background (*mi/mi*) results in a normalization of the thickness of the NRLL in the dorsal RPE [highlighted by the expression of Pax6 (arrow)]. RPE rescue in mice with a background of more than 90% *129/SvJ* (*n*=2/4). (G) In a second genetic background (see below), the *Mitf* mutation (*mi/mi*) results in a more dramatic NRLL in an animal heterozygous for the *Chx10*$^{or\text{-}J}$ mutation (*J/+*). *Pax6* expression is seen in both the NR (arrowhead) and NRLL (arrow). (H) Double *Mitf;Chx10* mutants (*mi/mi;J/J*) have a normalized RPE phenotype, highlighted by the loss of ectopic *Pax6*-expressing tissue in the RPE (arrow). RPE rescue in a mixed *129/SvJ;B6C3He* background (*n*=2/2). Scale bars: 80 μm in A-D; 20 μm in D′; 25 μm in E,F; 31 μm in G,H.

This hypothesis also raises the possibility that the ratio of *Mitf*:*Chx10* gene products in the developing eye, rather than the absolute level of expression of the *Mitf* or *Chx10* genes, is the crucial determinant of retinal cell identity. To examine these concepts, we asked whether the ectopic expression of transgenic human *MITF* in the developing NR, and of human *CHX10* in the developing RPE, were sufficient to determine RPE and neuroretinal cell identity, respectively (see Fig. S1 in the supplementary material).

The eyes of mice either hemizygous (see Fig. S1 in the supplementary material) or homozygous (data not shown) for the *NR-MITF* transgene were normal. Thus, in a wild-type background, the transgenic expression of *MITF* in the developing NR failed to alter retinal cell identity. We then considered that this failure resulted from the activity of the wild-type *Chx10* gene. We therefore increased the gene dosage of neuroretinal *MITF*:*Chx10* by crossing mice carrying the *NR-MITF/+* transgene to animals heterozygous or homozygous for mutant *Chx10*, and examined the resulting retinal phenotypes at P0. In animals hemi-or homozygous for the human *MITF* transgene and carrying one *Chx10* wild-type allele (*NR-MITF/+;Chx10*$^{or\text{-}J/+}$ and *NR-MITF/NR-MITF;Chx10*$^{or\text{-}J/+}$), no changes were observed in the NR (data not shown). By contrast, the NR in *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ individuals in two independent transgenic mouse lines changed dramatically, to a pigmented monolayer or PML (Fig. 4A-D). The PML resembled a mature RPE, not only in being a highly pigmented monolayer but also in not expressing the neuroretinal marker protein, Notch1 (see Fig. S2 in the supplementary material). By contrast, the cells of the PML were still partially neuroretinal in identity, because they expressed both the neural marker NCAM (Fig. 4E,F) and the neuroretinal protein Pax6 (Fig. 4G,H). In addition, the PML has not begun to differentiate into the earliest retinal cell type, the ganglion cell, as it did not express Islet1 (see Fig. S2 in the supplementary material).

To determine whether the formation of the PML in *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ mice represented a change in neuroretinal specification, or a later change in the identity of a properly specified NR because of a failure of maintenance of neuroretinal identity, we examined the developing retinas of *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ mice at E11.5. These retinas were histologically normal, and expressed the neuroretinal markers *Rax* (E11.5; Fig. 4I,J) and *Math5* (E16.5; see Fig. S2 in the supplementary material), but not the RPE marker *Dct* (E11.5; Fig. 4K,L). Thus, the change in neuroretinal phenotype resulted from the ‘transdifferentiation’ of a normally specified NR to a PML after E11.5. The transdifferentiation occurs incrementally, first noticeable as a decrease in the size of the NR and a subsequent increase in pigmentation that is apparent by about E16.5 (see Fig. S3 in the supplementary material) and

appears to progress from a peripheral-to-central fashion, expanding from the ciliary margin region. Taken together, these results demonstrate, first, that an increase in the level of *Mitf:Chx10* gene products in the developing optic cup can dramatically alter the identity of NR progenitors towards an RPE-like phenotype, and second, that the increased expression of *Mitf/MITF* gene products in the developing NR is insufficient to confer an RPE identity on cells that have been specified to become NR.

