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Ptf1a determines horizontal and amacrine cell fates during mouse retinal development

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The vertebrate neural retina comprises six classes of neurons and one class of glial cells, all derived from a population of multipotent progenitors. There is little information on the molecular mechanisms governing the specification of cell type identity from multipotent progenitors in the developing retina. We report that Ptf1a, a basic-helix-loop-helix (bHLH) transcription factor, is transiently expressed by post-mitotic precursors in the developing mouse retina. Recombination-based lineage tracing analysis in vivo revealed that Ptf1a expression marks retinal precursors with competence to exclusively produce horizontal and amacrine neurons. Inactivation of *Ptf1a* leads to a fate-switch in these precursors that causes them to adopt a ganglion cell fate. This mis-specification of neurons results in a complete loss of horizontal cells, a profound decrease of amacrine cells and an increase in ganglion cells. Furthermore, we identify *Ptf1a* as a primary downstream target for Foxn4, a forkhead transcription factor involved in the genesis of horizontal and amacrine neurons. These data, together with the previous findings on Foxn4, provide a model in which the *Foxn4-Ptf1a* pathway plays a central role in directing the differentiation of retinal progenitors towards horizontal and amacrine cell fates.

KEY WORDS: Retinal development, Basic helix-loop-helix, Amacrine cell, Horizontal cell, Ganglion cell, Lineage tracing, Ptf1a, Foxn4, Progenitor, Cell specification

INTRODUCTION

The vertebrate neural retina contains six types of neurons and one type of glial cells forming three cellular layers: (1) the rod and cone photoreceptors in the outer nuclear layer (ONL); (2) the horizontal, bipolar and amacrine neurons and Müller glia in the inner nuclear layer (INL); and (3) the ganglion and displaced amacrine cells in the ganglion cell layer (GCL). The molecular mechanisms governing generation of such diverse cell types in the developing neural retina are far from completely understood. Because retina has a relatively simple structure, mimics normal development in isolated explant cultures, and is easily accessible, it is a good model system for investigating the molecular mechanisms of neuronal subtype specification. The seven types of retinal cells are differentiated from common progenitors in an order largely conserved among many species: ganglion cells first and Müller glial cells last (Cepko et al., 1996; Young, 1985). It has been postulated that, in response to changes of intrinsic and extrinsic cues, retinal progenitors undergo a series of changes in competence to give rise to the various retinal cell types (Cepko, 1999; Harris, 1997; Livesey and Cepko, 2001). Recent advances in molecular genetic approaches have begun to unravel the molecular bases that underlie the determination and differentiation of different retinal cell types, including horizontal and amacrine cell.

Despite extensive studies, the precise molecular mechanism for horizontal cell type specification is far from completely elucidated. In particular, the bHLH genes that regulate horizontal cell differentiation remain to be determined. Horizontal cell genesis is significantly impaired in *Mash1*(*Ascl1*)*;Ngn2*(*Neurog2*)*;Math3*(*Neurod4*) and *Ngn2;Math3;Neurod1* triple mutant retinas, but not in single or double mutant retinas (Akagi et al., 2004). It is therefore likely that, in retinas containing single or double mutations for bHLH genes, other known and unknown neurogenic bHLH genes may compensate and allow horizontal cell development. The roles for two other transcription factors during horizontal cell development are more clearly defined. Prox1 is a crucial intrinsic factor that controls fate commitment of horizontal cells (Dyer et al., 2003). And we have shown that the forkhead/winged helix transcription factor Foxn4 plays an essential role in horizontal cell generation, as *Foxn4*-null retinas completely lack horizontal cells (Li et al., 2004a).

The specification of amacrine cells is found to depend on several transcription factors. Foxn4 appears to confer retinal progenitors with the competence for an amacrine cell fate in part by activating the expression of *Math3* (*Neurod4* – Mouse Genome Informatics)and *Neurod1* (Li et al., 2004a). In mice deficient for both *Neurod1* and *Math3*, a complete loss of amacrine cells is accompanied by a fate-switch of progenitors to ganglion and Müller cells (Inoue et al., 2002), whereas the formation of amacrine cells is essentially normal in single null mutants for either *Neurod1* or *Math3* (Inoue et al., 2002; Morrow et al., 1999). Therefore, the current model is that Math3 and NeuroD1 are redundantly required

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downstream of Foxn4 for specifying amacrine cells. Overexpression studies have also confirmed the important role of these three factors during amacrine cell development (Inoue et al., 2002; Li et al., 2004a; Morrow et al., 1999). Aside from the bHLH factors, the Pax6 and Barhl2 homeodomain factors are expressed by differentiating and mature amacrine cells, and have been implicated in the specification and/or differentiation of glycinergic amacrine cells (Marquardt et al., 2001; Mo et al., 2004).

Ptf1a (pancreas transcription factor 1a, also known as *Ptf1a-p48*), which encodes a bHLH factor, functions during pancreas development to drive undifferentiated foregut endoderm cells towards the pancreatic fate (Kawaguchi et al., 2002; Krapp et al., 1998). Recently, *Ptf1a* mutations were linked to the human permanent neonatal diabetes mellitus associated with cerebellar ataxia (Sellick et al., 2004). Mice mutant for *Ptf1a* result in cerebellar hypoplasia caused by the specific inhibition of GABAergic neuron production from the cerebellar ventricular zone (VZ) (Hoshino et al., 2005), and Ptf1a is also a crucial factor for the development of GABAergic neurons in the spinal cord dorsal horn (Glasgow et al., 2005). Expression of *Ptf1a* in developing zebrafish retinas suggests a possible role for Ptf1a in the specification and differentiation of retinal neurons (Lin et al., 2004; Zecchin et al., 2004).

Here, we report that Ptf1a is expressed by a subset of post-mitotic precursors in the developing mouse retina VZ and acts as a fate determinant to drive these precursors to differentiate into horizontal and amacrine neurons. Loss of *Ptf1a* function causes a conversion of horizontal/amacrine precursors into a ganglion cell fate. The essential role of Ptf1a in specifying horizontal and amacrine cells is thus non-redundant with other factors, and as such we have identified a key trigger factor that is a primary downstream target for Foxn4, which regulates progenitor competence for horizontal and amacrine neurons.

MATERIALS AND METHODS

Animals

Animal experiments were carried out in accordance with the guidelines of the Vanderbilt University Animal Care and Use Committee. *Ptf1a*^{Cre} mutant mice have a knock-in *Ptf1a* null allele, where the *Ptf1a*-coding region is replaced by Cre-recombinase (Kawaguchi et al., 2002). The *Ptf1a*^{Citrine} mutant mice also have a knock-in *Ptf1a null* allele, which drives citrine expression, instead of Ptf1a, under the control of the endogenous *Ptf1a* promoter. Details on construction of this allele will be described elsewhere (J. Burlison and M.A.M., unpublished). The reporter strains *R26R* and *R26R-EYFP* were used to visualize cells and progeny that were exposed to Cre recombinase activity (Soriano, 1999; Srinivas et al., 2001).

X-gal staining

Embryo tissues were fixed for 1 hour in 4% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C and washed three times with 0.1 M sodium phosphate buffer (pH 7.4) for 30 minutes. Tissues were sunk in 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C, embedded in OCT and cryosectioned at 16 μm. Adult mice were perfused (10 minutes) with 0.1 M sodium phosphate buffer (pH 7.4) containing 4% paraformaldehyde, 2% glutaraldehyde and 30% sucrose. Tissues were embedded in OCT and cryosectioned at 16 μm. Cryosections were incubated at 37°C for between 4 hours to overnight in X-gal staining solution [0.1 M sodium phosphate buffer (pH 7.4), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal].

Immunohistochemical analysis

Appropriately staged embryos and 3-week-old retinas were fixed for 30 minutes to 1 hour at 4°C in 4% paraformaldehyde. Tissues were washed several times with phosphate-buffered saline (PBS), sunk in 30% sucrose overnight, embedded in OCT and cryosectioned. Immunofluorescence was carried out on 14-16 μm sections using: mouse monoclonal antibodies specific to BrdU (Megabase Research Products), calbindin-D-28K (Sigma), syntaxin (HPC-1; Sigma), rhodopsin (RET-P1; Sigma), GAD65, Lim1 (Developmental Studies Hybridoma Bank; Iowa) and CRALBP (ABR); rabbit polyclonal antibodies to Ptf1a (Li and Edlund, 2001), recoverin (Chemicon), calretinin (Chemicon), GABA (Sigma), tyrosine hydroxylase (Chemicon), Prox1 (Chemicon) and GFP (Molecular Probes); goat polyclonal antibodies to Brn3b (Santa Cruz) and Glyt1 (Chemicon); and a sheep polyclonal antibody to Chx10 (Exalpha). Cy2- or Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Antibody concentrations and use available upon request (Y.F.). Some samples were counterstained with the nuclear dye, YO-PRO-1 (Molecular Probes) or DAPI (Vector Labs). Immunofluorescence was imaged on a Zeiss LSM 510 confocal microscope. For BrdU experiments, BrdU (Sigma; 200 μg/g body weight) was injected into pregnant mothers 1 hour before sacrifice. Double immunofluorescence of Pft1a and BrdU was performed by completing immunofluorescence of Ptf1a, followed by treating sections with 2.0 M HCl for 15 minutes, 0.1 M sodium borate (pH 8.5) for 20 minutes, before incubating with the primary BrdU antibody. TUNEL assays were performed using the *In situ* Apoptosis Detection Kit (Takara) according to the manufacturer's instruction.

