Development 134, 1151-1160 (2007) doi:10.1242/dev.02781

Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina

Hassan Nakhai[1,*,†], Saadettin Sel[2,*], Jack Favor[3], Lidia Mendoza-Torres[1], Friedrich Paulsen[4], Gernot I. W. Duncker[2] and Roland M. Schmid[1]

Basic helix-loop-helix (bHLH) transcription factors are important regulators of retinal neurogenesis. In the developing retina, proneural bHLH genes have highly defined expressions, which are influenced by pattern formation and cell-specification pathways. We report here that the tissue-specific bHLH transcription factor *Ptf1a* (also known as *PTF1-p48*) is expressed from embryonic day 12.5 of gestation (E12.5) to postnatal day 3 (P3) during retinogenesis in the mouse. Using recombination-based lineage tracing, we provide evidence that *Ptf1a* is expressed in precursors of amacrine and horizontal cells. Inactivation of *Ptf1a* in the developing retina led to differentiation arrest of amacrine and horizontal precursor cells in addition to partial transdifferentiation of *Ptf1a*-expressing precursor cells to ganglion cells. Analysis of late cell-type-specific markers revealed the presence of a small population of differentiated amacrine cells, whereas GABAergic and glycinergic amacrine cells, as well as horizontal cells, were completely missing in *Ptf1a*-knockout retinal explants. We conclude that *Ptf1a* contributes to the differentiation of horizontal cells and types of amacrine cells during mouse retinogenesis.

KEY WORDS: Amacrine cells, Cre-loxP method, Development, Ptf1a, Retina, Mouse

INTRODUCTION

The mammalian retina consists of six major neuronal cell types (cone photoreceptors, rod photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells) and one type of glia (Mueller cells) that originate during retinogenesis from a common population of undifferentiated multipotent retinal progenitor cells (RPCs) (Marquardt, 2003). Both the chronological sequence of cell generation and the spatial expansion of these diverse cell types are strictly regulated. In mice, retinal cell differentiation starts with the generation of ganglion cells at embryonic day 11.5 (E11.5), followed in a temporally overlapping sequence by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and finally Mueller glia cells (Marquardt, 2003). Retinogenesis in mice is completed at approximately postnatal day 10 (P10). These functionally different cells form three cellular (nuclear) layers and two synaptic (plexiform) layers in the mature retina. The outer nuclear layer (ONL) contains rod and cone photoreceptor cell bodies. The inner nuclear layer (INL) is comprised of horizontal, bipolar, amacrine and Mueller cell bodies. The ganglion cell layer (GCL) includes both nuclei of displaced amacrine cells and ganglion cells. The synaptic connections of these neuronal cells are localized in the outer plexiform layer (OPL) and the inner plexiform layer (IPL).

[1]II. Medizinische Klinik, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, D-81675 Munich, Germany. [2]Department of Ophthalmology, Martin-Luther-University Halle-Wittenberg, Ernst-Grube-Strasse 40, D-06120 Halle/Saale, Germany. [3]Institute of Human Genetics, GSF-National Research Center for Environment and Health, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany. [4]Department of Anatomy and Cell Biology, Martin-Luther-University Halle-Wittenberg, Grosse Steinstrasse 52, D-06097 Halle/Saale, Germany.

*These authors contributed equally to this work
[†]Author for correspondence (e-mail: hassan.nakhai@lrz.tum.de)

Accepted 14 December 2006

The molecular basis of pattern formation and cell-type specification in the vertebrate retina has been intensively investigated. Accumulating evidence indicates that cell-intrinsic regulators, such as transcription factors, and cell-extrinsic signals, such as neurotrophic factors, play important roles in progenitor cell fate determination and their subsequent differentiation (Malicki, 2004; Yang et al., 2003). Recent studies have demonstrated that basic helix-loop-helix (bHLH) transcription factors regulate the determination and differentiation of multiple neuronal cell types during retinogenesis (Akagi et al., 2004; Hatakeyama and Kageyama, 2004; Vetter and Brown, 2001). The gene *Ptf1a* (also known as *PTF1-p48*) encodes a bHLH protein of 48 kD. *Ptf1a*, together with two proteins [RBP-L and a common (type I) bHLH protein] comprises the three subunits of the pancreas transcription factor (PTF1) (Roux et al., 1989; Beres et al., 2006). *Ptf1a* plays a fundamental role in exocrine and endocrine pancreas development in mice (Kawaguchi et al., 2002). In a recent study the expression of *Ptf1a* has also been detected in the retina of developing zebrafish embryos by in situ hybridization (Zecchin et al., 2004). Using a positional candidate gene approach, Sellick et al. (Sellick et al., 2004) have identified mutations in the *Ptf1a* gene of patients with pancreatic and cerebellar agenesis, and Hoshino and colleagues (Hoshino et al., 2005) could demonstrate that *Ptf1a* is involved in GABAergic neuronal cell specification in the cerebellum. Very recently, it has been shown that *Ascl1* (previously *Mash1*) controls the expression of *Ptf1a*. Furthermore, *Mash1* is expressed in sensory interneuron progenitors and is involved in the switch between excitatory and inhibitory cell fates in the developing mouse spinal cord (Mizuguchi et al., 2006).

In the present study, we show that the bHLH transcription factor *Ptf1a* is expressed in the neuroretina of developing mice. Furthermore, inactivation of the *Ptf1a* gene produces severe cellular defects of the inner retina as a result of inhibition of differentiation of GABAergic and glycinergic amacrine precursor cells and horizontal precursor cells.

MATERIALS AND METHODS

Generation of *Ptf1a-Cre(ex1)* mice

The *Ptf1a* locus was targeted with a vector replacing part of exon 1 (ex1) with the Cre recombinase and neo-resistance genes. Following removal of the neo-resistance gene by transient transfection of a plasmid encoding the FLP recombinase, embryonic stem (ES) cells were injected into C57BL/6 E3.5 blastocysts. The integration of Cre at the ATG-start codon of the *Ptf1a* gene was verified directly by DNA sequencing. All procedures were approved by the local animal care committee.

Ptf1a-Cre(ex1) R26R and Ptf1a-Cre(ex1) Z/EG transgene

The *Gt(ROSA)26Sortm1Sor* (*R26R*) reporter mouse line was purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and the *Tg(ACTB-Bgeo/GFP)21Lbe* (*Z/EG*) reporter mouse line was kindly provided to us by Dr Mori Tetsuji, Institute of Stem Cell Research, GSF-National Research Center for Environment and Health (Soriano, 1999; Novak et al., 2000). Heterozygous *R26R* and *Z/EG* mice were crossed with *Ptf1a-Cre(ex1)* (*Ptf1a*$^{+/Cre(ex1)}$) to generate *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{+/Cre(ex1)}$ *Z/EG* mice.

Genotyping of transgenic mice

All offspring were genotyped by PCR of genomic DNA from mouse tail clips with primers specific for the *Ptf1a*, *Cre*, *R26R* and *Z/EG* transgenes as described by Krapp et al. (Krapp et al., 1996), Gu et al. (Gu et al., 1993), Soriano (Soriano, 1999) and Novak et al. (Novak et al., 2000) respectively.

Southern blot analysis

Genomic DNA from ES clones were digested with *Sac*I, separated by agarose gel electrophoresis, blotted to nylon membranes, and detected with the radioactive labeled 5′ flanking external probe.

Quantitative TaqMan PCR and data analysis

Real-time PCR was performed in a Perkin-Elmer 7700 Sequence Detection System. Total RNA was isolated by RNeasy Mini Kit (Qiagen) according to the manufacturer's manual. Two micrograms of total RNA was first reverse transcribed with SSIII (Invitrogen) and random primer in a total volume of 20 μl for 2 hours at 50°C. SSIII was inactivated by heating at 75°C for 15 minutes. The cDNA was diluted fivefold, and 5 μl was used for each 30 μl PCR using the SYBR GREEN PCR Master Mix (Applied Biosystems). The Taqman primers were designed using Primer Express Software (Applied Biosystems). The primer sequences are listed in Table 1. The PCR conditions for all genes were as follows: 50°C for 2 minutes hold, 95°C for 2 minutes hold and 40 cycles of 95°C, for 15 seconds and 60°C for 30 seconds. For each gene, the real-time PCR assay was performed four times with four different batches of total RNA. The *cyclophilin* gene served as an RNA input control. Relative gene expression ratio (ER) was calculated on the basis of differences between expression level of *cyclophilin* and genes analyzed using the following formula: $ER=2^{-\Delta Ct}$, where ΔCt is the difference of threshold cycles between the gene of interest and the control gene (*cyclophilin*).