We similarly found that the eyes and retinas of animals either hemizygous (see Fig. S1 in the supplementary material) or homozygous (data not shown) for the *RPE-CHX10* transgene in a wild-type background were also normal. Thus, either the absolute level of *CHX10* expression achieved in the developing RPE of wild-type mice, or the ratio of *CHX10:Mitf* gene products, was insufficient to alter cell identity. To evaluate the latter possibility, we increased the ratio of *RPE-CHX10* transgene:wild-type *Mitf* by crossing mice carrying the *RPE-CHX10* transgene onto mice heterozygous for a semi-dominant mutant allele of *Mitf*, the *mi* allele. At P0, the retinas of *RPE-CHX10/+;Mitf*$^{mi/+}$ mice had a significant decrease in the overall level of RPE pigmentation compared with *Mitf*$^{mi/+}$ mice (Fig. 4M,N). Most notable, however, was the transformation of the dorsal RPE in *RPE-CHX10/+;Mitf*$^{mi/+}$ animals to a thickened multicellular layer (Fig. 4O-R). This thickened structure was a neuroretinal-like layer (NRLL), as it expressed the progenitor markers Pax6, *Rax*, mouse *Chx10* (Fig. 4S-V) and Notch1 (see Fig. S2 in the supplementary material) and had begun to differentiate, as shown by the presence of Islet1-expressing cells (see Fig. S2 in the supplementary material). The similarity of the NRLL to the transdifferentiated neuroretinal phenotype found in the dorsal RPE of mice with a severe loss of *Mitf* function is striking (Nguyen and Arnheiter, 2000; Packer, 1967). All of the above findings suggest that *Mitf* and *Chx10* act antagonistically in the developing eye, and that the