Quantification of Brn3b-positive cells

Embryonic day 18.5 (E18.5) retinal sections were immunostained with goat anti-Brn3b antiserum. Five animals were analyzed for each genotype (wild type versus *Ptf1a*-null). For each animal, cell counting was performed on four nonadjacent optic-nerve-containing sections, to avoid double scoring. Three non-overlapping fields (230 μm × 230 μm) of central retina, with the inner-most edge of GCL included at one edge of each field were photographed from each section using the confocal microscope and printed at 400× magnification to count Brn3b-positive cells. Comparison of Brn3b$^+$ cell numbers was made by unpaired *t* test. Quantification of TUNEL-positive cells was also performed in the same method.

In situ hybridization

RNA in situ hybridization (Sciavolino et al., 1997) used digoxigenin-labeled riboprobes prepared following the manufacturer's protocol (Roche Diagnostics). The full-length cDNAs of *Foxn4, Math3, Neurod1, Math5* (Li et al., 2004a), and a partial 3′ *Ptf1a* cDNA were used as probes. The *Ptf1a* probe was subcloned after PCR amplification using the following primers: 5′-AGTCCATCAACGACGCCTTCGA-3′ and 5′-ACAAA-GACGCGGCCGACCCGATGTGAG-3′.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from four each of E14.5 *Foxn4*$^{+/-}$ and *Foxn4*$^{-/-}$ retinas as described (Li et al., 2004b). qRT-PCR was performed in duplicate for each RNA sample (100 ng) using the QuantiTect SYBR green one-step RT-PCR kit (Qiagen). The following sequence-specific primers were designed using the MacVector software (Accelrys): *Foxn4*, 5′-CGA-CAAGATGGAGGAGGAGAT-3′ and 5′-CTTGTCCAACTCCTCAGG-GTT-3′; *Ptf1a*, 5′-GCACTCTCTTTCCTGGACTGA-3′ and 5′-TCCAC-ACTTTAGCTGTACGGA-3′; *β-actin*, 5′-AGAGGGAAATCGTGCGT-GAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′; and *Gapdh*, 5′-TCACCACCATGGAGAAGGC-3′ and 5′-GCTAAGCAGTTGGTGGT-GCA-3′. PCR products were monitored in real time (Mx4000 multiplex quantitative PCR system; Stratagene), and the threshold cycles (Ct) were determined using the Mx4000 software. Relative quantities were calculated for a target gene transcript in comparison to a reference gene (*β-actin*) transcript as described previously (Pfaffl, 2001). All data were tested for significance using two-sample Student's *t*-test with unequal variances.

Retinal explant culture

The retinal explant culture was performed, as described (Mo et al., 2004; Tomita et al., 1996). The neural retina, with pigment epithelium stripped away, was placed on a chamber filter (Whatman: 25 mm diameter, 0.2 μm pore size) with the ganglion cell layer upwards, which was then transferred to a six-well culture plate. Each well contained 1 ml of culture medium (50% MEM with HEPES, 25% Hank's solution, 25% heat-inactivated horse serum, 200 μM L-glutamine and 5.75 mg/ml glucose). Explants were cultured at 34°C in 5% CO_2, with medium changed every other day.

RESULTS

Expression pattern of Ptf1a during retinogenesis

The spatiotemporal expression pattern of Ptf1a during mouse retinogenesis was assessed by immunofluorescence analysis with a specific anti-Ptf1a antibody (Li and Edlund, 2001), for which the signal specificity was established by the absence of signal on stage-matched retinal tissues from *Ptf1a*$^{Cre/Cre}$ (null) embryos (Fig. 1E,H). Ptf1a expression was not observed at E11.5 (Fig. 1A), and was detectable at E12.5 in the central region of the retina (Fig. 1B). By E14.5, Ptf1a expression had expanded from the center to the entire retina (Fig. 1C and data not shown). At this stage, the developing retina comprises two discrete zones: the outer neuroblastic layer (onbl) and inner neuroblastic layer (inbl). Ptf1a expression was rarely observed in the inbl. Between E16.5 and postnatal day 1 (P1), Ptf1a continued to be expressed strongly in a subset of cells within the outer neuroblastic layer (Fig. 1D,E). Ptf1a expression began to be downregulated by P2 (Fig. 1F) and was undetectable in P6 retinas (Fig. 1G). Ptf1a expression was not detected in late postnatal and adult retinas. The observed time-course of Ptf1a expression is consistent with the transient expression of *Ptf1a* mRNA previously reported in the zebrafish eye (Lin et al., 2004; Zecchin et al., 2004), suggesting an evolutionarily conserved temporal expression profile for this gene.

The onbl of the developing retina consists of a mixture of dividing progenitor cells and newly generated postmitotic neurons/glial cells. To determine the cell-cycle status of Ptf1a-expressing cells, we pulse-labeled S phase cells in the E14.5 retina with BrdU just before fixation and analysis (1 hour labeling period) and performed immunostaining using Ptf1a and BrdU antibodies. Ptf1a-expressing cells appeared to have exited the cell cycle, judged by the absence of co-labeling with BrdU (Fig. 1I). The post-mitotic status of Ptf1a-expressing cells is in contrast to the Prox1-expressing cell population, in which a significant proportion of cells are still dividing at E14.5 (Dyer et al., 2003). Prox1 and Ptf1a most probably mark distinct progenitor/precursor populations, as no co-expression of Ptf1a and Prox1 was observed in the E14.5 retina (data not shown). At E18.5 and P1, newborn amacrine cells in the inner half of the retina, which were identified based on expression of differentiation markers such as calretinin, did not express Ptf1a (data not shown). Taken together, these data indicate that the expression of Ptf1a is restricted to post-mitotic cells in the VZ of the developing retina, and in a pattern suggestive of a neuronal precursor population.

Ptf1a-expressing precursors are restricted to horizontal and amacrine cell lineages

To gain insight into the cell types in the adult retina that are derived from *Ptf1a*-expressing precursors, we performed genetic lineage tracing by Cre-mediated reporter-gene activation. We previously generated a *Ptf1a*^{Cre} knock-in allele in which the *Ptf1a* protein-coding region was precisely replaced by nuclear-targeted Cre recombinase (Kawaguchi et al., 2002). We crossed *Ptf1a*$^{Cre/+}$ with *Gt(ROSA)26Sor*^{tm1sor}*(R26R)* mice, which carry a modified *lacZ* gene driven by the cell type-independent *ROSA26* promoter (Soriano, 1999). In offspring obtained from this cross, *Ptf1a*-driven expression of Cre excises a stop cassette upstream of *lacZ* and activates cell-type-independent expression of β-galactosidase (β-gal), which is maintained specifically in *Ptf1a*-expressing cells and their progeny (Kawaguchi et al., 2002). *R26R-EYFP* reporter mice, which carry the enhanced *YFP* gene driven by the *Rosa26* promoter (Srinivas et al., 2001), were also used for lineage tracing studies. The *Rosa26* promoter is active in all retinal cell types in adult mice (Rowan and Cepko, 2004).

In the adult retina, the majority of β-gal-expressing *Ptf1a*-lineage-labeled cells were found in the inner half of the INL, and the remainder was sparsely distributed in the GCL and along the outer boundary of the INL (Fig. 2A). To characterize the mature cell fate of cells derived from *Ptf1a*-lineage-labeled precursors, we co-labeled the retinas of 3-week-old *Ptf1a*$^{Cre/+}$*;R26R-EYFP* mice with GFP antibody and antibodies against specific cellular markers. The photoreceptor markers recoverin (Fig. 2D) and rhodopsin (data not shown) were not co-expressed with YFP, suggesting that *Ptf1a*-

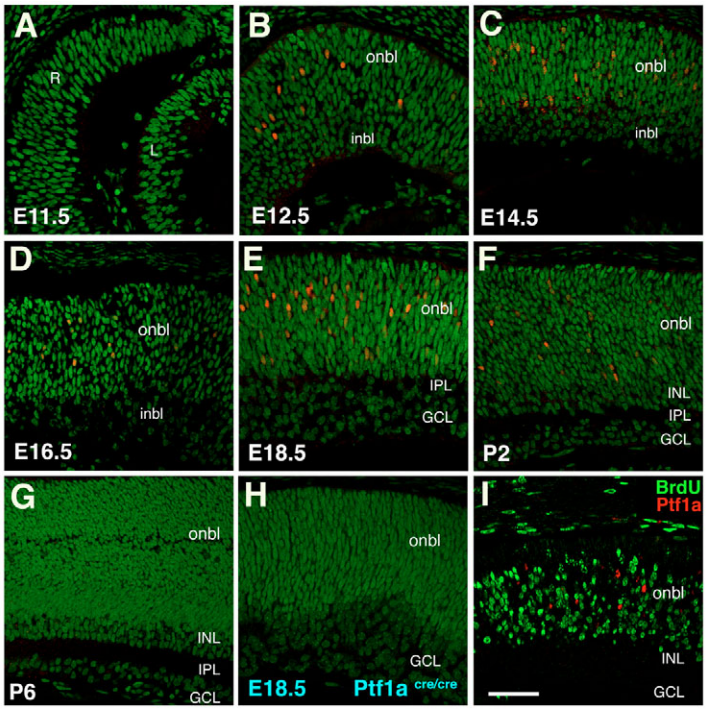

Fig. 1. Spatial and temporal expression patterns of Ptf1a protein during mouse retinogenesis. (**A-G**) Retinal sections from the indicated developmental stages were immunostained with an anti-Ptf1a antibody (red) and nuclei were counterstained with YoPro-1 (green). The expression of Ptf1a commences at E12.5 in the central retina (B), peaks around E14.5 in the outer neuroblastic layer (C) and continues to be strong around E18.5 (E). It begins to be downregulated significantly at P2 (F) and eventually disappears around P6-P7. At P6, Ptf1a expression is completely downregulated from the central retina. (**H**) Retinal sections from E18.5 *Ptf1a*$^{Cre/Cre}$ embryos immunostained with an anti-Ptf1a antibody. (**I**) Retinal sections from BrdU-labeled E14.5 embryos were double immunostained with anti-Ptf1a (red) and anti-BrdU (green) antibodies. There was no colocalization between Ptf1a-immunoreactive nuclei and the S-phase nuclei immunoreactive for BrdU. Scale bar: 50 μm. Abbreviations: R, retina; L, lens; onbl, outer neuroblastic layer; inbl, inner neuroblastic layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer.