Retinal explant culture

Eyes were isolated from E18.5 embryos. Dissected explants of neural retina were placed into a Millicell CM chamber (Millipore), with the ganglion cell layer upward. These retinal explants were cultured in 50% DMEM (Gibco) supplemented with HEPES, 25% Hank's solution, 25% heat-inactivated horse serum, 200 μmol/l L-glutamine and 6.75 mg/ml glucose at 34°C in a 5% CO_2 incubator (Hatakeyama and Kageyama, 2002).

X-Gal staining

Sections were air-dried for 20 minutes, fixed in paraformaldehyde, washed in PBS and stained with X-Gal reaction buffer (2 mmol/l $MgCl_2$, 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 0.5 mg/ml X-Gal in PBS) at 37°C overnight.

BrdU labeling

Pregnant mice were injected with 0.14 mg/g body weight BrdU 2 hours before they were sacrificed. BrdU incorporation was detected on 10 μm cryostat sections by antibody directed against BrdU (Sevotech), and at least four different eyes from wild-type embryos were analyzed.

Samples, histology, immunohistochemistry, tunnel assay, microscopy and imaging

After enucleating the mouse eyes, we quickly removed the anterior segment (cornea and lens) leaving half eyecups. We analyzed sections, which were through the central retina. Histological analysis of retinal explant cultures was as previously described (Hatakeyama and Kageyama, 2002). Tissues and whole mounts were fixed in 4% paraformaldehyde for 30 minutes on ice, equilibrated overnight in 30% sucrose at 4°C, frozen in OCT compound (Leica) and stored at –80°C. Tissues were sectioned (8-10 μm) with a cryostat (MICROM, Laborgeraete GmbH, Walldorf, Germany).

Immunohistochemical and immunofluorescence analyses were carried out as previously described (Hsu, 1990). Briefly, sections were incubated with cell-type-specific antibodies in blocking serum overnight at 4°C. We used the following primary antibodies: anti-Ptf1a (a kind gift from R. J. MacDonald), anti-Thy1.2 (CD90; Promega), anti-syntaxin HPC 1 (Sigma), anti-γ-amino butyric acid (GABA; Sigma), anti-glycine transporter 1 (GlyT1; Chemicon), anti-Lhx1 (anti-Lim1; Chemicon), anti-Hu/D (Elavl4; Molecular Probes), anti-Brn3 (clone C-13; Santa Cruz) anti-Pax6 (Developmental Studies Hybridoma Bank), anti-N-cadherin (Transduction Laboratories), anti-recoverin (Chemicon), anti-β-galactosidase (from rabbit; ICN), anti-β-galactosidase (from mouse; Developmental Studies Hybridoma Bank), anti-GFP (Molecular Probes), anti-calbindin-D28K (Sigma), anti-PKCα (Zymed) and biotin-conjugated secondary antibodies (Dianova), as well as the avidin-biotin-peroxidase and avidin-biotin-alkaline phosphatase complex (Vectorstain ABC and ABC-AP), according to the manufacturer's instructions (Vector Labs). Antibodies were detected by BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium), TSA (Tyramide Signal Amplification) (Molecular Probes). The cell nuclei were stained with DAPI (4′,6-diamidine-2-phenylindol-dihydrochloride; Boehringer, Mannheim). Tunnel assay was performed using the In Situ Cell Death Detection Kit TMR red (Roche Diagnostics) following the manufacturer's protocol, except that all sections were counterstained with DAPI. At least three to five independent retinae from knockout and wild-type mice were collected and analyzed. For each retina, positive cells were counted under the microscope from four different sections in an area extending from the outer segments of the photoreceptor cells (osp) to the inner limiting membrane (ilm). The length of osp and ilm was 200 μm. Images were acquired using a Zeiss ApoTome microscope (Axiovert 200M) and captured by a CCD cool digital camera (Zeiss). Software module AnxioVision (Zeiss) was used for subsequent 3D reconstruction of the images.

Statistical analysis

Data from quantitative PCR and immunohistochemistry were analyzed by Student's *t*-test or Mann-Whitney *U*-test, as appropriate. A *P*-value of <0.05 was considered statistically significant. All analyses were performed using SPSS 12.0 software (SPSS, Chicago, IL).

RESULTS

Generation of *Ptf1a-Cre(ex1)* mice

To study the function of the *Ptf1a* gene in the developing mouse, we generated a *Ptf1a-Cre* knock-in mouse line by homologous recombination in ES cells. To prevent a loss of potential regulatory elements in the first intron of *Ptf1a*, we replaced precisely only the coding sequence in exon1 (ex1) with the coding region of a *Cre* recombinase targeted to the nucleus (Gannon et al., 2000) and obtained a *Ptf1a* null allele [*Ptf1a*$^{tm1(Cre)Nak}$ or *Ptf1a-Cre(ex1)*] (see Fig. S1 in the supplementary material). After verifying germline transmission, we crossed *Ptf1a*$^{+/Cre(ex1)}$ heterozygous mice with *Gt(ROSA)26Sortm1Sor* (*R26R*) or *Tg(ACTB-Bgeo/GFP)21Lbe* (*Z/EG*) reporter mouse lines (Soriano, 1999; Novak et al., 2000) to determine Cre activity. The *R26R* mice carry a modified *lacZ* gene driven by the cell-type-independent *ROSA26* promoter and the *Z/EG* reporter mouse line contains a CMV enhancer/chicken β-actin promoter. In *Ptf1a*$^{+/Cre(ex1)}$ *R26R* animals, *Ptf1a*-driven expression of Cre excises a stop cassette upstream from the *lacZ* gene and thereby activates the expression of β-galactosidase. Expression of β-galactosidase therefore marks all the cells in which *Ptf1a* is activated

Table 1. Primer sequences for real-time PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Foxn4	GCACAAGTGGAAACGAAAGGA	CGGGCGGTCTGAGATGAG
Gad1	GTTTACGGAGCATTCGATCCA	CATGCAGCCAAAGGTTGTATTTC
Lim1	TTATCTCCGGATTCCCAAGATC	CTGAGACGTTGGCACTTTCAGA
Neurod1	GAACACGAGGCAGACAAGAAAGA	CTCCCCCGTTTCTCAGAGAGT
Neurod4	GAGTCAAGGCCAATGCTAGAGAA	CCTAAGATTATCCAAGGCATCATTC
Barhl2	TCATCACACCCCGAAGCA	TCAAGCTTTGGCCTGAAGCT
Ptf1a	GGGTGAGCGCGATGATG	CTGCAAGAGGAGGGAGACCATA

and the active β-galactosidase expressing locus is inherited by their descendant cells. These cells can be efficiently detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). In offspring from the cross between $Ptf1a^{+/Cre(ex1)}$ and *Z/EG* mice line, *lacZ* expression is replaced with the enhanced green fluorescent protein (GFP) expression in cells expressing Cre.

Ptf1a is expressed during retinogenesis

Examination of $Ptf1a^{+/Cre(ex1)}$ *R26R* mice, in which all cells carry a *Ptf1a* heterozygous null allele, revealed that the *Ptf1a-Cre*-mediated *R26R* recombination and the subsequent activation of β-galactosidase expression was not restricted to the developing pancreas (Kawaguchi et al., 2002), neural tube (Obata et al., 2001) and cerebellum (Hoshino et al., 2005; Sellick et al., 2004) (see Fig. S2A,B in the supplementary material). Moreover, we detected β-galactosidase-expressing cells in the neuroretina of developing mice (see Fig. S2C-F in the supplementary material). These X-Gal positive cells were first detectable at E14.5 (see Fig. S2C,E in the supplementary material). At this early stage of embryonic development, the mouse retina consists of an inner and an outer neuroblastic layer (NBL), which contains RPCs (Dyer et al., 2003). X-Gal labeled cells of the *Ptf1a* lineage were located within the inner NBL. At E18.5, β-galactosidase expressing cells were primarily found in the inner NBL of the developing retina. Small numbers of X-Gal positive cells were also detected in the GCL and the outer region of the neuroretina (data not shown). In adult mice, most of the cells expressing β-galactosidase were localized in the innermost zone of the INL. Some β-galactosidase-expressing cells were found in the GCL and in the outermost zone of the INL (see Fig. S2D,F in the supplementary material). These data led us to a detailed analysis of the expression and function of the *Ptf1a* gene during retinal development in mice.