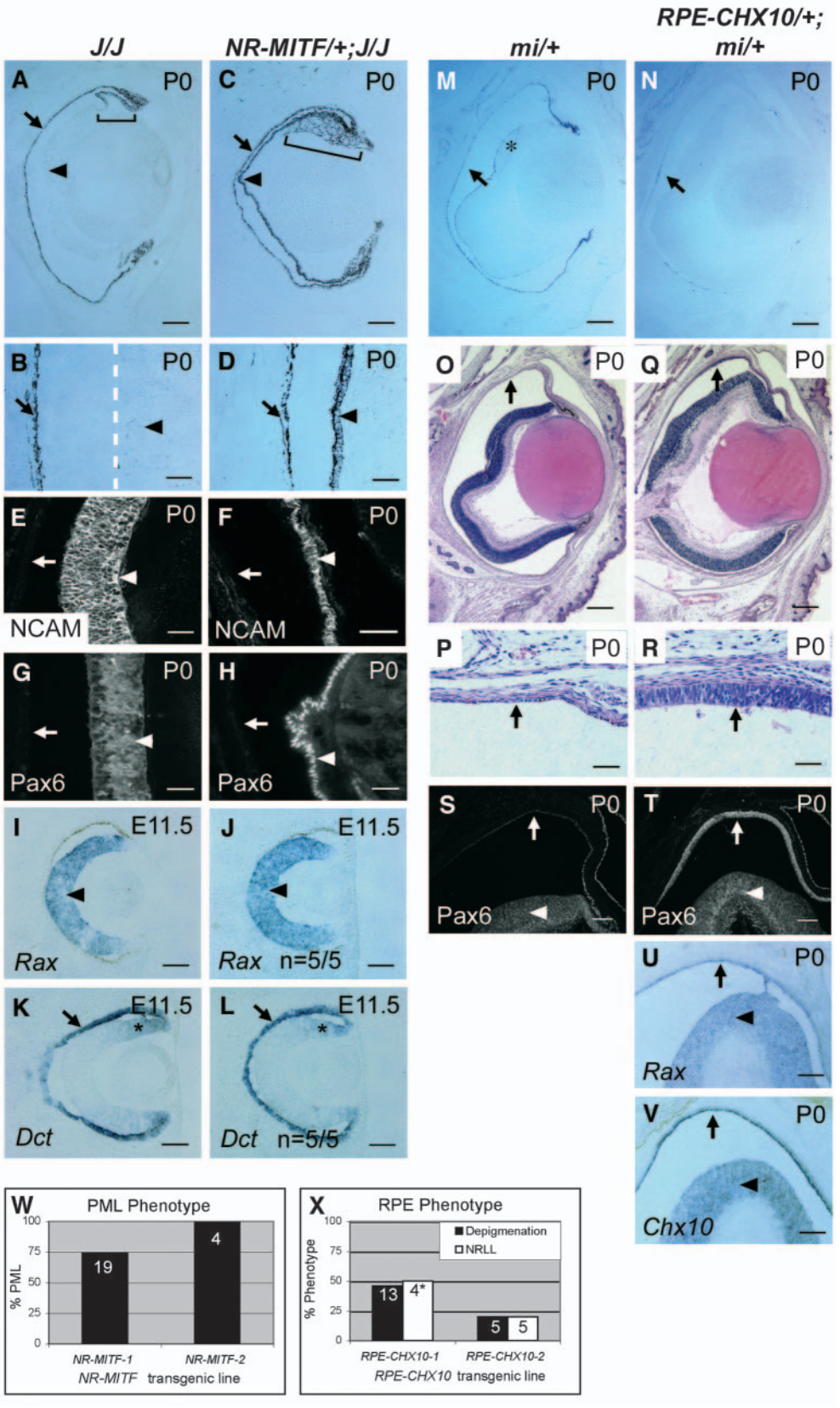

Fig. 4. *Mitf* and *Chx10* function together in a dose-dependent antagonistic fashion to regulate retinal cell identity. (A) An unstained control *Chx10*$^{or\text{-}J/or\text{-}J}$ retinal section at P0 (labeled *J/J* above the column of panels) shows pigmentation only in the RPE monolayer (arrow) and in the ciliary margin (square bracket). The NR (arrowhead) is unpigmented. (B) An enlargement of (A) displays the pigmented RPE (arrow) and non-pigmented NR (arrowhead). The broken white line indicates the edge of the NR. (C) *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ littermates have a normal RPE (arrow), greatly expanded ciliary margin (square bracket) and a pigmented monolayer (PML) instead of a NR (arrowhead). (D) An enlargement of (C) shows the pigmented RPE (arrow) and the PML (arrowhead). (E) The *Chx10*$^{or\text{-}J/or\text{-}J}$ NR (arrowhead) expresses the neural-specific cell-adhesion molecule NCAM, while the RPE does not express NCAM (arrow). (F) The PML also expresses NCAM (arrowhead); the RPE does not (arrow). (G) The *Chx10*$^{or\text{-}J/or\text{-}J}$ NR expresses Pax6 (arrowhead), while the RPE does not (arrow). (H) The nuclei of the PML contains the neuroretinal marker Pax6 (arrowhead) in contrast to the RPE, which lacks Pax6 (arrow). Pax6 subcellular localization changes compared with that in a *Chx10*$^{or\text{-}J/or\text{-}J}$ NR (G), which we verified with a second independent Pax6 antibody (data not shown). The mechanism mediating the differential localization is unknown, and its significance is unclear. (I) In situ hybridization of a *Chx10*$^{or\text{-}J/or\text{-}J}$ eye at E11.5 shows normal neuroretinal *Rax* expression (arrowhead). (J) A *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ littermate also expresses *Rax* in the NR. Normal *Rax* expression (arrowhead) at E11.5 in *NR-MITF-1, n*=5/5. (K) A *Chx10*$^{or\text{-}J/or\text{-}J}$ animal expresses *Dct* in the RPE (arrow) and presumptive ciliary margin (asterisk). (L) An identical *Dct* expression pattern is seen in *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ littermates. Normal *Dct* or *Tyr* (not shown) expression at E11.5 in *NR-MITF-1, n*=5/5. (M) An unstained P0 coronal section of an *Mitf*$^{mi/+}$ eye (labeled *mi/+*) showing RPE pigmentation. The pigmentation present in the NR (asterisk) is an artifact of the dissection; it is RPE tissue that has adhered to the NR. (N) *RPE-CHX10/+;Mitf*$^{mi/+}$ littermates have a dramatic decrease in pigmentation in the RPE (arrow). (O) Hematoxylin and Eosin-stained P0 coronal section of an *Mitf*$^{mi/+}$ eye has a normal RPE (arrow). (P) A higher magnification view of (O) shows the RPE. (Q) *RPE-CHX10/+;Mitf*$^{mi/+}$ littermates have a morphological change in the dorsal RPE; the RPE monolayer has become a thickened, multicellular structure (arrow). (R) An enlargement of Q illustrates the thickened multicellular dorsal RPE (arrow). (S) The *Mitf*$^{mi/+}$ NR expresses the homeodomain protein Pax6 (arrowhead), while the RPE expresses very low levels of Pax6 (arrow). (T) Pax6 is present in the thickened RPE (arrow) as well as the NR (arrowhead) in *RPE-CHX10/+;Mitf*$^{mi/+}$ mice, suggesting that this RPE structure is a NRLL. (U) Both the NR (arrowhead) and the NRLL (arrow) in *RPE-CHX10/+;Mitf*$^{mi/+}$ mice express *Rax* mRNA. (V) The NRLL in *RPE-CHX10/+;Mitf*$^{mi/+}$ mice expresses mouse *Chx10* (arrow), as does the NR (arrowhead). (W) Graphical representation of the percentage frequency of the PML phenotype in different *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ transgenic lines. The numbers within the bars indicate the number of individuals examined for each transgenic line. *NR-MITF-1*, *n*=14/19; *NR-MITF-2*, *n*=4/4. (X) Graphical representation of the percent frequency of changes in RPE phenotype in the *RPE-CHX10/+;Mitf*$^{mi/+}$ transgenic lines. The depigmentation and NRLL phenotypes are shown. Numbers within the bars indicate the number of individuals tested. *RPE-CHX10-1*, depigmentation, *n*=6/13; four animals with no pigment were tested for the presence of an NRLL: *n*=2/4 (denoted by the asterisk). *RPE-CHX10-2,* both depigmentation and NRLL phenotypes: *n*=1/5. Scale bars: 50 μm in A,C; 25 μm in B,D-L,S,T; 80 μm in M,N,O,Q; 12.5 μm in P,R,U,V.

