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## Overlapping Expression Patterns and Redundant Roles for AP-2 Transcription Factors in the Developing Mammalian Retina

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### Abstract

**Background**—We have previously shown that the transcription factor AP-2 $\alpha$  (*Tcfap2a*) is expressed in postmitotic developing amacrine cells in the mouse retina. Although retina-specific deletion of *Tcfap2a* did not affect retinogenesis, two other family members, AP-2 $\beta$  and AP-2 $\gamma$ , showed expression patterns similar to AP-2 $\alpha$ .

**Results**—Here we show that, in addition to their highly overlapping expression patterns in amacrine cells, AP-2 $\alpha$  and AP-2 $\beta$  are also co-expressed in developing horizontal cells. AP-2 $\gamma$  expression is restricted to amacrine cells, in a subset that is partially distinct from the AP-2 $\alpha$ / $\beta$ -immunopositive population. To address possible redundant roles for AP-2 $\alpha$  and AP-2 $\beta$  during retinogenesis, *Tcfap2a/b*-deficient retinas were examined. These double mutants showed a striking loss of horizontal cells and an altered staining pattern in amacrine cells that were not detected upon deletion of either family member alone.

**Conclusions**—These studies have uncovered critical roles for AP-2 activity in retinogenesis, delineating the overlapping expression patterns of *Tcfap2a*, *Tcfap2b*, and *Tcfap2c* in the neural retina, and revealing a redundant requirement for *Tcfap2a* and *Tcfap2b* in horizontal and amacrine cell development.

### Keywords

AP-2 transcription factors; retinal development; horizontal cell; amacrine cell; *Tcfap2a/b* double mutant; redundant roles

## INTRODUCTION

The vertebrate neural retina (NR) is composed of six principal types of neurons and the Müller glia cells. During development, retinal cells are generated from multipotent progenitors that are instructed by both intrinsic and extrinsic factors to form the correct types and proportions of cells. Throughout neurogenesis, the differential expression of transcription factors is a defining feature of developing retinal cells as they proceed through cell fate determination and terminal differentiation (Livesey and Cepko, 2001). Amacrine and horizontal cells are retinal interneurons with tightly linked developmental pathways (Poche and Reese, 2009). Horizontal cell bodies reside in the outermost inner nuclear layer (INL) and make up only 3% of mouse INL cells (Jeon et al., 1998). Amacrine cells, a highly diverse class with approximately 30 morphologically distinct subtypes, are found in the inner portion of the INL as well as the ganglion cell layer (GCL), and in the mouse retina comprise approximately 40% of cells in both of these layers (Jeon et al., 1998; MacNeil et al., 1999). Nearly all amacrine cells contain either  $\gamma$ -aminobutyric acid (GABA) or glycine inhibitory neurotransmitters, and a range of additional neurotransmitters and neuropeptides have been localized to GABAergic amacrine cells (Vaney, 1990; Lam, 1997).

Gain- and loss-of-function studies in various animal models have identified key factors in the transcriptional hierarchy regulating amacrine and horizontal cell development. For example, *Foxn4* is required for the generation of all horizontal and most amacrine cells and lies at the top of the hierarchy, activating expression of the downstream factors *Math3* (*Neurod4*), *NeuroD* (*Neurod1*) and *Ptf1a* (Li et al., 2004; Fujitani et al., 2006). *Math3/NeuroD* double knockout (KO) mice exhibit a severe loss of amacrine cells, as do *Ptf1a*-null mice (Inoue et al., 2002; Fujitani et al., 2006; Nakhai et al., 2007). In the horizontal cell branch of the pathway, *Ptf1a* positively regulates the expression of *Prox1*, which is specifically required for horizontal cell genesis, and *Lim1* (*Lhx1*), which is necessary for horizontal cell migration and differentiation (Dyer et al., 2003; Fujitani et al., 2006; Poche et al., 2007). Horizontal cell positioning and morphology are similarly affected by loss of the zinc finger transcription factor *Sall3*, which is required to maintain LIM1 expression in horizontal cells (de Melo et al., 2011). Downstream of early amacrine cell specification genes, additional transcription factors including *Barhl2*, *Bhlhb5* (*Bhlhe22*), and *Isl1* regulate the development of amacrine subpopulations (Feng et al., 2006; Elshatory et al., 2007b; Ding et al., 2009). Although much has been learned about the early regulators of retinal cell specification, retinogenesis is by no means complete upon generation of these retinal cell types and many of the regulators controlling the subsequent layering and mosaic spacing of retinal cell populations are not known.