To identify the cells expressing *Ptf1a* in the developing retina, we immunolabeled retinal cryosections of wild-type mice at embryonic and postnatal stages of development. Ptf1a-expressing cells were first detected at E12.5 (Fig. 1A,D). From E12.5 to P1, Ptf1a was expressed in the nuclei of retinal precursor cells scattered in the NBL (Fig. 1A-F). The number of Ptf1a-expressing cells increased from E12.5 to P1. After P1, the number of Ptf1a-expressing cells decreased gradually, and from P3 onward, Ptf1a expression was not discernible in the retina.

Ptf1a is expressed in precursors of amacrine, displaced amacrine and horizontal cells

To identify the specific neuroretinal cells that are derived from *Ptf1a*-expressing cells in the developing mouse, we analyzed the adult retina of $Ptf1a^{+/Cre(ex1)}$ *R26R* mice by double-labeling experiments using antibodies against β-galactosidase and cell-type-specific markers: Thy1.2 (ganglion cell marker), syntaxin (amacrine cell marker), GABA (GABAergic amacrine and displaced amacrine cell marker), glycine transporter 1 (GlyT1, glycinergic amacrine cell marker), calbindin (horizontal cell marker) and PKC-α (bipolar cell marker). These results showed that *Ptf1a* expression, as assayed by *Cre*-mediated β-galactosidase expression, was observed in the amacrine, displaced amacrine and horizontal cells (Fig. 2A-R). β-Galactosidase was not coexpressed with vimentin, a marker for Mueller glia (data not show). These lineage-tracing data, taken together with the immunolocalization results of Ptf1a, indicate that *Ptf1a* is expressed in precursors of amacrine, displaced amacrine and horizontal cells.

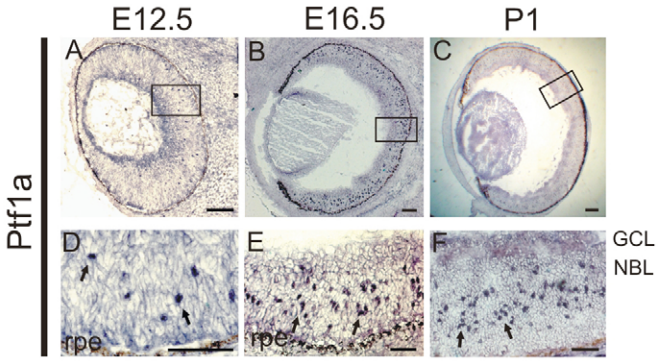

Fig. 1. Expression pattern of Ptf1a protein during mouse retinogenesis. Ptf1a protein was visualized by immunostaining with an anti-Ptf1a antibody of wild-type retina at E12.5 (**A**,**D**), E16.5 (**B**,**E**) and P1 (**C**,**F**). The boxed areas in A,B,C are shown at higher magnifications in D,E,F, respectively. The expression of Ptf1a protein is first seen at E12.5 in cells scattered in the NBL (D, arrows). As development proceeds, the number of *Ptf1a*-expressing cells increases and some cells reach out to the innermost zone of the NBL (E,F). Scale bars: 100 μm in A-C; 50 μm in D-F. GCL, ganglion cell layer; NBL, neuroblastic layer.

The *Ptf1a*-expressing cells are postmitotic

To determine whether cells expressing Ptf1a were actively dividing, we pulse-labeled retinae of wild-type mice with the thymidine analog BrdU for 2 hours and carried out double-immunofluorescence staining using anti-Ptf1a and anti-BrdU antibodies at E13.5 and P1. We found that neither E13.5 nor P1 S-phase cells were Ptf1a immunopositive (see Fig. S3 in the supplementary material). Thus, in summary, *Ptf1a* expression is initiated in the postmitotic amacrine, displaced amacrine and horizontal cell precursors.

Ptf1a-deficient retinae lack the IPL

To study the biological role of the *Ptf1a* gene in retinal development, we then investigated the retina of $Ptf1a^{Cre(ex1)/Cre(ex1)}$ *R26R* mice. In these mice, all cells are homozygous for the *Ptf1a* null mutation, but the retinal cells in which *Ptf1a* is transcriptionally activated can be identified by their expression of *lacZ* derived from *Ptf1a-Cre-*

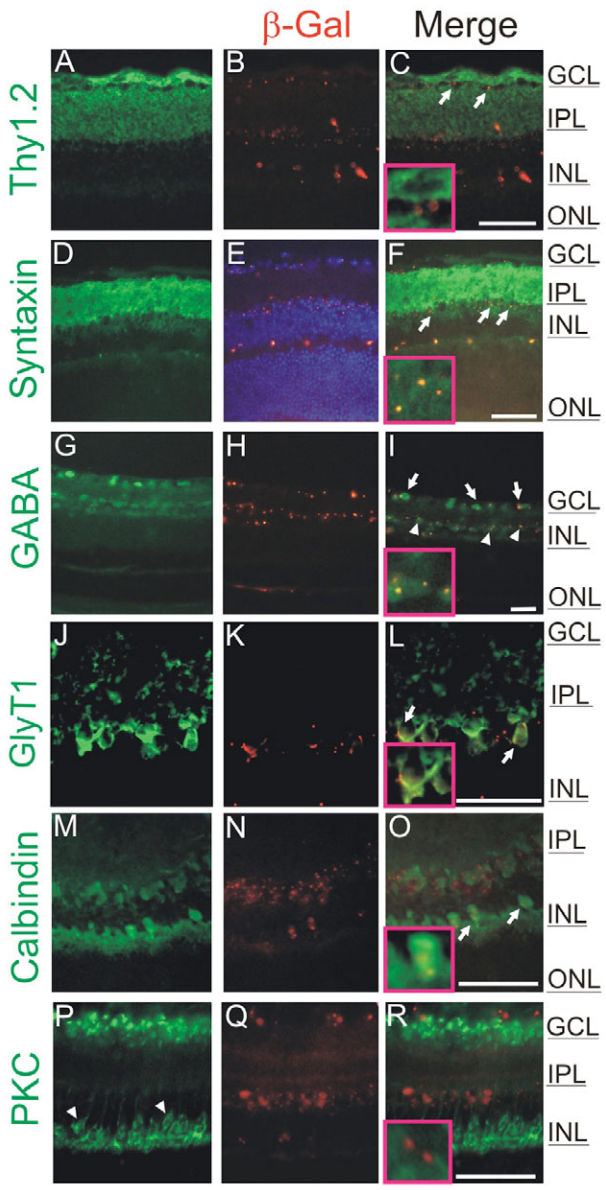

Fig. 2. Identification of *Ptf1a* cell lineage in *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinae of adult mice. *Ptf1a*-mediated β-galactosidase expression is co-localized with markers of specific classes of retinal neurons in the adult *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina. (**A**,**D**,**G**,**J**,**M**,**P**) Expression of selected markers is shown with Alexa 488 (green). (**B**,**E**,**H**,**K**,**N**,**Q**) Expression of β-galactosidase is shown by immunostaining with Alexa 568 (red). (**C**,**F**,**I**,**L**,**O**,**R**) In merged images, the yellow color represents co-localization of β-galactosidase with a specific marker. (C) β-galactosidase is present in the GCL (arrows) but does not co-localize with Thy1.2. (F) β-galactosidase coexpression with syntaxin, a general marker of amacrine cells, is evident in INL (arrows). (I) GABA coexpression with β-galactosidase is demonstrated in GABAergic amacrine (arrowheads, yellow) and displaced amacrine cells (arrows, yellow). (L) Coexpression of β-galactosidase with glycine transporter 1 (GlyT1) is found in glycinergic amacrine cells (arrows). (O) β-galactosidase coexpression with calbindin is present in horizontal cells (arrows, yellow). PKC-α-expressing bipolar interneurons (P, arrowheads) do not co-localize with β-galactosidase expression (R, inset). Insets in merge images (C,F,I,L,O,R) represent a 2.67-fold enlargement. DAPI staining in E shows the retinal nuclear layers. Scale bars: 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

mediated *R26R* activation. As the *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* pups die approximately three hours after birth, we examined the morphology of the retina in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* embryos at E18.5. After verifying the absence of Ptf1a protein in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae (Fig. 3A,E) by immunohistochemistry, we compared cryosections of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* with *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae. In retinae of heterozygous *Ptf1a*$^{+/Cre(ex1)}$ *R26R* mice, the IPL formed a continuous layer between the GCL and NBL (Fig. 3B,C,D). X-Gal staining of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina showed that the majority of β-galactosidase-expressing cells were localized in the innermost zone of the NBL (Fig. 3B,C). By contrast, in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina, the GCL and NBL were fused, resulting in loss of the IPL (Fig. 3F-H). We confirmed these results using immunostaining with anti-N-cadherin antibody and 3D reconstruction by ApoTome microscopy (Fig. 3D,H). In addition, the β-galactosidase-expressing cells were scattered in the inner retina of *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* mice (Fig. 3G). These data suggested a misplacement of β-galactosidase-expressing cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* toward the inner retina.