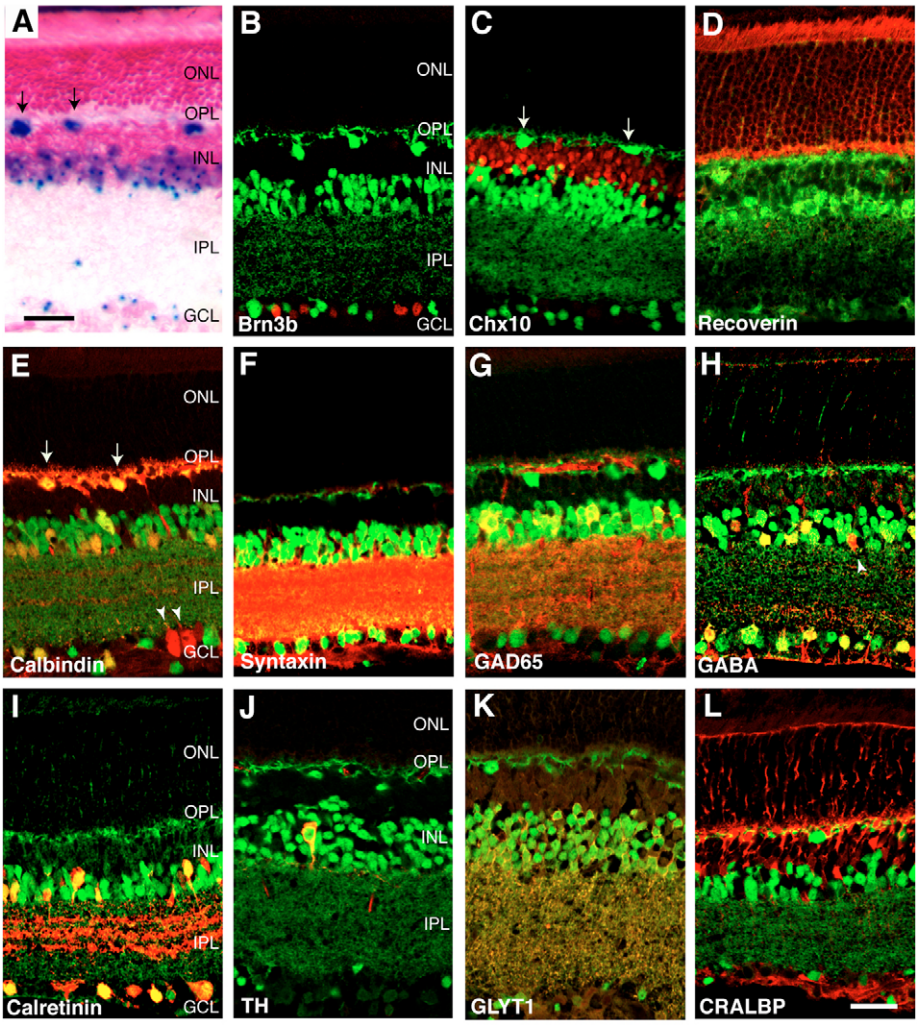

Fig. 2. Lineage tracing analysis in the retina. All retinal sections were examined at 3 weeks of age. (**A**) X-gal staining of retinal sections from *Ptf1a*$^{Cre/+}$*;R26R* pups counterstained with Fast Red. No β-gal activity was detected in the ONL. (**B-L**) *Ptf1a*$^{Cre/+}$*;R26R-EYFP* retinal sections double-labeled with anti-GFP (green) and the indicated antibodies (red). Brn3b (ganglion cells, B); Chx10 (bipolar cells, C); recoverin (rods and cones, D); calbindin (horizontal and amacrine cells, E); syntaxin (horizontal and amacrine cells, F); Gad65 (GABAergic amacrine cells, G); GABA (GABAergic amacrine cells, H); calretinin (amacrine subclass and ganglion cells, I); tyrosine hydroxylase (Th, dopaminergic amacrine cells, J); glycine transporter 1 (Glyt1, glycinergic amacrine cells, K); Cralbp (RPE and Müllar glia, L). Arrows in A,C,E indicate representative mature horizontal cells; arrowheads in E,H indicate non-colocalized cells. Scale bars: in A, 25 μm for A; in L, 25 μm for B-L. Abbreviations: INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer; IPL, inner plexiform layer.

expressing precursors did not differentiate into photoreceptor cells. Similarly, there was no overlap of YFP-positive cells with the bipolar cell marker Chx10 (Fig. 2C) (Burmeister et al., 1996). *Ptf1a*-lineage-labeled cells in the GCL (Fig. 2A) were not labeled with the ganglion cell marker Brn3b (Fig. 2B), but were positive for amacrine markers, including calbindin and GABA (Fig. 2E,F,H,I). Thus, *Ptf1a*-lineage-labeled cells in the GCL were displaced amacrine cells, which have been previously identified in the GCL (Barnstable and Drager, 1984). The glial markers Cralbp (cellular retinaldehyde-binding protein; Rlbp1 – Mouse Genome Informatics) (Fig. 2L) (Bunt-Milam and Saari, 1983) and vimentin (data not shown) showed no colocalization with YFP, indicating that glial cells were not derived from *Ptf1a*-expressing precursors. By contrast, all amacrine markers examined [calbindin, syntaxin, Gad65 (Gad2 – Mouse Genome Informatics), GABA, glycine transporter 1 (Glyt1; Slc6a9 – Mouse Genome Informatics), calretinin, tyrosine hydroxylase (Th), choline acetyltransferase (Chat) and the vesicular glutamate transporter type 3 (Vglut3; Slc17a8 – Mouse Genome Informatics)] showed variable but high proportions of colocalization with YFP (Fig. 2E-K; data not shown). For example, some of the amacrine markers labeled a few cells that were YFP negative (e.g. arrowheads in Fig. 2E,H). The percentage of amacrine marker-positive cells that were labeled with anti-YFP varied depending on the subclass of amacrine cells (Table 1). Horizontal cells, which are located on the outer border of the INL and are characterized by a relatively large soma and calbindin immunoreactivity, were labeled with anti-YFP without exception (Fig. 2E), suggesting that all horizontal cells were derived from *Ptf1a*-expressing precursors. Thus, our lineage tracing data suggest that *Ptf1a*-expressing cells represent a subset of precursors that are restricted to production of the horizontal and amacrine cells.

Retinal dysplasia in *Ptf1a*$^{-/-}$ mice

We found evidence that the expression of *Ptf1a* in precursors of the horizontal and amacrine cell lineages plays a function in regulating the production of these cell types. Morphological and molecular

Table 1. The proportions of *Ptf1a*-lineage-labeled retinal cells (GABA-, GLYT1-, TH-, calretinin- and calbindin-expressing cells) in *Ptf1a*$^{Cre/+}$*;R26R-EYFP* mice

	GABA	GLYT1	TH	Calretinin	Calbindin (horizontal cell)
Ptf1a$^{Cre/+}$*;R26R-EYFP*	79%	90%	64%	76%	63% (100%)

Horizontal cells (calbindin-positive cells along the outer boundary of the INL) are 100% lineage labeled by *Ptf1a*^{Cre}.

maturation of the retina is not completed until 2-3 weeks of age, but mice null for Ptf1a (*Ptf1a*$^{Cre/Cre}$) die just after birth (Kawaguchi et al., 2002; Krapp et al., 1998). We therefore explanted E18.5 retinas and cultured them for up to 2 weeks to study the role of Ptf1a in retinal differentiation and maturation. After 2 weeks of culture, retinas from wild-type embryos exhibited well-developed laminar structures containing the three cellular layers: GCL, INL and ONL (Fig. 3). By contrast, explants from *Ptf1a*$^{Cre/Cre}$ embryos, although initially of the same thickness as wild type, were significantly thinner after 2 weeks and often exhibited substantial focal perturbations in laminar organization. Rosette-like structures (e.g. Fig. 3L,P) were often observed in *Ptf1a*$^{Cre/Cre}$ retinas. Immunostaining by antibodies against the photoreceptor markers rhodopsin (Fig. 3A) and recoverin (data not shown) revealed normal generation of photoreceptor cells in *Ptf1a*$^{Cre/Cre}$ retinal explants. In addition, cells in the rosette-like structures were labeled by photoreceptor markers (data not shown), which strongly suggested a photoreceptor identity. These rosettes may be analogous to similar structures previously identified in explant cultures of *Hes1*-null mutant retinas (Tomita et al., 1996). There was no general deficiency in the emergence of Chx10-positive bipolar cells (Fig. 3C,D) or Cralbp-positive glial cells (Fig. 3O,P) in *Ptf1a*$^{Cre/Cre}$ retinas. Thus, *Ptf1a* is dispensable for the genesis of bipolar cells and Müller glia, which is consistent with the absence of *Ptf1a*-lineage labeling in these cell types. Calbindin-positive cells in the outer border of the INL were entirely absent in *Ptf1a*$^{Cre/Cre}$ retinas (Fig. 3E,F), indicating a complete loss of horizontal cells. Lack of calbindin-positive and syntaxin-positive cells in the mutant retina indicated also that amacrine cells, including the displaced amacrine cells in the GCL, were profoundly missing (Fig. 3E-H). Immunofluorescence labeling for the specific amacrine subtypes (e.g. GABA and Gad65 for GABAergic neurons; Th for dopaminergic neurons, Glyt1 for glycinergic neurons, Chat for cholinergic neurons, and Vglut3 for glutamatergic neurons) (Fig.