levels of *Mitf:Chx10* gene products in the optic cup are major determinants of retinal development.

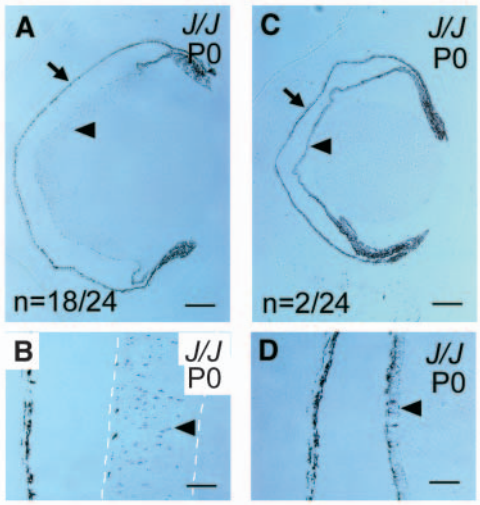

Fig. 5. *Chx10* is necessary to maintain neuroretinal cell identity. (A) The majority (*n*=18/24) of *Chx10*$^{or\text{-}J/or\text{-}J}$ (*J/J*) individuals from a mixed genetic background (*129/SvJ;C57BL/6*) have a 'salt-and-pepper' neuroretinal phenotype consisting of pigmented and non-pigmented cells (arrowhead) and a normal RPE (arrow). (B) A higher magnification view of A clearly shows the cellular pigment phenotype in the NR (arrowhead). The broken white lines indicate the edges of the NR. For comparison, a *129/SvJ Chx10*$^{or\text{-}J/or\text{-}J}$ non-pigmented NR is shown in Fig. 4A,B. (C) A subset of *Chx10*$^{or\text{-}J/or\text{-}J}$ mice (*n*=2/24) from a mixed genetic background have a PML (arrowhead) instead of a NR and a normal RPE (arrow). (D) An enlargement of (C) focusing on the PML (arrowhead). Scale bars: 50 μm in A,C; 12.5 μm in B,D.

Chx10 is necessary for neuroretinal cell identity

The modification by *Mitf* of the ability of *Chx10* to define the phenotype of the NR, together with the importance of genetic background in the phenotype of the *Chx10* mutant eye (Bone-Larson et al., 2000; Green et al., 2003), suggested that in some genetic backgrounds the failure to maintain neuroretinal identity may result from the loss of *Chx10* function alone. To evaluate this possibility, we examined the eyes of P0 mice with a mixed *129/SvJ;C57Bl/6;SJL* (~75%;20%;5%) mouse background that resulted from crossing *NR-MITF/+* and *Chx10*$^{or\text{-}J/or\text{-}J}$ animals. The majority of *Chx10*$^{or\text{-}J/or\text{-}J}$ mice in this mixed background had neuroretinas with a mixture of pigmented and non-pigmented cells (a 'salt and pepper' phenotype) (Fig. 5A,B), suggesting that the RPE and neuroretinal cells are properly specified, but are incorrectly organized within the OV. Remarkably, however, in a small fraction (~8%) of *Chx10*$^{or\text{-}J/or\text{-}J}$ mice with this mixed background, the NR was replaced by a PML phenotype comparable with the PML of *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ animals (Fig. 5C,D). Altogether, these results establish, first, that *Chx10* is required, at least in some genetic contexts, not for the specification of neuroretinal cell identity, but for neuroretinal organization and maintenance; and, second, our work demonstrates that the level of expression of the MITF protein is critical in transforming the NR to an RPE-like structure, as this phenotype is seen infrequently in *Chx10*$^{or\text{-}J/or\text{-}J}$ mice, but in most *Chx10*$^{or\text{-}J/or\text{-}J}$ mice carrying a *MITF* transgene.