Ptf1a knockout retinae lack differentiated amacrine cells

To identify the cells expressing β-galactosidase in *Ptf1a*-deficient retinae and to further elucidate the defect in formation of the IPL in the *Ptf1a*-deficient retinae, we labeled retinal cryosections with the cell-specific antibodies Pax6 and Hu/D.

At E18.5, Pax6 is mainly expressed in differentiated amacrine cells and to a lower level in differentiated ganglion cells, as well as in RPCs (Marquardt et al., 2001). We found that Pax6 and β-galactosidase were coexpressed in differentiated amacrine cells of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* mice (Fig. 4A-C). By contrast, in *Ptf1a*-deficient retinae, the expression of Pax6 was restricted to cells in the NBL and showed no coexpression with β-galactosidase (Fig. 4D-F). These data indicate that retinae of *Ptf1a*-deficient mice fail to generate differentiated amacrine cells.

During retinal development, the RNA-binding protein Hu/D is expressed in differentiating amacrine and ganglion cells, but not in differentiated amacrine cells (Link et al., 2000). At E18.5, immunostaining with anti-Hu/D antibody of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinae showed Hu/D expression mainly in the GCL, which was not co-localized with β-galactosidase-expressing cells (Fig. 4G-I). However, in *Ptf1a*-deficient retinae we found that the large majority of the cells expressing β-galactosidase also were positive for Hu/D (Fig. 4J-L). These results suggest that β-galactosidase-expressing cells are amacrine cell precursors and/or ganglion cells in *Ptf1a* knockout retina.

Based on these data, we hypothesized that in *Ptf1a* knockout retina, the β-galactosidase-expressing cells, which would differentiate to amacrine or horizontal cells in the course of normal retinal development, were either inhibited in their differentiation or transdifferentiated to ganglion cells.

A proportion of *Ptf1a*-deficient precursor cells transdifferentiated to ganglion cells

To test the hypothesis that *Ptf1a*-deficient precursor cells transdifferentiated to ganglion cells, we analyzed the cell fate of these cells by using the *Z/EG* reporter mouse line, which expresses β-galactosidase before Cre excision and enhanced GFP after Cre excision. The double immunostaining of the *Ptf1a*$^{+/Cre(ex1)}$ *Z/EG* retinae with anti-GFP and anti-Brn3 showed no co-labeling (Fig. 5A-C). However, in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* retinae, GFP and Brn3 were coexpressed in approximately 30% of the cells (Fig. 5D-F,H). Taken together, these data provide evidence that a proportion of

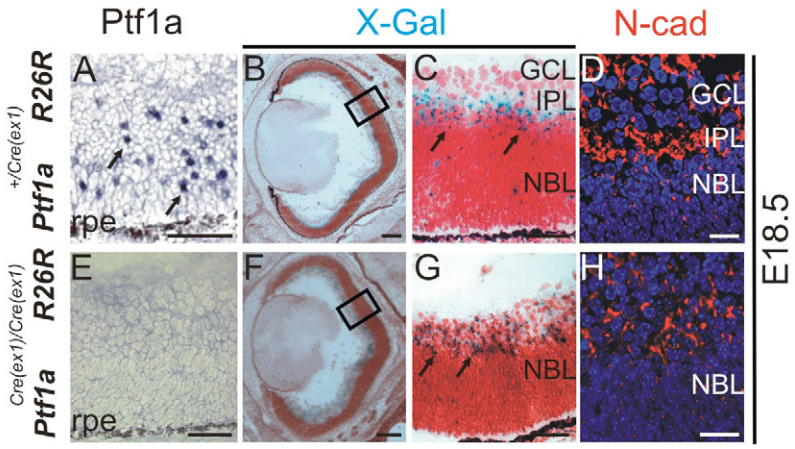

Fig. 3. Morphological comparison of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae. (**A**,**E**) To confirm the absence of Ptf1a protein in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae, retinal sections from E18.5 embryos were immunostained with an antibody against Ptf1a (arrows). (E) No immunoreactivity can be detected in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina. (**B**,**C**,**F**,**G**) X-Gal staining of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae at E18.5. *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina shows morphological change in the inner retina (F) compared with the *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina (B). Higher magnification of boxed areas in B and F are shown in C and G, respectively. At E18.5, the GCL and IPL are formed in *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina (B,C). β-galactosidase-expressing cells are primarily restricted to the inner zone of the NBL (C, arrows). By contrast, in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina (F,G), the GCL and NBL are fused and the IPL is lost. The β-galactosidase-positive cells are scattered in the inner retina (G, arrows). In both *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinae some isolated cells are found in the outermost zone of the NBL. (B,C,F,G) Background is stained by neutral red. (**D**,**H**) Images are taken in ApoTome mode (Zeiss) for the purpose of 3D reconstruction showing immunohistochemistry for N-cadherin (red) with a DAPI counterstain (blue). (D) In *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina, N-cadherin stains GCL and IPL. (H) By contrast, N-cadherin-positive processes are strongly reduced in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R*, resulting in loss of the IPL. Scale bars: 50 μm in A,C,E,G; 100 μm in B,F; 10 μm in D,H. GCL, ganglion cell layer; IPL, inner plexiform layer; NBL, neuroblastic layer; rpe, retinal pigment epithelium.

Ptf1a-deficient precursor cells transdifferentiate to ganglion cells. In line with these results, we observed a significant increase of Brn3-positive cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ (Cre/Cre) compared with wild-type retinae (Fig. 5G).

GABAergic and glycinergic amacrine cells are missing in *Ptf1a*-deficient retinal explants

To further determine the cell fate of *Ptf1a*-deficient precursor cells that did not transdifferentiate to ganglion cells, we examined *Ptf1a* knockout cells at the postnatal stage of development. As homozygous null mice for the *Ptf1a* gene die about three hours after birth with pancreatic and cerebellar agenesis (Hoshino et al., 2005), retinal explants were prepared from E18.5 embryos and cultured for 12 days [E18.5+12 days in culture (DIC)]. We analyzed cryosections of retinal explants from *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* mice using specific antibodies against each retinal cell type (Fig. 6, Table 2).