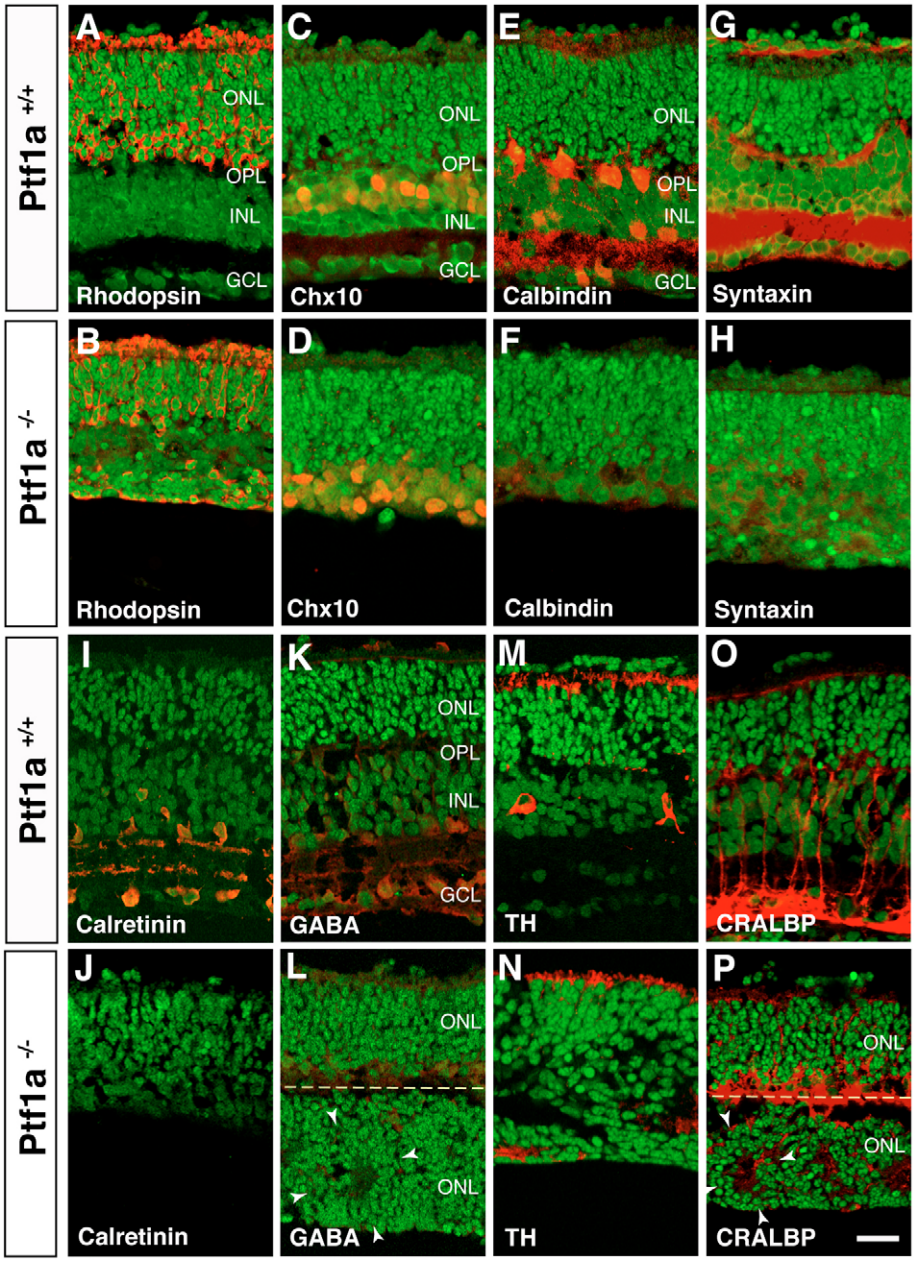

Fig. 3. Effect of targeted *Ptf1a* deletion on the formation of different retinal cell types. (A-P) Retinal explants were prepared from wild-type (A,C,E,G,I,K,M,O) and *Ptf1a*$^{Cre/Cre}$ (B,D,F,H,J,L,N,P) embryos at 18.5 and then cultured for 2 weeks. The neuronal subtypes were examined by immunohistochemistry (red) for rhodopsin (rods; **A**,**B**), Chx10 (bipolar cells; **C**,**D**), calbindin (horizontal and amacrine cells; **E**,**F**), syntaxin (horizontal and amacrine cells; **G**,**H**), calretinin (amacrine cells and RGCs; **I**,**J**), GABA (GABAergic amacrine cells; **K**,**L**), tyrosine hydroxylase (Th, dopaminergic amacrine cells; **M**,**N**) and Cralbp (RPE and Müller glia; **O**,**P**). In retinal explants, there is postnatal degeneration of pre-existing RGCs, owing to RGC axon severance resulting from optic nerve transection during tissue preparation. During explant culture, folding of retinal tissue usually results in mirror-image-like duplication on the periphery (broken lines in L and P). Arrowheads in L,P indicate rosette-like structure formation. Scale bars: 25 μm. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer.

3E-N; data not shown) revealed a dramatic decrease of all amacrine subtypes. Occasionally, very small numbers of residual amacrine cells were observed in some sections of retinal explant, irrespective of which amacrine marker was examined. Taken together, these findings provide evidence that *Ptf1a* function is selectively required for proper specification of the vast majority of horizontal and amacrine cells.

Defects in Ptf1a mutant retina at embryonic stages

So far, we have shown that horizontal and amacrine cells are generated from *Ptf1a*-expressing precursors and are absent (horizontal cells) or severely reduced in number (amacrine cells) in later stage *Ptf1a*-null mutant retinas. To gain insight into the cellular mechanism underlying retinal defects in *Ptf1a null* retinas, we characterized the mutant retina at embryonic stages. As early as E16.5, abnormal laminar organization of the $Ptf1a^{Cre/Cre}$ retina was noticeable (data not shown), and by E18.5 the inner plexiform layer (IPL), which was well demarcated in wild-type retinas, was absent from the mutant (Fig. 4G-J). Although cells belonging to the onbl were confined to the ventricular zone in the wild type, they tended to invade into the GCL in $Ptf1a^{Cre/Cre}$ retinas (Fig. 4H,J). The GCL in $Ptf1a^{Cre/Cre}$ retinas was significantly thicker than that of controls (Fig. 4G,H), which was also manifested by the increased number and expanded spatial localization of cells expressing the ganglion cell marker Brn3b (Fig. 4K-M; discussed below). In E18.5 $Ptf1a^{Cre/+}$;*R26R* retinas, two rows of β-gal-expressing *Ptf1a*-lineage cells were observed (Fig. 4E). As discussed above, our lineage analysis of adult $Ptf1a^{Cre/+}$;*R26R* retinas showed that *Ptf1a*-lineage-labeled cells contribute exclusively to the horizontal and amacrine cell populations. Based upon the presumptive location of nascent horizontal and amacrine cells at this stage, the outer row of β-gal-expressing cells in the onbl most probably corresponds to maturing horizontal cells, and the inner row encompassing the INL and IPL probably represents forming amacrine cells in $Ptf1a^{Cre/+}$;*R26R* retinas (Fig. 4E). At the same developmental stage (E18.5), the positioning of lineage-labeled cells was abnormal in the $Ptf1a^{Cre/Cre}$;*R26R* retina. The majority of the β-gal-positive cells were dispersed in the enlarged GCL, and some of them aggregated adjacent to the optic nerve layer (Fig. 4F). The altered location of lineage-labeled cells in the retina of $Ptf1a^{Cre/Cre}$;*R26R* mice implies that the migratory behavior associated with the altered specification of retinal cells was affected by the absence of Ptf1a expression. From E14.5 onwards, β-gal-expressing fibers were reproducibly observed in the optic nerve and retinal nerve layer of $Ptf1a^{Cre/Cre}$;*R26R* mice, but not in those of $Ptf1a^{Cre/+}$;*R26R* animals (Fig. 4A-D). Given that the ganglion cell is the only cell type extending axons through the optic nerve, β-gal-positive fibers in the optic nerve are likely to be the axons of ganglion cells. Thus, the observation that optic nerve fibers are labeled only in the absence of *Ptf1a* suggests that Ptf1a expression may suppress ganglion cell differentiation.