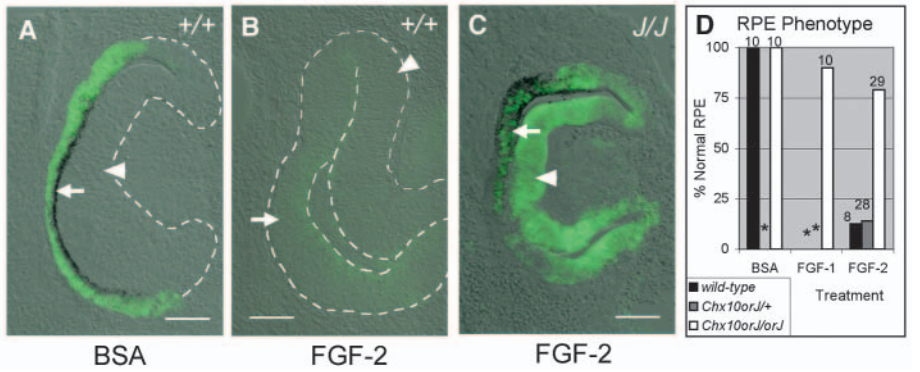

Fig. 6. *Chx10* lies downstream of FGF in mouse neuroretinal cell identity decisions. (A) An OV culture from a wild-type animal (+/+) dissected at E9-9.5 and grown for 3 days in the presence of a bovine serum albumin-coated bead (BSA) results in normal development of the NR (arrowhead) and RPE (arrow), including pigmentation and expression of Mitf protein (green). (B) By contrast, a culture of wild-type OV in the presence of an FGF2-coated bead results in a change in cell identity from an RPE to a NR (arrow), as evidenced by a loss of pigmentation and greatly reduced Mitf expression, although the NR appears normal (arrowhead). (C) A $Chx10^{or\text{-}J/or\text{-}J}$ OV cultured with an FGF2-coated bead has no effect on the identity of the RPE, as shown by the pigmentation of the RPE and the expression of Mitf (arrow). The ectopic Mitf is clearly apparent in the $Chx10^{or\text{-}J/or\text{-}J}$ NR (arrowhead). (D) Graphical representation of the percent frequency of normal RPE pigmentation in optic vesicle cultures incubated with beads coated in BSA, FGF1 or FGF2. Genotype is indicated by bar color (black, wild-type; grey, $Chx10^{or\text{-}J/+}$; white, $Chx10^{or\text{-}J/or\text{-}J}$). The asterisks represent experiments that were not performed, rather than a value of zero. The numbers above the bars indicate the number of individuals tested. RPE pigmentation: +/+ BSA, 10/10; +/+ FGF2, 1/8; *J/J* FGF2, 23/29; *J/J* BSA, 10/10; *J/J* FGF1, 9/10; *J/+* FGF2, 4/28. Scale bars: 20 μm in A-C.

Chx10 lies downstream of FGF in neuroretinal cell identity maintenance

FGF has been shown to induce and organize neuroretinal cells (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Park and Hollenberg, 1989), and has recently been shown to repress the expression of *Mitf* in the OV (Nguyen and Arnheiter, 2000). Our demonstration that *Chx10* is also required to repress *Mitf* expression in the developing NR suggested that FGF lies upstream of *Chx10* and *Mitf* in regulating retinal identity decisions, and that FGF represses *Mitf* by first activating the expression of *Chx10* in the OV. This model is supported by the fact that the removal of surface ectodermal FGF leads to the formation of 'salt and pepper' neuroretinas (Hyer et al., 1998), similar to those seen in the absence of *Chx10* (Fig. 5A,B). To examine this model, we exposed OV cultures to FGF1 (data not shown) or FGF2 (Fig. 6) through the implantation of FGF-coated beads (Nguyen and Arnheiter, 2000), and examined the expression of *Mitf* in OVs derived from wild-type and $Chx10^{or\text{-}J/or\text{-}J}$ mice. In wild-type mouse OV cultures, the exposure to FGF leads the developing RPE to become an ectopic NR that does not express the Mitf protein (Fig. 6A,B). By contrast, FGF fails to induce an ectopic NR in the OV of $Chx10^{or\text{-}J/or\text{-}J}$ mice, which develops instead into a pigmented RPE that expresses Mitf (Fig. 6C). The dependency of the FGF-induced repression of *Mitf* on *Chx10* suggests that *Chx10* lies downstream of FGF1 or FGF2 in a regulatory pathway that defines neuroretinal identity.