In both heterozygous and homozygous *Ptf1a* null mutant retinal explants, Thy1.2 (Fig. 6A,B), a marker expressed in ganglion cells, and Hu/D (Fig. 6C,D), a marker for ganglion and amacrine

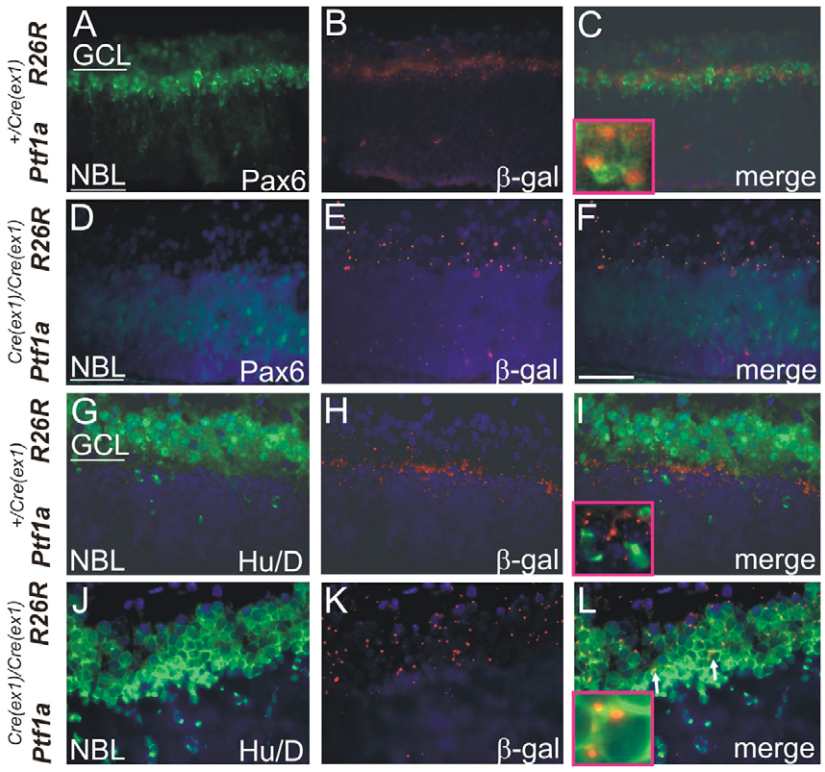

Fig. 4. Effect of *Ptf1a* absence on generation of retinal cell types at E18.5. Retinal sections were prepared from *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* and control *Ptf1a*$^{+/Cre(ex1)}$ *R26R* embryos at E18.5. Co-immunostaining for Pax6 (green, **A**) and β-galactosidase (red, **B**) shows the expression of both genes in the inner NBL of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina (**C**, inset). In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina, Pax6 is expressed at a low level in the NBL (**D**), whereas β-galactosidase-positive cells are in the GCL (**E**). The merged image shows no coexpression of Pax6 and β-galactosidase (**F**). Immunohistochemistry on cryosections from *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retina shows the expression of Hu/D antigen in the GCL (green, **G**) and β-galactosidase in the inner NBL (red, **H**). β-galactosidase-positive cells do not coexpress Hu/D (**I**, inset). In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retina, both Hu/D (**J**) and β-galactosidase (**K**) are expressed in the GCL. Merged image shows the coexpression of Hu/D and β-galactosidase in the GCL (**L**, inset, arrows). Insets in C,I and L represent a 2.67× enlargement. Background was stained by DAPI (blue). Scale bars: 50 μm. GCL, ganglion cell layer; NBL, neuroblastic layer.

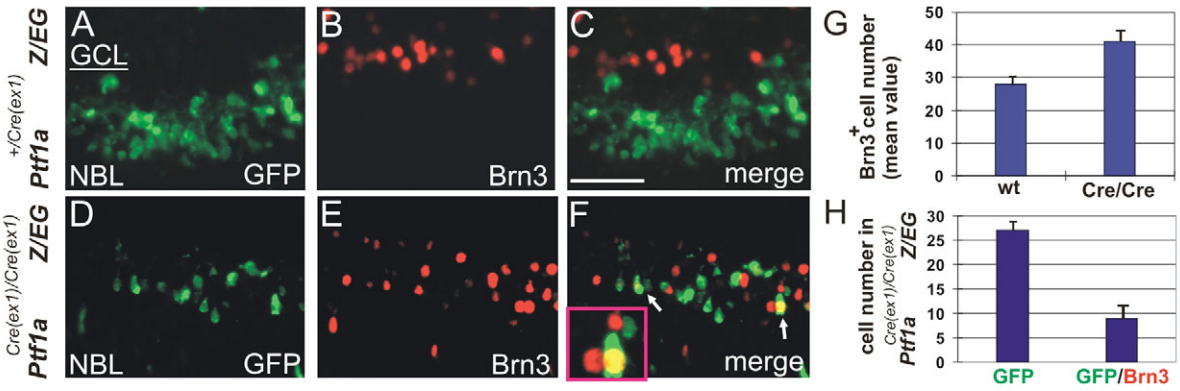

Fig. 5. Identification of transdifferentiated precursor cells in *Ptf1a*-deficient retina at E18.5. (**A-F**) Sections from E18.5 retinae of *Ptf1a*$^{+/Cre(ex1)}$ *Z/EG* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* were double-immunostained with anti-GFP and anti-Brn3 antibodies. (A,D) *Ptf1a*-mediated GFP expression is present in *Ptf1a*$^{+/Cre(ex1)}$ *Z/EG* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* retinae (green). (B,E) Ganglion cells are marked with Brn3 (red). (C,F) Yellow in merged images represents co-localization of GFP with Brn3. GFP coexpression with Brn3 is not present in *Ptf1a*$^{+/Cre(ex1)}$ *Z/EG* retina (C). By contrast, in about one-third of *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* retinal cells, GFP is coexpressed with Brn3 (F, arrows, inset). (**G**,**H**) Quantification of Brn3-positive cells in wild-type (wt) and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ (Cre/Cre) retinae (G) and of GFP and GFP/Brn3-positive cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* retinae (H). Each histogram represents the mean±SD for four retinae (Number of Brn3-positive cells in wt are 28.5±2.08 and in Cre/Cre are 40.75±3.40. In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *Z/EG* retinae, the number of GFP-positive cells are 27.2±1.70 and GFP/Brn3-positive cells are 9.2±3.59). Positive cells were scored in an area that was above a line of 200 μm in length parallel to the retinal pigment epithelium. Scale bars: 50 μm. GCL, ganglion cell layer; NBL, neuroblastic layer.

cells, were present. However, the number of these cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants was significantly decreased.

In retinal explants of *Ptf1a*-deficient mice, we found almost no GABAergic (Fig. 6E,F) and glycinergic-positive cells (Fig. 6G,H). In addition, in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants, the number of X-Gal-positive cells was significantly reduced (P=0.001, Fig. 6Q,R), whereas that of apoptotic cells was significantly increased (P=0.001, Fig. 6S,T) compared with *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explants. Therefore, we suggest that in *Ptf1a*-deficient retinae, the precursors of GABAergic and glycinergic amacrine cells either transdifferentiate to ganglion cells or undergo apoptotic cell death during postnatal development. If transdifferentiation occurs, it should be accompanied by an increase of X-Gal-positive cells. We detected only a few X-Gal positive cells in *Ptf1a*-deficient retinal explants, and we conclude that most of the precursors of GABAergic and glycinergic amacrine cells undergo apoptotic cell death rather than transdifferentiate to ganglion cells.

To further characterize the defects of *Ptf1a*-deficient retinae, we investigated the genesis and differentiation of the remaining retinal cells. The presence of syntaxin- and calbindin-positive cells in *Ptf1a*-deficient retinal explants (Fig. 6J,L) indicates that not all of the amacrine precursor cells undergo apoptotic cell death or transdifferentiate to ganglion cells. Thus, we propose that a subtype of amacrine precursor cells do not need *Ptf1a* for differentiation.

Furthermore, we observed a disorganization of syntaxin-positive cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* compared with *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explants (Fig. 6I,J). The same was true for calbindin (amacrine and horizontal cells) and PKCα (bipolar cells) (Fig. 6K-N) immunoreactive cells. Recoverin staining showed less evident changes in *Ptf1a*-deficient retinal explants (Fig. 6O,P).

In summary, retinal explant results show: (1) a small population of differentiated amacrine cells are present in the *Ptf1a* null mutant; (2) GABAergic and glycinergic amacrine cells are absent in the *Ptf1a*-deficient mice; (3) a disorganization of the inner neuroretina; and (4) an increase of apoptotic cells in *Ptf1a* knockout mice.