Loss of *Ptf1a* function results in mis-specification of *Ptf1a*-expressing retinal precursors as ganglion cells

Loss of horizontal and amacrine cells concomitant with the abnormal location of *Ptf1a*-lineage labeled cells in the optic nerve fibers suggested that precursors that would normally produce horizontal and amacrine cells might have changed fate to become ganglion cells in the absence of *Ptf1a* function. Consistent with this hypothesis, E18.5 mutant retinas contained 54.7% more cells expressing *Brn3b*, which encodes a POU-domain transcription factor specific for differentiated retinal ganglion cells (RGCs) (Gan et al., 1996; Xiang et al., 1993), with no change in the total thickness of the retina (Fig.

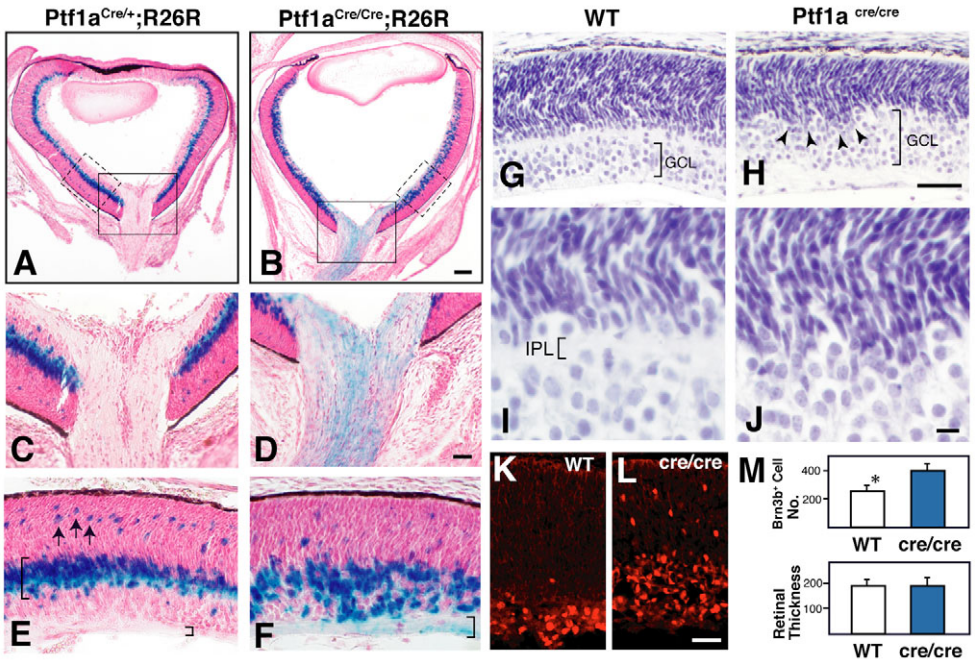

Fig. 4. Defects in laminar structure and cell specification in *Ptf1a* mutant retinas. (**A-F**) X-gal staining of retinal sections from E18.5 $Ptf1a^{Cre/+}$;*R26R* embryos (A) and $Ptf1a^{Cre/Cre}$;*R26R* embryos (B) counterstained with Fast Red. (C,E) Higher-magnification images of the areas in the solid and broken rectangles in A, respectively. (D,F) Higher-magnification images of the areas in the solid and broken rectangles in B, respectively. Arrows in E indicate representative developing horizontal cells and the bracket in E indicates normal location of maturing amacrine cells in the E18.5 retina. The bracket in F indicates *lacZ*-positive nerve fiber layer, which is thicker than the control *lacZ*-negative nerve fiber layer (indicated by the small bracket in E). (**G-J**) Hematoxylin and Eosin-stained sections of wild-type and mutant retinas at E18.5. Higher-magnification pictures of G and H are shown in I and J, respectively. Arrowheads in H indicate a front-line of cells in the outer neuroblastic layer invading into the GCL. (**K**,**L**) Wild type and $Ptf1a^{Cre/Cre}$ E18.5 retinal sections were immunostained with an anti-Brn3b antibody. Scale bars: in B, 100 μm for A,B; in H, 50 μm for E-H; in D, 25 μm for C,D; in L, 25 μm for K,L; in J, 10 μm for I,J. (**M**) Top, increased ganglion cell number in $Ptf1a^{Cre/Cre}$ retinas. Bottom, no significant change in central retinal thickness in the mutant. Histograms represent the mean±s.d. of measurements from five animals of each genotype. *P<0.001. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer.

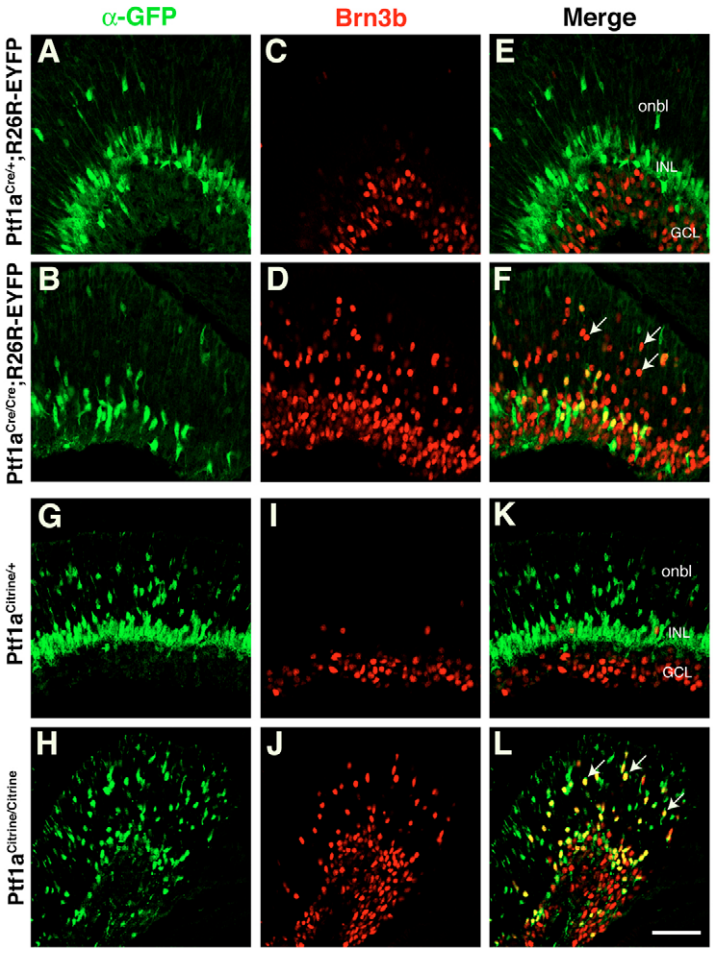

Fig. 5. *Ptf1a* lineage-labeled cells are transfated to RGCs in Ptf1a-deficient embryos. (**A-F**) An anti-GFP antibody was used to detect YFP in E18.5 *Ptf1a*^{Cre}*;R26R-EYFP* retinas and an anti-Brn3b antibody was used to detect differentiated ganglion cells. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. YFP (green) does not co-localize with Brn3b (red) in the presence of Ptf1a, but they do co-localize in *Ptf1a*-null embryos (compare E with F). (**G-L**) An anti-GFP antibody was used to detect citrine, a modified version of GFP, in E18.5 *Ptf1a*^{Citrine} embryos. Citrine serves as a lineage marker for cells that are expressing or have expressed the *Ptf1a* locus during the past couple of days. Citrine (green) does not co-localize with Brn3b (red) in the presence of Ptf1a, but they do co-localize in *Ptf1a*-null embryos (compare K with L). Arrows in F indicate Brn3b^{+}YFP^{-} 'newly specified RGCs' in the onbl. Arrows in L indicate Brn3b^{+}citrine^{+} *Ptf1a* lineage-labeled RGCs. Scale bar: 50 μm. Abbreviations: onbl, outer neuroblastic layer; INL, inner nuclear layer; GCL, ganglion cell layer.

4K-M). The supernumerary Brn3b-expressing cells were confirmed to be RGCs based on expression of the additional ganglion markers Brn3a and islet 1 (data not shown). Double-immunolabeling analysis on E18.5 *Ptf1a*^{Cre}*;R26R-EYFP* heterozygous and mutant retinas provided some evidence that the additional RGCs arose via a switch in fate of the postmitotic precursors. The ganglion marker Brn3b was very seldom co-expressed with YFP in retinas heterozygous for *Ptf1a* (Fig. 5E), consistent with the results obtained from 3-week-old heterozygous mice (Fig. 2B). In *Ptf1a*$^{Cre/Cre}$*;R26R-EYFP* retinas, however, YFP and Brn3b were often co-localized (Fig. 5F) providing direct evidence that the precursors that normally produce horizontal and amacrine cells switch fate in the absence of Ptf1a and contribute to the RGC lineage. This cell fate switch was observed as early as E16.5 (data not shown).