Discussion

Chx10, *Mitf* and the $Chx10^{or\text{-}J/or\text{-}J}$ mutant phenotype

The ectopic expression of *Mitf* in the $Chx10^{or\text{-}J/or\text{-}J}$ NR establishes that *Chx10* represses the neuroretinal transcription of *Mitf*, either directly or indirectly. Chx10-mediated repression of *Mitf* would account for the specific downregulation of *Mitf* expression in the presumptive NR in normal eye development, at the time that *Chx10* expression is initiated (Liu et al., 1994; Nguyen and Arnheiter, 2000). Although we could not demonstrate it, the Chx10-mediated repression of *Mitf* may indeed be direct, but might require eye-specific co-factors. Alternatively, Chx10 may act in the NR through another *Mitf* promoter (Oboki et al., 2002; Takeda et al., 2002; Takemoto et al., 2002).

The absence of ectopic neuroretinal expression of the Mitf-regulated pigmentation enzyme transcripts in the $Chx10^{or\text{-}J/or\text{-}J}$ mouse may reflect low levels of *Mitf* expression, or to the absence of essential co-factors. The lack of ectopic *Otx1* and *Otx2* expression in the *Chx10* mutant NR argues that Chx10 may specifically repress *Mitf*, rather than downregulating an entire RPE developmental program. However, the expansion of the expression domains of *Mitf*, *Tyr*, *Tyrp1* and *Dct* in the ciliary margin of the $Chx10^{or\text{-}J/or\text{-}J}$ eye indicates that *Chx10* is required not only to repress the neuroretinal expression of *Mitf*, but also to constrain the size of the presumptive ciliary margin. Thus, *Chx10* and *Mitf* may regulate ciliary margin size in an antagonistic manner.

We confirmed and extended earlier findings (Konyukhov and Sazhina, 1966) that the expression of *Mitf* modifies the *Chx10* mutant phenotype and contributes to the failure of lamination and the hypocellularity of the *Chx10* mutant. We propose that the proliferation defects in the $Chx10^{or\text{-}J/or\text{-}J}$ NR may result from the shift towards an RPE cell identity, an identity normally associated with decreased proliferation (at least compared to the NR) (Green et al., 2003; Packer, 1967). The Chx10-mediated regulation of proliferation is mediated, at least partially, through $p27^{kip1}$ (Green et al., 2003), and may also be Mitf dependent. Consistent with a possible Mitf-dependent regulation of $p27^{kip1}$, $p27^{kip1}$ is expressed in the developing RPE (Defoe and Levine, 2003) and Mitf directly stimulates p21 (a $p27^{kip1}$ family member) (Goding et al., 2004).

We also demonstrated that *Chx10* is required for the RPE-hyperproliferation phenotype of the $Mitf^{mi/mi}$ mutant, suggesting that Mitf may normally repress *Chx10* activity in the developing RPE. However, *Chx10* is not ectopically expressed throughout the entire RPE in *Mitf* mutants early in eye development (data not shown), arguing that the repression of Chx10 activity in the $Mitf^{mi/mi}$ RPE is not mediated by the repression of *Chx10* expression. Mitf may therefore repress neuroretinal cell identity in the developing RPE by some other mechanisms that repress both Chx10 activity indirectly and the

function and/or expression of other neuroretinal specification genes also expressed in the *Mitf*$^{mi/mi}$ RPE (Nguyen and Arnheiter, 2000). Finally, the relatively normal eye that results from the *Chx10:Mitf* double mutant emphasizes that although *Chx10* and *Mitf* are essential for the maintenance of retinal identity, other genes must also be involved in these processes and can at least partially compensate for loss of *Chx10* and *Mitf*.

Chx10 and *Mitf* function antagonistically in a pathway with FGF to regulate retinal cell maintenance