Genes involved in amacrine and horizontal cell genesis are differentially regulated in *Ptf1a*-deficient retinae

To consolidate our findings, we performed real-time PCR analysis on total RNA extracted from wild-type and *Ptf1a*-deficient retinae at E18.5 using a set of marker genes that regulate amacrine [*Foxn4*, *Neurod1* (formerly *NeuroD*), *Neurod4* (formerly *Math3*), *Barhl2*] and horizontal [*Foxn4*, *Lhx1* (also known as *Lim1*)] cell development plus glutamic acid decarboxylase 1 (*Gad1*), an enzyme that catalyzes the synthesis of the inhibitory neurotransmitter GABA (Fig. 7I). *Foxn4* controls the genesis of amacrine and horizontal cells by activating the expression of *Neurod1* and *Neurod4* (Li et al., 2004). The expression level of *Foxn4* remained unchanged in wild-type and *Ptf1a*-deficient retinae, indicating that *Ptf1a* acts chronologically later on amacrine cell genesis than *Foxn4*. Both bHLH transcription factors, *Neurod1* and *Neurod4*, are expressed in differentiating amacrine cells and regulate amacrine cell fate specification (Inoue et al., 2002). The absence of *Ptf1a* does not alter *Neurod1* and *Neurod4* expression, suggesting that the involvement of *Ptf1a* to amacrine cell fate specification takes place at later stages of amacrine cell development, as is the case for *Neurod1* and *Neurod4*. By contrast, in *Ptf1a*-deficient retinae the expression of *Barhl2* and *Gad1* transcripts were downregulated, indicating that *Ptf1a* is involved in the molecular mechanism governing the specification of subtype identity of amacrine cells. The result that the expression level of *Barhl2* in *Ptf1a*-deficient retinae is only slightly decreased is consistent with the fact that *Barhl2* is also expressed by ganglion cells. Also in line with our immunohistochemical data was the finding that the transcription factor *Lim1* (horizontal cell marker) is not detectable in *Ptf1a*-deficient retinae (Fig. 7II).

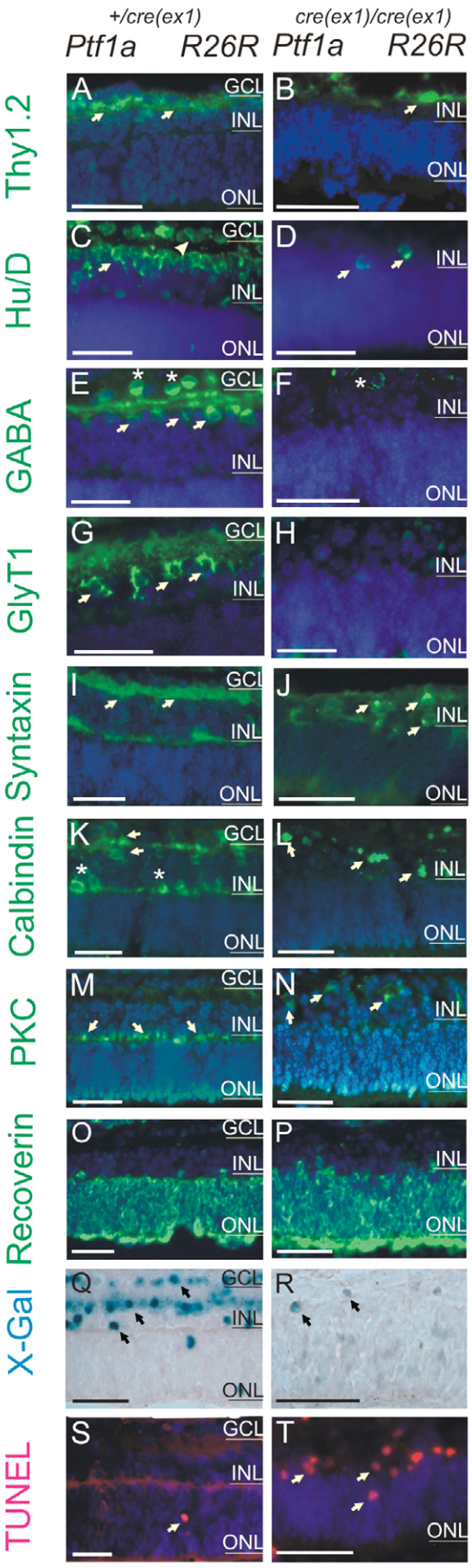

Fig. 6. Effect of *Ptf1a* absence on the generation and formation of different cell types in retinal explants. Phenotypic comparison of retinal explants from *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* mice after 12 days in culture (DIC). Retinae from two siblings were dissected at E18.5 and were maintained in culture as whole retinae without destroying their cellular integrity for 12 days. Retinal explants were cryosectioned and immunolabeled with antibodies to cell-specific markers of all major classes of retinal cells. Immunofluorescence analysis of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants at E18.5+12DIC with Thy1.2 (**A**,**B**) Hu/D (**C**,**D**), GABA (**E**,**F**), Gly-T1 (**G**,**H**), syntaxin (**I**,**J**), calbindin (**K**,**L**), PKCα (**M**,**N**) and recoverin (**O**,**P**). (**Q**,**R**) Expression of β-galactosidase. (**S**,**T**) Results of the TUNEL assay. (A) In *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant, Thy1.2 is expressed in the GCL (green, arrows). (B) In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explant, Thy1.2-positive cells are located in the innermost zone of the INL and do not form a distinct cell layer (green, arrow). (C) In *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant, Hu/D is expressed in the GCL (arrowhead) and INL (arrow). (D) In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explant, Hu/D positive cells are strongly reduced (arrows). (E) In *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant, GABA is expressed in amacrine (arrows) and displaced amacrine cells (asterisks). (F) By contrast, GABA-positive cells (asterisk) are virtually missing in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants. (G) Glycinergic amacrine cells express GlyT1 in *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant (arrows). (H) GlyT1-positive cells are not found in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants. (I) Syntaxin is found in the inner plexiform (IPL) and in amacrine cells (arrows, green) of *Ptf1a*$^{+Cre(ex1)}$ *R26R* retinal explant. (J) By contrast, the IPL is missing in retinal explant from *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* embryos and the number of amacrine cells (arrows) is strongly reduced. (K) Calbindin is expressed in the OPL, horizontal cells (asterisk), amacrine cells (arrows) and displaced amacrine cells (arrows) of the *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant. (L) In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explant only reduced number of calbindin-positive cells (arrows) can be detected. (M) Retinal explant from *Ptf1a*$^{+/Cre(ex1)}$ *R26R* mice expresses PKC-α in bipolar cells (arrows, green). (N) In retinal explant from *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R*, there are few PKC-α labeled cells (arrows, green), which do not form a cell layer and show a disarrayed structure. Both *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants are positive for recoverin in the ONL (O,P, green). Cells expressing β-galactosidase (arrows) are significantly reduced in retinal explant of *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* (R) in comparison with *Ptf1a*$^{+/Cre(ex1)}$ *R26R* (Q). In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explant (S, arrows), compared with *Ptf1a*$^{+/Cre(ex1)}$ *R26R* retinal explant (T, arrows), the number of apoptotic nuclei is significantly increased in both INL and ONL. In A-P and S,T the background was stained by DAPI (blue), showing the location of nuclei. Scale bar: 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

DISCUSSION

Ptf1a is expressed in precursors of postmitotic amacrine and horizontal cells during retinogenesis

Using a novel *Ptf1a-Cre* knock-in mutation in combination with a *ROSA26* reporter transgene, we could analyze the expression of *Ptf1a* in the mouse retina. The experiments reported here show that Ptf1a expression is first detectable at around E12.5 in the developing retina. As development proceeds, Ptf1a-positive cells spread from the centre to the inner retina and become primarily localized to the outer NBL. However, Ptf1a expression is absent in adult retina. Our cell-lineage-tracing experiments revealed that transcriptional activation of *Ptf1a-Cre* occurs in amacrine and horizontal precursor cells. Analysis of the cell cycle state of Ptf1a-expressing cells showed that these cells were postmitotic. Our data suggest that, in neuroretinal cells, *Ptf1a* expression commences at a developmental state in which these cells are specialized but not terminally

Table 2. Quantification of cells in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* retinal explants

Cell type	Antibody used	Cell number (mean±s.d.)	
		Ptf1a$^{+/Cre(ex1)}$ *R26R*	*Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R*
X-Gal$^+$ cells	–	26.82±7.75	1.83±0.89
GABAergic amacrine cells	GABA	19.16±1.17	1.66±0.81
Glycinergic amacrine cells	GlyT1	8.83±1.16	0.33±0.51
Calbindin$^+$ amacrine cells	Calbindin	13.33±1.50	6.50±1.87
Syntaxin$^+$ amacrine cells	Syntaxin	15.60±0.81	3.81±0.75
Bipolar cells	PKC-α	9.30±1.50	2.50±1.04

Immunostaining was performed with retinal-cell-specific antibodies from four independent retinal explants of *Ptf1a*$^{+/Cre(ex1)}$ *R26R* and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ *R26R* each. The number of positive cells was counted from three different sections of each retinal explant. Data are presented as mean±s.d.