Although many Brn3b-positive cells in the *Ptf1a*$^{Cre/Cre}$*;R26R-EYFP* GCL co-expressed YFP, most ectopic RGCs in the onbl were YFP negative (Fig. 5F). Because Brn3b-positive cells are not normally found in the onbl at this stage (e.g. Fig. 5C), we reasoned that the Brn3b^{+}/YFP^{-} cells in the onbl either originated from a non-cell-autonomous overproliferation of *Ptf1a*-negative RGC precursors, or from trans-fated *Ptf1a*-positive precursors that had not yet activated the YFP reporter, perhaps because of the time needed to accumulate sufficient Cre protein. The latter possibility seemed reasonable in light of the observation that the onset of enzymatically detectable β-gal protein in *Ptf1a*$^{Cre/+}$*;R26R* embryos was delayed by ~1 day compared with endogenous Ptf1a expression (data not shown). Furthermore, considering that RGCs are first specified in the onbl soon after exit from the cell cycle, and then migrate inwards to the GCL, Brn3b^{+} cells within the onbl should represent recently specified RGCs (Xiang, 1998). As Ptf1a expression was seen only post-mitotically (Fig. 1I), recently specified RGCs in the onbl may not have had enough time to activate the *R26R-EYFP* reporter. To test this possibility, we used a novel *Ptf1a* null allele, *Ptf1a*^{citrine}, in which citrine (another yellow variant of GFP) is directly expressed from the *Ptf1a* locus. The onset of citrine expression in *Ptf1a*$^{Citrine/+}$ mice coincided with the onset of endogenous Ptf1a protein expression, and citrine protein was detectable for ~2 days after disappearance of endogenous Ptf1a (data not shown), presumably because of its slow turnover. The sustained expression of citrine allows 'partial lineage tracing', wherein citrine protein presence marks Ptf1a-expressing cells and their progeny for ~2 days after shutdown of *Ptf1a* expression. Although only a small fraction of Brn3b^{+} cells in the onbl co-localized with YFP in *Ptf1a*$^{Cre/Cre}$*;R26R* retinas, most of the Brn3b^{+} cells in the onbl of *Ptf1a*$^{Citrine/Citrine}$ retinas co-localized with Citrine (Fig. 5F,L). Based on these results, we conclude that the Brn3b^{+} cells detected in the mutant onbl at E18.5 are derived from precursors that can activate expression from the *Ptf1a* locus but are unable to produce Ptf1a protein. Therefore, conversion of *Ptf1a*-active amacrine/horizontal precursors into ganglion precursors is likely to be the primary contributor to increased production of RGCs in *Ptf1a* mutants.

Ptf1a as a downstream target for Foxn4

Targeted deletion of *Foxn4* also causes the loss of all horizontal cells and the great majority of amacrine cells (Li et al., 2004a). Given the similar retinal phenotypes in *Ptf1a* and *Foxn4* mutant

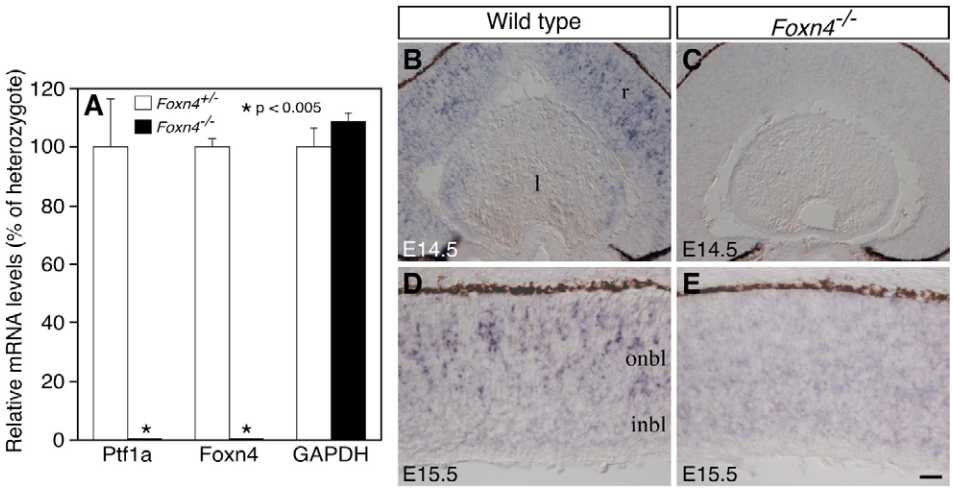

Fig. 6. Downregulation of *Ptf1a* expression in the *Foxn4*$^{-/-}$ retina. (**A**) Real-time qRT-PCR analysis of *Ptf1a, Foxn4* and *Gapdh* mRNA levels in E14.5 *Foxn4*$^{+/-}$ and *Foxn4*$^{-/-}$ retinas. Each histogram represents the mean±s.d. for four retinas. (**B-E**) Retinal sections of the indicated stages from wild-type and mutant mice were hybridized with a *Ptf1a* riboprobe. The absence of *Ptf1a* signals from *Foxn4*$^{-/-}$ retinas (C,E). Scale bar: 50 μm in B,C; 25 μm in D,E. Abbreviations: onbl, outer neuroblastic layer; inbl, inner neuroblastic layer.

mice, we investigated their inter-regulatory relationship. A microarray analysis revealed that *Ptf1a* expression was dramatically downregulated in E14.5 *Foxn4*$^{-/-}$ retina, by 37-fold compared with the wild type (F.Q. and M.X., unpublished). To confirm this result, we performed real-time qRT-PCR, using total RNA isolated from E14.5 *Foxn4*$^{+/-}$ and *Foxn4*$^{-/-}$ retinas (Fig. 6A). We found that *Foxn4*$^{-/-}$ tissue contained no *Ptf1a* mRNA and, as expected, *Foxn4* mRNA was absent from the *Foxn4*$^{-/-}$ sample. RNA in situ hybridization with a *Ptf1a* riboprobe detected prominent signals scattered within the onbl of wild-type retina at E14.5 and E15.5 (Fig. 6B,D), and the absence of these signals in *Foxn4*$^{-/-}$ tissue (Fig. 6C,E), further confirming the complete downregulation of *Ptf1a* expression by the loss of *Foxn4* function. In reciprocal experiments, in situ hybridization revealed no alteration of *Foxn4* expression in the *Ptf1a null* retina (Fig. 7). We conclude that Foxn4 functions upstream of *Ptf1a* to activate its expression, thereby regulating the generation of amacrine and horizontal cells during retinogenesis.

Downregulation of Prox1 and Lim1, but not Math3 and Neurod1 expression in *Ptf1a*$^{-/-}$ retinas

To further determine the molecular basis of the defect in horizontal and amacrine cells in *Ptf1a*-null retinas, we investigated whether loss of *Ptf1a* function would affect the expression of genes involved in the genesis of these two cell types. The Prox1 homeodomain factor has been shown to play a crucial role in the commitment towards the horizontal cell fate (Dyer et al., 2003). Compared with wild-type controls, Prox1 expression was severely downregulated in *Ptf1a*-null retinas at E14.5 (Fig. 7K,L) and E16.5 (data not shown), whereas the strong lens expression was not altered. Loss of horizontal cell specification was further demonstrated by a loss of Lim1 expression in E18.5 *Ptf1a*-null retinas (Fig. 7M,N). The bHLH factors Math3 and Neurod1 are redundantly required for the determination of amacrine cells (Inoue et al., 2002). Unexpectedly, the expression of both genes did not exhibit any alteration in the mutant retina (Fig. 7E-H). Thus, Ptf1a does not appear to act upstream of Math3 or Neurod1 for the fate determination of amacrine cells (Fig. 8C). Expression of *Math5*, a bHLH factor required for ganglion cell genesis (Wang et al., 2001), becomes downregulated at E18.5 in the wild-type retina. Consistent with the increase of RGCs (Figs 4, 5) in the *Ptf1a*-null retinas, *Math5* expression in the mutant was moderately upregulated compared with wild-type controls (Fig. 7I-J).

DISCUSSION

Retinal progenitor competence revealed by lineage tracing

Although it has been postulated that retinal progenitors undergo a series of changes in competence to give rise to the various retinal cell types (Cepko, 1999; Livesey and Cepko, 2001), there have been no studies that provide rigorous evidence for fate-restricted precursors in vivo. By using recombination-based lineage tracing analysis, we have demonstrated that *Ptf1a*-expressing cells represent a subset of precursors that are restricted to the production of horizontal and amacrine cells. Transient and/or low expression of Cre from the Ptf1a locus often results in 'escapers' in Cre-loxP lineage tracing systems (Gu et al., 2002; Hoshino et al., 2005; Kawaguchi et al., 2002). The variable penetrance of the lineage-labeling among the amacrine cell subtypes (Table 1) could be most probably explained by Cre not rising to high enough levels in all cells, despite all precursors expressing the *Ptf1a* gene, or that for unknown reasons the reporter gene locus is not equally sensitive in all cells. Our lineage analysis has revealed that Ptf1a-expressing precursors contribute to all amacrine subtypes analyzed. We cannot, however, formally rule out the possibility that a minor population of Ptf1a-non-expressing precursor cells can contribute to amacrine cells, or that some amacrine subclasses may be generated in a Ptf1a-independent manner.