Through the use of transgene expression in *Chx10* and *Mitf* mutant mice, we established that *Chx10* and *Mitf* act antagonistically during development to regulate the acquisition and/or maintenance of retinal cell identity. However, neither transgenic RPE *CHX10* nor neuroretinal *MITF* were sufficient to alter retinal cell identity in a wild-type context. The levels of the *NR-MITF* and *RPE-CHX10* transgenes, their spatiotemporal expression patterns, or the cellular environment may have limited the effects of the transgenes. Although high levels of *Mitf* expression are sufficient to establish pigment cell identity in some contexts (Lister et al., 1999; Planque et al., 2004; Planque et al., 1999; Tachibana et al., 1996), the levels of ectopic *Mitf* expression achieved in those studies (using strong promoters) are likely to have been higher than those obtained in the NR from the human *PAX6* enhancer we used (Kammandel et al., 1999). Similarly, the *Dct* promoter-enhancer *RPE-CHX10* construct we used was unable to change RPE identity in wild-type mice. This result contrasts with the finding that a *Dct* promoter-enhancer construct driving RPE *Fgf9* or *Ras* conferred an RPE-to-NR change in a wild-type background (Zhao and Overbeek, 1999). The differences between these results may be due to variations in transgene expression levels or to differences in the roles of *Chx10* versus *Fgf9* and *Ras* in neuroretinal development. For example, ectopic RPE *Fgf9/Ras* may mimic the combined role of various FGFs or other ligands activating receptor tyrosine kinases [e.g. epidermal growth factor (EGF), see below] that confer neuroretinal cell identity.

We established that *Chx10* is required for neuroretinal maintenance, at least in some genetic backgrounds, as a PML instead of a differentiated NR formed in *Chx10*$^{or\text{-}J/or\text{-}J}$ mutants. A similar PML was observed in the great majority (74-100%) of *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ animals in which the dose of *MITF:Chx10* genes was high. In these transgenic mice the NR was properly specified and initially normal in appearance, but after E11.5 it transdifferentiated into a PML. We suggest that the transdifferentiation to a PML of most of the *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ neuroretinas is likely to reflect the higher level of Mitf expression from the combined neuroretinal expression of the endogenous *Mitf* gene and the *MITF* transgene, in contrast to the formation of a PML in only a small fraction (8%) of *Chx10*$^{or\text{-}J/or\text{-}J}$ mutants lacking the *NR-MITF* transgene. Furthermore, the formation of a PML in the majority of *NR-MITF/+;Chx10*$^{or\text{-}J/or\text{-}J}$ animals suggests that the levels and/or activity of the Mitf pathway may be the major or sole variable in the *Chx10*$^{or\text{-}J/or\text{-}J}$ genetic background that led to the formation of a PML in some animals. The failure to obtain a fully differentiated RPE from the transgenic expression of *MITF* may be due to the fact that the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR has been specified before the onset of *MITF* expression from the transgene at ~E9.5 (Kammandel et al., 1999). The mixed RPE/neuroretinal cellular phenotype of the PML may be that of an OV-type cell. Alternatively, it may indicate an additional role for Mitf and Chx10 in the development of the ciliary margin, which forms the boundary of the NR and RPE. In any case, these results provide strong evidence that *Chx10* and *Mitf* act antagonistically in the regulation of retinal cell identity.

The inability of FGF to change the RPE to a NR in *Chx10*$^{or\text{-}J/or\text{-}J}$ optic vesicles indicates that Chx10 transduces the surface ectodermal FGF signal and represses *Mitf* in the OV. Our proposal that FGF and *Chx10* act within a single pathway is supported by the similar neuroretinal phenotypes – a 'salt and pepper' mixture of pigmented (presumed RPE) cells and un-pigmented (presumed NR) cells – observed in both the majority of *Chx10*$^{or\text{-}J/or\text{-}J}$ mutants in a mixed genetic background and in developing chick eyes from which the proposed FGF source (the surface ectoderm) was removed (Hyer et al., 1998). That cells of the OV lacking either *Chx10* or an FGF signal appear to become either NR or RPE cells further argues that the FGF→Chx10⊣Mitf pathway we identified is necessary for neuroretinal maintenance rather than specification. The disorganization of the neuroretinal and RPE