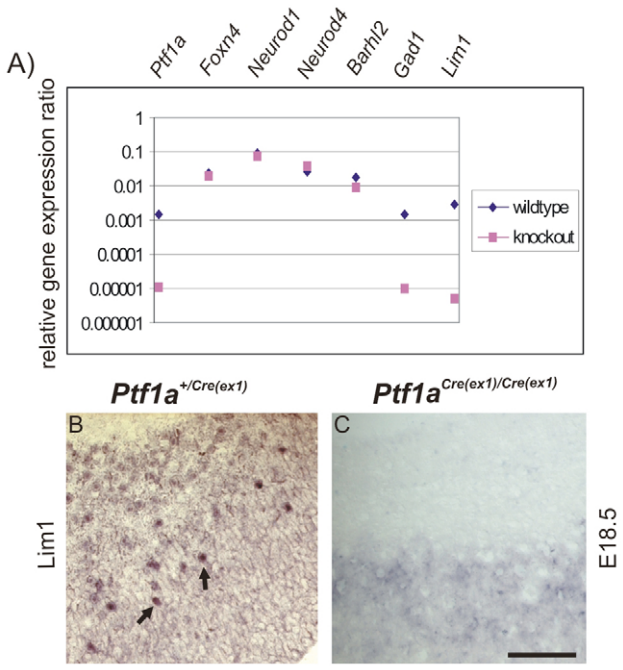

Fig. 7. Amacrine- and horizontal cell-specific factors are downregulated in *Ptf1a*-deficient retina. (**A**) Quantification of differentially expressed genes in *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ retina by real-time PCR at E18.5. Real-time PCR was performed on cDNA samples prepared from four independent retinae of wild type and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ each. Mean values of relative gene ER (see Materials and methods) for wild-type and *Ptf1a* knockout retinae are shown for each gene. Differences in ER between wild-type and *Ptf1a* knockout retinae were statistically significant for *Ptf1a*, *Gad1* and *Lim1* (P<0.001). (**B**,**C**) Immunostaining of horizontal cells in mice retina. Retinal sections from E18.5 of *Ptf1a*$^{+/Cre(ex1)}$ and *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ embryos were immunolabeled with an antibody to Lim1. (B) Arrows indicate Lim1 immunopositive cells in *Ptf1a*$^{+/Cre(ex1)}$ retina. In *Ptf1a*$^{Cre(ex1)/Cre(ex1)}$ retina, no Lim1-positive cells are detectable (C). Scale bar: 50 μm.

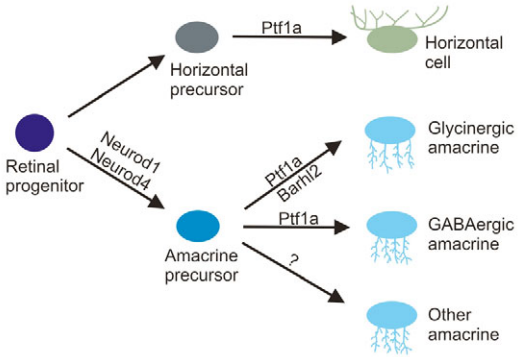

Fig. 8. Schematic depicting the role of *Ptf1a* in retinal cell development. During retinogenesis, expression of *Neurod1* and *Neurod4* initiates specification of RPCs to amacrine precursor cells, after which they migrate to the innermost zone of the NBL. *Ptf1a* induces amacrine cell precursors to differentiate into GABAergic and glycinergic amacrine cells. In addition, *Ptf1a* is essential in the specification of horizontal precursor cells to horizontal cells.

differentiated. This is in line with the finding that Ptf1a expression is absent in terminally differentiated amacrine and horizontal cells. The early and cell-specific expression of *Ptf1a* suggests an important role for *Ptf1a* in inducing the differentiation of amacrine and horizontal cells.

Ptf1a controls terminal differentiation of GABAergic and glycinergic amacrine cells

Amacrine cells can be divided into two major nonoverlapping subpopulations classified by neurotransmitter production. In the mouse retina, GABAergic amacrine cells comprise ~35% and glycinergic amacrine cells ~40% of all amacrine cells (Mo et al., 2004). We presented several lines of evidence that *Ptf1a* is involved in the terminal differentiation of GABAergic and glycinergic amacrine cells. First, birth-dating studies of retinal cells have revealed that during mouse retinogenesis amacrine cells are generated over a time period from E11 to P4 (Young, 1985). We demonstrated that *Ptf1a* expression starts at around E12.5 and ceases gradually at P3 in the developing retina of wild-type mice, which is concomitant to amacrine cell genesis. Second, we could show that inactivation of the *Ptf1a* gene leads to downregulation of genes (*Barhl2*, *Gad1*) contributing to mature amacrine cell phenotype and to complete absence of GABAergic and glycinergic amacrine cells in retinal explants.

Recent studies have shown that the bHLH transcription factor *Ptf1a* is involved in driving neural precursors to differentiate into GABAergic neurons in the cerebellum, and that *Ptf1a* is required for the formation of GABAergic neurons in the dorsal horn of the spinal cord. The absence of *Ptf1a* expression in this tissue causes a transdifferentiation of GABAergic neurons to glutamatergic neural cells (Glasgow et al., 2005; Hoshino et al., 2005). By contrast to spinal cord, lack of *Ptf1a* activity lead to differentiation arrest of amacrine precursor cells and partial transdifferentiation of amacrine precursor cells to ganglion cells, which results in loss of GABAergic and glycinergic amacrine cells in mature retina. The fact that in the *Ptf1a*-deficient retinae the number of differentiated amacrine cells is significantly decreased and the number of ganglion cells is increased could lead to loss of IPL. These data are in line with the results that in *Math3/NeuroD* double-mutant retina the IPL is not formed (Inoue et al., 2002).

Horizontal cell development is impaired in *Ptf1a*-deficient retinae

The amacrine and horizontal cells are important interneurons that process and transmit visual input in the retinal circuitry. It has been demonstrated that amacrine and horizontal cells begin to exit the cell cycle at E11 (Young, 1985). In our analysis, *Ptf1a* expression first appears at E12.5 in postmitotic cells, indicating that *Ptf1a* is unlikely to be involved in initial generation of amacrine and horizontal cells. We have also followed the cell fate of horizontal cells in *Ptf1a*-deficient retina. *Ptf1a* is important for differentiation of horizontal precursor cells. First, our lineage-tracing analysis revealed that amacrine and horizontal cells express *Ptf1a*. Second, in *Ptf1a*-deficient retina the expression of Lim1 is totally absent, which indicates that horizontal cells are not generated. Our observation of calbindin-positive cells in *Ptf1a* mutant retinal explants, although the horizontal cells are deficient, may be attributed to the presence of a subtype of amacrine cells as previously reported (Uesugi et al., 1992).

Foxn4 is involved in the genesis of horizontal cells (Li et al., 2004). As the absence of *Ptf1a* does not affect the expression level of *Foxn4* (Fig. 7I), we conclude that *Ptf1a* acts downstream in the development of horizontal cells, then *Foxn4*.

In *Caenorhabditis elegans*, genetic analyses of Lim and homeodomain genes have demonstrated a prominent role for them in terminal differentiation of specific neurons. For instance, *mec-3* is required for differentiation of mechanosensory neurons, and *lim-6* regulates neurite outgrowth and function of GABAergic motoneurons (Hobert et al., 1999; Way and Chalfie, 1988).