Ptf1a, a bHLH factor implicated in the cell fate determination in various organs

Originally, Ptf1a was identified as a transcriptional regulator of pancreatic exocrine-specific genes such as elastase 1 (Krapp et al., 1996). Mice null for *Ptf1a* exhibit a complete loss of exocrine tissue and severe defects in the formation and spatial organization of endocrine cells (Kawaguchi et al., 2002; Krapp et al., 1998). Dramatic phenotypes have also been observed in the development of the cerebellum and dorsal spinal cord. The requirement for Ptf1a to specify multiple GABAergic cell-types, including Purkinje, stellate and basket cells, all derived from Ptf1a-expressing precursors, is responsible for the cerebellar hypoplasia in *Ptf1a* mutants (Hoshino et al., 2005). The *Ptf1a*-null mouse also develops a dorsal spinal cord with nearly a complete loss of inhibitory GABAergic neurons. In dorsal spinal cord, loss of *Ptf1a* function results in a reciprocal increase of excitatory glutamatergic neurons. These findings lead to the conclusion that Ptf1a functions as a selector molecule that determines GABAergic over glutamatergic cell fate in these neural tissues (Glasgow et al., 2005). Defects in

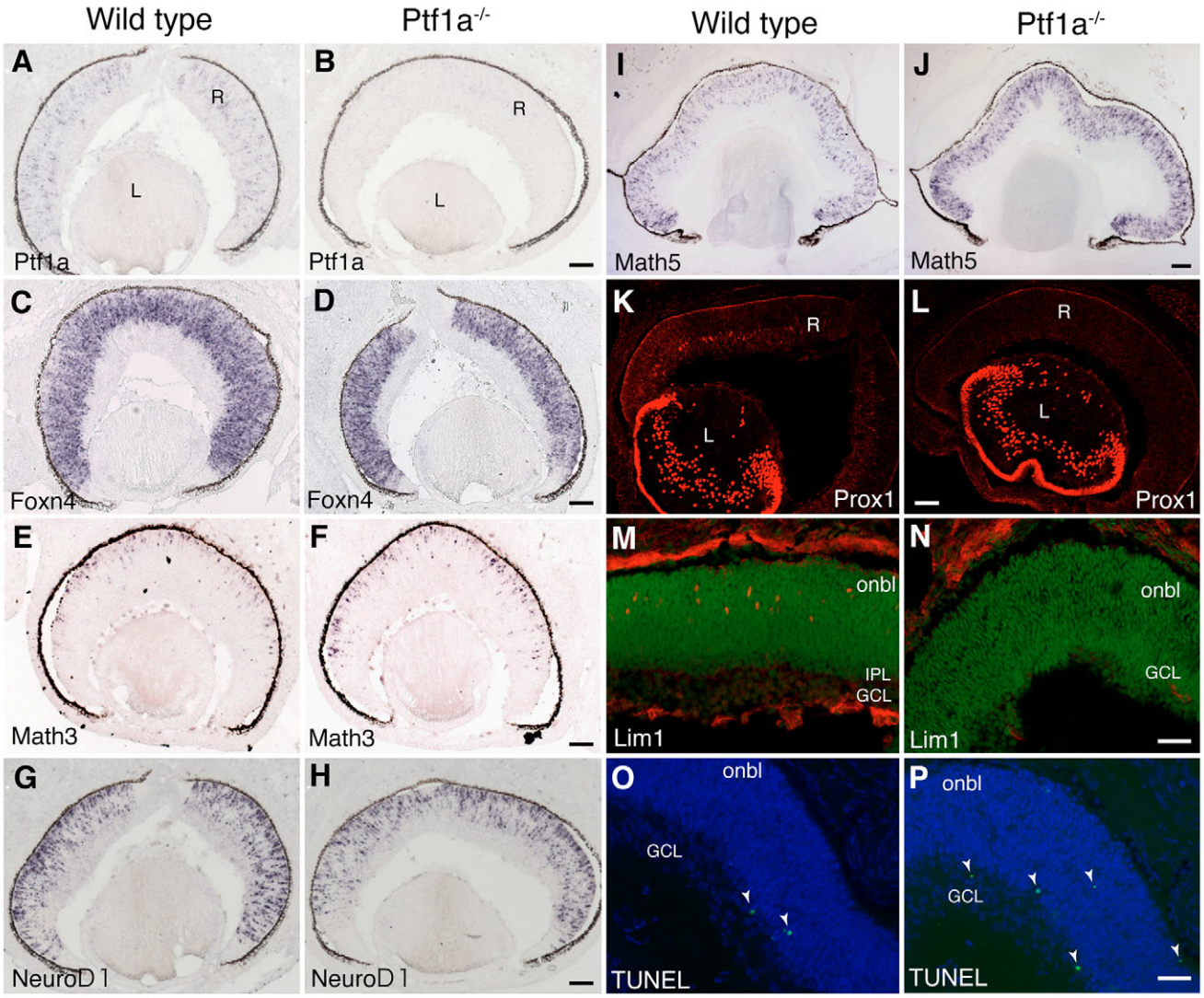

Fig. 7. Effect of targeted *Ptf1a* deletion on the expression of retinogenic factors. (A-J) Sections from E14.5 (A-H) or E18.5 (I,J) wild-type and *Ptf1a*$^{Cre/Cre}$ retinas were hybridized with a *Ptf1a* (**A**,**B**), *Foxn4* (**C**,**D**), *Math3* (**E**,**F**), *Neurod1* (**G**,**H**) or *Math5* (**I**,**J**) riboprobes. Similar levels of *Foxn4, Math3 and Neurod1* expression are seen in wild-type and null retinas. *Math5* expression is upregulated in the null retina at E18.5. (K-N) Sections from E14.5 (K,L) or E18.5 (M,N) wild-type and null retinas were immunostained with antibodies against Prox1 (**K**,**L**) or Lim1 (**M**,**N**). Prox1 and Lim1 signals are absent from *Ptf1a*$^{Cre/Cre}$ retinas (L,N). (**O**,**P**) Cells undergoing apoptosis (green) were TUNEL-labeled in wild-type and null retinas at E18.5. A moderate increase of apoptotic cells is seen in the *Ptf1a*-null retina. Sections in M-P were weakly counterstained with Yo-Pro-1 (M,N) or DAPI (O,P). Scale bar: 50 μm. Abbreviations: R, retina; L, lens; onbl, outer neuroblastic layer; GCL, ganglion cell layer; IPL, inner plexiform layer.

Ptf1a-null retinas, however, do not seem to be restricted to GABAergic neurons. Our results that all amacrine subtypes examined were overall affected in the absence of *Ptf1a*, suggesting the involvement of Ptf1a in the genesis for all amacrine neurons. We are unable to rule out completely the possibility that some amacrine subtypes were lost as an indirect consequence of the massive loss of the cells in the INL during explant culture. Horizontal and amacrine cells, which are deficient in *Ptf1a*-null retinas, are two classes of interneurons that modulate and integrate visual signals in the retinal circuitry. Thus, Ptf1a in the developing retina may be involved in the determination of two interneuron cell fates, rather than in regulating transmitter-subtype specification, as proposed for a Ptf1a function in the cerebellum and the spinal cord (Glasgow et al., 2005).

Several lines of evidence suggest that Ptf1a regulates the genesis of amacrine and horizontal cells primarily by controlling their fate commitment. First, during mouse retinogenesis, Ptf1a exhibits transient expression over a time course spanning from E12 to P5, and this temporal pattern of expression closely correlates with the birthdates of amacrine and horizontal cells (Young, 1985). Second, we have demonstrated the requirement of Ptf1a in the generation of the overwhelming majority of amacrine neurons and all horizontal cells. Lineage tracing experiments based on *Ptf1a*^{Cre}-mediated activation of R26R and R26R-EYFP revealed that Ptf1a expression marks lineage-restricted precursors that exclusively produce horizontal and amacrine cells. Furthermore, the *Ptf1a*-active precursors, normally fated to amacrine and horizontal cells, switched to ganglion cell fates in the absence of Ptf1a function. These observations clearly demonstrate that activation of *Ptf1a* is tightly associated with the acquisition of the amacrine and horizontal cell fate. The function of Ptf1a in switching cell fates in the retina is similar to the role attributed to Ptf1a in the development of pancreas and dorsal