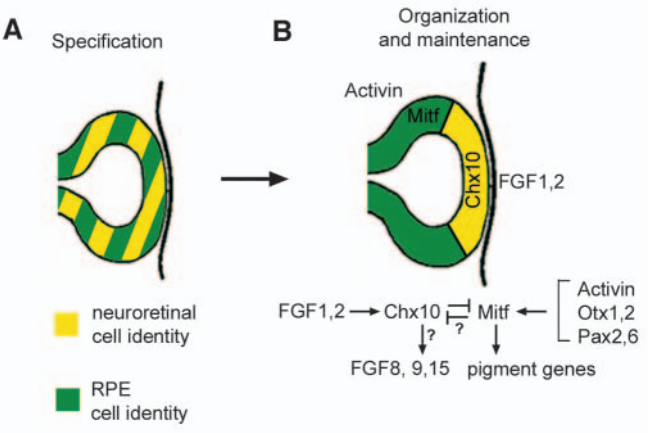

Fig. 7. A model of the specification, organization and maintenance of vertebrate retinal cells. (A) Retinal cell type specification occurs prior to the OV stage. By the OV stage depicted here, retinal cells are already biased to become either RPE or neuroretinal cells, as shown by the striped green and yellow region. (B) When the surface ectoderm comes in close contact with the OV, FGF1 and/or FGF2 from the surface ectoderm signals through Chx10 to organize the NR, adjacent to the future lens. Chx10 then represses the expression of *Mitf* (directly or indirectly) to maintain neuroretinal cell identity, perhaps through the regulation of *Fgf8*, *Fgf9* and/or *Fgf15* and other neuroretinal genes. Cells that are distant from the surface ectoderm, and thus from FGF1 and/or FGF2, do not express Chx10, allowing *Mitf* expression to continue, thus organizing the RPE at the back of the developing eye. The RPE, by contrast, appears to be organized by activin signals from the posterior ocular mesenchyme (Fuhrmann et al., 2000), signals that may act through *Mitf* and other genes essential for RPE formation, such as *Otx1*, *Otx2* (Martinez-Morales et al., 2001), *Pax2* and *Pax6* (Baumer et al., 2003). Mitf appears to maintain RPE cell identity by the activation of downstream genes, such as pigmentation enzyme-encoding genes. Finally, although the mechanism is unknown, Mitf may also negatively regulate *Chx10* to maintain RPE cell identity.

cells in optic vesicles without FGF or *Chx10* also highlights the need for this pathway in organizing and patterning the already-specified NR and RPE in the developing OV.

The role of FGF in patterning and organizing the OV was defined by in vitro loss-of-function studies (Hyer et al., 1998; Nguyen and Arnheiter, 2000). By contrast, FGF overexpression induces the transdifferentiation of RPE into NR (Fig. 6B) (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Park and Hollenberg, 1989). This contradiction may be resolved by a model in which FGFs function redundantly in eye development, consistent with the lack of phenotypes in *Fgf1* and/or *Fgf 2* mutants (Dono et al., 1998; Miller et al., 2000; Ortega et al., 1998). In this model, some FGFs, including at least FGF1 and FGF2 from the surface ectoderm (Nguyen and Arnheiter, 2000) organize and pattern the OV by activating *Chx10*. Subsequently, *Chx10* or other FGF-induced genes in the NR may then activate *Fgf8* (Vogel-Hopker et al., 2000), *Fgf9* (Zhao et al., 2001) and/or *Fgf15* (McWhirter et al., 1997; Thut et al., 2001) to further develop and maintain the NR. It is entirely conceivable, however, that FGF8, FGF9 or FGF15 also act upstream of Chx10, as they will activate the same signaling cascades as FGF1 or FGF2. Indeed, FGFs themselves may not be the surface ectodermal signals that organize the OV. Rather, the FGFs may mimic another surface ectodermal ligand by activating the FGF receptor tyrosine kinase signaling pathway. However, no other known ligand is capable of causing RPE-to-NR transdifferentiation (including EGF, insulin, NGFβ, IGF1, IGFII and TGFβ1) (Nguyen and Arnheiter, 2000; Park and Hollenberg, 1991; Pittack et al., 1991), although EGF can substitute for FGF or surface ectoderm to organize the OV (Nguyen and Arnheiter, 2000). We suggest, therefore, that FGFs or other unidentified molecule(s) organize the OV by activating Chx10, which then represses *Mitf*.

The mechanisms regulating retinal cell identity

On the basis of our work, we propose that major function of Chx10 is to prevent *Mitf* from imposing an RPE cell identity on the developing NR (Fig. 7). Alternatively, Chx10 may also have pro-neuroretinal functions apart from the repression of *Mitf*, and the balance of the Chx10-dependent neuroretinal and Mitf-dependent RPE regulatory activities is a key determinant of retinal cell identity. Although *Chx10* and *Mitf* are essential for the maintenance of retinal cell identity, it has been established that other genes participate in regulating cell identity, including the early eye transcription factor *Six6/Optix* (Toy et al., 1998), FGF family members, *Gas1* (Lee et al., 2001) and *Ap2a* (West-Mays et al., 1999).

A recent study, complimentary to the work reported here, has also demonstrated that the *Chx10*$^{or\text{-}J/or\text{-}J}$ NR undergoes transdifferentiation to become an RPE-like structure (Rowan et al., 2004).

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Supplementary material

Supplementary material for this article is available at
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