Inactivation of *Ptf1a* causes a switch in cell fate

In *Ptf1a* knockout retinae, we observed a co-localization of GFP and Brn3 expression in approximately 30% of GFP-positive cells at E18.5 (Fig. 5). This strongly indicates that a proportion of *Ptf1a*-deficient precursor cells transdifferentiate to ganglion cells. As the number of apoptotic cells in *Ptf1a* knockout retina is increased, we assume that *Ptf1a*-deficient cells that do not transdifferentiate undergo apoptotic cell death. Regarding the transdifferentiation of retinal cells, *Ptf1a* knockout resembles *Math3/NeuroD* double-mutant retina (Inoue et al., 2002). However, there are several significant differences concerning amacrine cell differentiation and the number of retinal cells. First, in INL of *Ptf1a*-deficient retinal explants, some amacrine cells (syntaxin$^+$, calbindin$^+$) were generated. By contrast, in *Math3/NeuroD* null mutant retinal explants, amacrine cells are completely missing (Inoue et al., 2002). Second, whereas the number of ganglion cells was significantly increased in *Math3/NeuroD* double-mutant retinal explants, that of *Ptf1a*-deficient retinal explants was strongly reduced. Third, in *Ptf1a* null mutant retinal explants, the number of apoptotic cells was increased, bipolar cells were significantly reduced and the architecture of the INL was disrupted. In *Math3/NeuroD* double-mutant retinal explants, bipolar cells were normally generated and positioned and the number of apoptotic cells was not increased. Therefore, we conclude that there are more defects in *Ptf1a*-deficient retina compared with *Math3/NeuroD* double-mutant retina.

To summarize previous results and our present study, we propose a hypothetical model for cell differentiation in the retinae of embryonic mice (Fig. 8). The transcription factors *Neurod1* and *Neurod4* specify RPCs to amacrine precursor cells. It has been demonstrated that the *Barhl2* homeobox gene is involved in the specification of glycinergic amacrine cells (Mo et al., 2004). As the inactivation of *Ptf1a* leads to loss of GABAergic and glycinergic amacrine cells, as well as to the downregulation of *Barhl2* and *Gad1* transcripts, *Ptf1a* promotes the differentiation of amacrine cell precursors to GABAergic and glycinergic amacrine cells. Furthermore, we found that in the *Ptf1a* null retina a small number of amacrine precursor cells differentiated to amacrine cells. Thus, we conclude that *Ptf1a* contributes to the specification of amacrine cell subtypes rather than to the generation of amacrine cells. *Ptf1a* is expressed in postmitotic horizontal precursor cells. As the expression level of *Foxn4* in *Ptf1a*-deficient retinae did not change (Fig. 7I), we propose that *Ptf1a* acts downstream to *Foxn4* in the cell signaling cascade regarding the generation of horizontal cells. Even though there are gaps in our knowledge about retinogenesis, we provide evidence that *Ptf1a* plays an important role in retinal cell differentiation.

We are grateful to Raymond J. MacDonald for providing the Ptf1a antibody (Department of Molecular Biology, the University of Texas Southwestern Medical Center, Dallas). We thank Peter K. Wellauer (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) for the genomic *Ptf1a* sequence, Klaus Rajewsky (CBR Institute for Biomedical Research, Harvard Medical School, Boston) for the *Cre* construct and Bernhard Luecher (Institute of Pharmacology, University of Zurich, Switzerland) for the *neomycin-resistance* cassette flanked by *FRT* sites. We are grateful to T. Marquardt for helpful discussions and A. Dietrich as well as N. Buentig for comments on the manuscript. This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 518) and the Wilhelm-Roux program (FKZ 10/41) from Martin Luther University Halle-Wittenberg.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/6/1151/DC1

References

Akagi, T., Inoue, T., Miyoshi, G., Bessho, Y., Takahashi, M., Lee, J. E., Guillemot, F. and Kageyama, R. (2004). Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. *J. Biol. Chem.* **279**, 28492-28498.

Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M. and Macdonald, R. J. (2006). PTF1 Is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or Its paralogue, RBP-L. *Mol. Cell. Biol.* **1**, 117-130.

Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat. Genet.* **34**, 53-58.

Gannon, M., Herrera, P. L. and Wright, C. V. (2000). Mosaic Cre-mediated recombination in pancreas using the pdx-1 enhancer/promoter. *Genesis* **26**, 143-144.

Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. and Johnson, J. E. (2005). Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development* **132**, 5461-5469.

Gu, H., Zou, Y. R. and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73**, 1155-1164.

Hatakeyama, J. and Kageyama, R. (2002). Retrovirus-mediated gene transfer to retinal explants. *Methods* **28**, 387-395.

Hatakeyama, J. and Kageyama, R. (2004). Retinal cell fate determination and bHLH factors. *Semin. Cell Dev. Biol.* **15**, 83-89.

Hobert, O., Tessmar, K. and Ruvkun, G. (1999). The Caenorhabditis elegans lim-6 LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons. *Development* **126**, 1547-1562.

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M. et al. (2005). Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* **47**, 201-213.

Hsu, S. M. (1990). Immunohistochemistry. *Meth. Enzymol.* **184**, 357-363.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J. E. and Kageyama, R. (2002). Math3 and NeuroD regulate amacrine cell fate specification in the retina. *Development* **129**, 831-842.

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J. and Wright, C. V. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat. Genet.* **32**, 128-134.

Krapp, A., Knofler, M., Frutiger, S., Hughes, G. J., Hagenbuchle, O. and Wellauer, P. K. (1996). The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. *EMBO J.* **15**, 4317-4329.

Li, S., Mo, Z., Yang, X., Price, S. M., Shen, M. M. and Xiang, M. (2004). Foxn4

controls the genesis of amacrine and horizontal cells by retinal progenitors. *Neuron* **43**, 795-807.
Link, B. A., Fadool, J. M., Malicki, J. and Dowling, J. E. (2000). The zebra fish young mutation acts non-cell-autonomously to uncouple differentiation from specification for all retinal cells. *Development* **127**, 2177-2188.
Malicki, J. (2004). Cell fate decisions and patterning in the vertebrate retina: the importance of timing, asymmetry, polarity and waves. *Curr. Opin. Neurobiol.* **14**, 15-21.
Marquardt, T. (2003). Transcriptional control of neuronal diversification in the retina. *Prog. Retin. Eye Res.* **22**, 567-577.
Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* **105**, 43-55.
Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q. and Goulding, M. (2006). Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nat. Neurosci.* **9**, 770-778.
Mo, Z., Li, S., Yang, X. and Xiang, M. (2004). Role of the Barhl2 home box gene in the specification of glycinergic amacrine cells. *Development* **131**, 1607-1618.
Novak, A., Guo, C., Yang, W., Nagy, A. and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon cre-mediated excision. *Genesis* **28**, 147-155.
Obata, J., Yano, M., Mimura, H., Goto, T., Nakayama, R., Mibu, Y., Oka, C. and Kawaichi, M. (2001). p48 subunit of mouse PTF1 binds to RBP-Jkappa/CBF-1, the intracellular mediator of Notch signaling, and is expressed in the neural tube of early stage embryos. *Genes Cells* **6**, 345-360.
Roux, E., Strubin, M., Hagenbuchle, O. and Wellauer, P. K. (1989). The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA. *Genes Dev.* **10**, 1613-1624.
Sellick, G. S., Barker, K. T., Stolte-Dijkstra, I., Fleischmann, C., Coleman, R. J., Garrett, C., Gloyn, A. L., Edghill, E. L., Hattersley, A. T., Wellauer, P. K. et al. (2004). Mutations in PTF1A cause pancreatic and cerebellar agenesis. *Nat. Genet.* **36**, 1301-1305.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Uesugi, R., Yamada, M., Mizuguchi, M., Baimbridge, K. G. and Kim, S. U. (1992). Calbindin D-28k and parvalbumin immunohistochemistry in developing rat retina. *Exp. Eye Res.* **54**, 491-499.
Vetter, M. L. and Brown, N. L. (2001). The role of basic helix-loop-helix genes in vertebrate retinogenesis. *Semin. Cell Dev. Biol.* **12**, 491-498.
Way, J. C. and Chalfie, M. (1988). mec-3, a homoebox-containing gene that specifies differentiation of the touch receptor neurons in C. elegans. *Cell* **54**, 5-16.
Yang, M., Donaldson, A. E., Jiang, Y. and Iacovitti, L. (2003). Factors influencing the differentiation of dopaminergic traits in transplanted neural stem cells. *Cell. Mol. Neurobiol.* **23**, 851-864.
Young, R. W. (1985). Cell differentiation in the retina of the mouse. *Anat. Rec.* **212**, 199-205.
Zecchin, E., Mavropoulos, A., Devos, N., Filippi, A., Tiso, N., Meyer, D., Peers, B., Bortolussi, M. and Argenton, F. (2004). Evolutionary conserved role of ptf1a in the specification of exocrine pancreatic fates. *Dev. Biol.* **268**, 174-184.