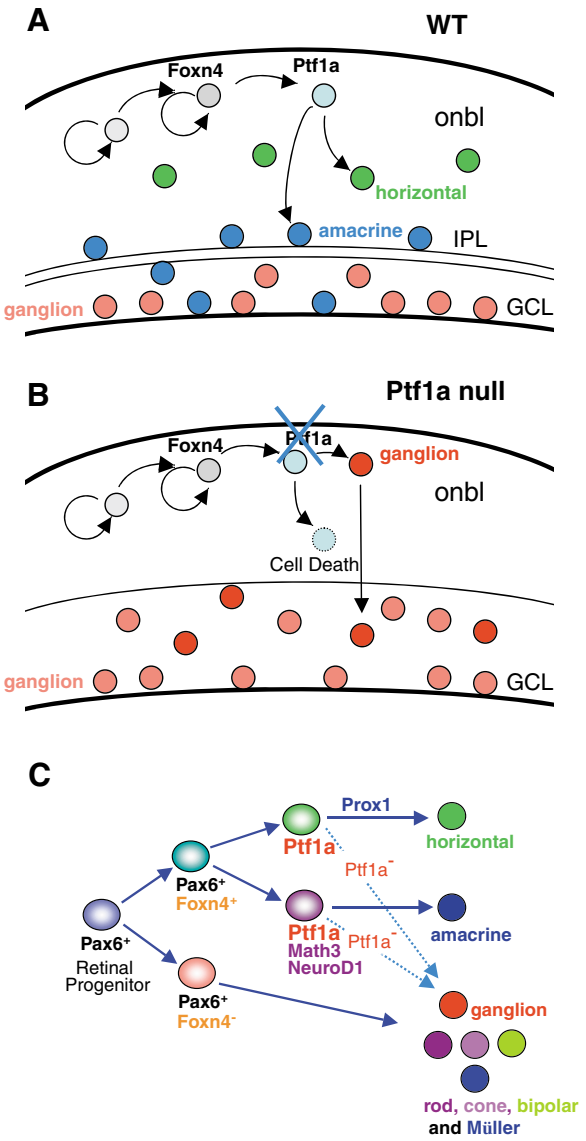

Fig. 8. A model for directed differentiation of retinal progenitors towards horizontal and amacrine fates by the *Foxn4-Ptf1a* pathway. (**A**) Foxn4-expressing cells in the outer neuroblastic layer represent progenitors biased towards horizontal and amacrine fates (Li et al., 2004a). Subsets of Foxn4$^+$ cells activate Ptf1a after exit from the cell cycle. Activation of Ptf1a depends on the Foxn4 function (Fig. 6). Ptf1a-expressing post-mitotic cells differentiate to horizontal or amacrine cells. (**B**) In the absence of Ptf1a, retinal precursors, which would normally activate Ptf1a, adopt ganglion cell fates or otherwise undergo apoptotic cell death (referred also in C). The cell fate switch to RGC takes place relatively quickly in the onbl after exit from the cell cycle. Green, horizontal cells; blue, amacrine cells; red, RGCs. Dark-red cells in B represent RGCs originating from Ptf1a-lineage-labeled precursor populations. (**C**) Foxn4 and Ptf1a control the genesis of amacrine and horizontal cells in cooperation with other retinogenic factors. Foxn4-expressing progenitors require sequential activation of Ptf1a and Prox1 for the genesis of horizontal cells. Foxn4 confers progenitors with the competence for the genesis of amacrine cells by activating the expression of Ptf1a, Math3 and Neurod1. Activation of Math3 or Neurod1 does not require Ptf1a function. The rod, cone, bipolar, ganglion and Müller cells are likely to be largely derived from the Pax6$^+$ Foxn4$^-$ progenitors via activation of other retinogenic factors, which are not shown here for simplicity. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer.

spinal cord (Glasgow et al., 2005; Kawaguchi et al., 2002). By using the same lineage tracing method, we showed that Ptf1a is expressed by early progenitors for the pancreatic ducts, acinar and endocrine cells, and that Ptf1a-deficient pancreatic progenitors adopt duodenal cell fates (Kawaguchi et al., 2002). Similarly, in the spinal cord dorsal horn, inactivation of *Ptf1a* switches dI4 and dIL^A fates (GABAergic neuron lineage) into the alternative fates dI5 and dIL^B (glutamatergic neurons). It remains to be determined whether Ptf1a also codes for shared characteristics in cell fate determination in other Ptf1a-expression domains, such as cerebellum and the ventral hypothalamus (Glasgow et al., 2005).

Ptf1a in the horizontal cell specification pathway

Although efforts to unravel the transcription factor code for the specification of retinal neurons have revealed a complex interplay of spatial and temporal control between bHLH and homeodomain transcription factors, no single bHLH factor alone has been identified that is required for the specification of horizontal neurons. To our knowledge, this is the first report for the identification of a bHLH factor essential for the genesis of all horizontal cells. In *Ptf1a$^{Cre/Cre}$* retinas, we have observed a failure in the generation of horizontal cells in an explant culture as assessed by cell-type specific markers, including calbindin and syntaxin. To address whether horizontal cells had been specified in the absence of *Ptf1a*, we examined the embryonic expression of two additional horizontal markers, Prox1 (Dyer et al., 2003) and Lim1 (Liu et al., 2000). As Prox1 is believed to be the major intrinsic factor that is both necessary and sufficient for the promotion of horizontal cells from competent progenitors (Dyer et al., 2003), we tested whether Ptf1a controls specification of horizontal cells by regulating Prox1 expression. As expected, our analyses have indeed revealed that Ptf1a positively regulates the expression of Prox1 during horizontal cell ontogeny, beginning as early as E14.5 (Fig. 7). Given that Prox1 expression is regulated also by Foxn4 (Li et al., 2004a), Foxn4 most probably controls Prox1 expression through the activation of Ptf1a in the horizontal cell specification cascade (Fig. 8). At E18.5, expression of Lim1, which is exclusively confined within the horizontal cell type in the adult retina, was entirely absent from *Ptf1a$^{Cre/Cre}$* retinas, providing yet more evidence for the lack of specification of the horizontal cells (Fig. 7).

A Foxn4-Ptf1a pathway determines horizontal and amacrine cell fate

The complete downregulation of *Ptf1a* expression in *Foxn4*-null retinas, together with the unaltered expression of Foxn4 in *Ptf1a$^{Cre/Cre}$* retina, places Ptf1a downstream of Foxn4 in the transcriptional cascade that leads to amacrine/horizontal cell specification in the developing retina. These findings are in agreement with several of our observations. First, the timing of Ptf1a expression closely follows the transient expression pattern of Foxn4 in progenitor cells. Initiation of Ptf1a expression at E12.5 is ~0.5-1 day later than that of Foxn4 at E11.5, and expression of both genes is largely downregulated by about the same time at P5-P6 (Fig. 1) (Li et al., 2004a). Second, Foxn4 is expressed by a subset of progenitor cells whose progeny are significantly biased toward amacrine and horizontal cell fates (Li et al., 2004a), whereas Ptf1a is expressed by postmitotic precursors that are almost exclusively fated for horizontal and amacrine cells. The onbl of E11.5-E13.5 embryonic retina carries substantial numbers of Foxn4$^+$ mitotic progenitors, many of which have competence to preferentially differentiate to horizontal or amacrine cells (Li et al., 2004a). Our results suggest that these Foxn4$^+$ progenitors require Ptf1a function

after exit from the cell cycle to acquire completely their fates as mature amacrine or horizontal cells; these ideas are summarized in the model presented in Fig. 8. In *Ptf1a*$^{Cre/Cre}$ retinas, these precursors undergo reallocation to the ganglion fate relatively quickly after exit from the cell cycle, otherwise presumably being pushed into an apoptotic program. This possibility is supported by a modest increase of cell death in *Ptf1a*$^{Cre/Cre}$ retinas (48.6±5.8 cells/mm^2) at E18.5 compared with wild-type (22.4±1.7 cells/mm^2; $P<0.01$) littermates as assessed by TUNEL analysis (Fig. 7).

The cell fate switch in *Ptf1a*-null retina is considered to be analogous to the fate switch of amacrine to ganglion cells in *Math3-Neurod1* double mutant retinas, as reported previously (Inoue et al., 2002). The function of Ptf1a in postmitotic cells highlights another important example in which cell fate allocation is regulated after cell cycle exit. This agrees well with previous reports where postmitotic precursors fated to become photoreceptor cells can be respecified to become amacrine cells in the absence of Otx2 (Baas et al., 2000; Nishida et al., 2003), or become bipolar cells in the presence of Cntf (Ezzeddine et al., 1997).

Given its necessity, we tested whether Ptf1a was also sufficient to promote the fates of horizontal and amacrine cells from retinal progenitors by a gain-of-function approach. Overexpression of Ptf1a in retinal progenitors by an expression vector revealed that expression of Ptf1a alone was not sufficient to promote the generation of horizontal and amacrine cells (data not shown). The other intrinsic factors that are required together with Ptf1a for horizontal and amacrine cell genesis remain to be elucidated. Recently RBP-L (Rbpsuhl – Mouse Genome Informatics) was identified as one of the subunits to constitute a heterotrimeric transcription factor Ptf1 together with Ptf1a (Beres et al., 2006). Although Ptf1a alone failed to activate its reporter gene, addition of RBP-L significantly potentiated its transcriptional activity. Thus, co-factors for Ptf1a, such as RBP-L, might be required for full activation of Ptf1 to induce horizontal and amacrine cell genesis.

In spite of the selective deficiency in horizontal and amacrine cells that is shared by *Ptf1a* and *Foxn4* mutants, these mutants show some phenotypical differences. Although *Ptf1a*-null cells preferentially trans-differentiate to ganglion cells, *Foxn4*$^{-/-}$ cells appear to primarily adopt a photoreceptor fate (Li et al., 2004a). The switch of the horizontal/amacrine precursors to a ganglion fate rather than a photoreceptor fate in the *Ptf1a*-null retinas may reflect their ongoing competence based upon the presence of Foxn4 within these cells (Fig. 7). Foxn4 may have a suppressive role over photoreceptor differentiation given that *Foxn4*$^{-/-}$ cells preferentially differentiated to photoreceptor lineages, and that the expression of the photoreceptor differentiation factor *Crx* was upregulated in *Foxn4*$^{-/-}$ retinas (Furukawa et al., 1997; Furukawa et al., 1999; Li et al., 2004a). There is a formal possibility that some *Foxn4*$^{-/-}$ cells may indeed switch fates to become ganglion cells at early stages, but that they quickly degenerate because of elevated apoptosis during postnatal stages in *Foxn4*$^{-/-}$ retinas (Li et al., 2004a).

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