The activating protein-2 (AP-2) transcription factors are a developmentally important family of genes that have been shown by our laboratory and others to play important roles in eye development (West-Mays et al., 1999; Dwivedi et al., 2005; Pontoriero et al., 2008; Bassett et al., 2010). The family currently includes five members, named *Tcfap2a* to *Tcfap2e* (encoding AP-2 $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ) in mice (Eckert et al., 2005). We have previously shown that AP-2 $\alpha$  and AP-2 $\beta$  proteins are expressed in developing and mature mouse retinal amacrine cells of the INL and GCL, and we also detected transcripts of *Tcfap2c* (AP-2 $\gamma$ ) and *Tcfap2d* (AP-2 $\delta$ ) in the embryonic and adult murine retina (Bassett et al., 2007). *Tcfap2c* appeared to be expressed in a pattern similar to *Tcfap2a* and *Tcfap2b*, whereas *Tcfap2d*, the most divergent AP-2 family member, was largely confined to the GCL (Bassett et al., 2007). Likewise, in the developing chick retina, AP-2 $\delta$  is expressed in a subset of ganglion cells and shows no overlap with AP-2 $\alpha$  and AP-2 $\beta$  expression (Bisgrove and Godbout, 1999; Li et al., 2008).

To examine a potential intrinsic role for AP-2 $\alpha$  in retinogenesis, we previously generated a conditional KO of *Tcfap2a* in the developing retina (“*Re-AP-2 $\alpha$* ” mice; Bassett et al., 2007). In *Re-AP-2 $\alpha$*  mice, no apparent retinal defects were observed, suggesting that AP-2 $\alpha$  alone is not intrinsically required for NR development. We therefore considered that the loss of AP-2 $\alpha$  may have been compensated for by other AP-2 family member(s), the most likely candidates being AP-2 $\beta$  and/or AP-2 $\gamma$ . The purpose of this study was to investigate the potentially redundant roles of *Tcfap2a*, *Tcfap2b*, and *Tcfap2c* in the developing NR, through detailed expression analyses and generation of a double *Tcfap2a/b* mutant mouse model that was studied until its death at postnatal day 0 (P0). We show that, in addition to amacrine cells, AP-2 $\alpha$  and AP-2 $\beta$  are also co-expressed in developing horizontal cells. AP-2 $\gamma$  expression is restricted to amacrine cells, in a subset that is partially distinct from the AP-2 $\alpha/\beta$ -immunopositive population. *Tcfap2a/b*-deficient retinas show both amacrine and horizontal cell defects that are not detected upon deletion of either family member alone, uncovering critical roles for AP-2 activity in the development of these cell types.

## RESULTS

### AP-2 $\alpha$ and AP-2 $\beta$ Are Expressed in Postmitotic Amacrine and Horizontal Cells

We previously showed that AP-2 $\alpha$  is expressed in postmitotic developing amacrine cells, and that AP-2 $\alpha$  and AP-2 $\beta$  are expressed in overlapping populations of cells in the neonatal and adult retina (Bassett et al., 2007). Here, we extend our previous work by examining earlier coexpression of AP-2 $\alpha$  and AP-2 $\beta$ , where we detected extensive colocalization by embryonic day (E) 15.5 (Fig. 1A). We performed double immunostains for AP-2 $\beta$  and the proliferative markers phosphohistone H3 (PH3), which labels cells in late G<sub>2</sub> and M phase (Hendzel et al., 1997), and proliferating cell nuclear antigen (PCNA), which labels cells in early G<sub>1</sub> and S phase (Bravo et al., 1987). In E13.5, E15.5 and postnatal day (P) 0 retinas, AP-2 $\beta$ -positive cells did not colocalize with PCNA or PH3 (Fig. 1B and not shown). We also quantified the proportion of AP-2 $\alpha$ -positive and AP-2 $\beta$ -positive cells colocalizing with Ki67, which is expressed by cells in all phases of the cell cycle (Scholzen and Gerdes, 2000; Supp. Fig. S1A, which is available online). The vast majority (>98%) of AP-2 $\alpha$  or AP-2 $\beta$ -positive cells did not express Ki67, suggesting that both family members are a feature of postmitotic retinal cells. The synaptic vesicle docking protein syntaxin-1 (STX1A) is expressed in retinal progenitor cells (RPCs) that are heavily biased toward an amacrine or horizontal cell fate, in addition to postmitotic amacrine and horizontal cells in the developing and mature retina (Alexiades and Cepko, 1997). At E15.5, cells with AP-2 $\beta$ -positive nuclei also expressed STX1A (Fig. 1C). At birth, AP-2 $\beta$  and STX1A colocalized to amacrine cells in the INL and GCL, as well as a row of cells in the presumptive horizontal cell layer with the characteristic radial morphology of horizontal cells at this stage (Huckfeldt et al., 2009; Fig. 1D). By P7, AP-2 $\beta$  was clearly localized to STX1A-positive amacrine and horizontal cells (Fig. 1E). Amacrine cells can be broadly divided into two major subsets, glycinergic and GABAergic (Vaney, 1990), which can be detected by anti-GLYT1 (*Slc6a9*) and anti-GAT-1 (*Slc6a1*) antibodies, respectively. As is the case for AP-2 $\alpha$  (Bassett et al., 2007), AP-2 $\beta$  expression was detected in both glycinergic and GABAergic amacrine cells in the adult retina (Fig. 1F,G).

To further examine the expression of AP-2 proteins in horizontal cells, we used antibodies for known horizontal cell markers: homeobox transcription factors LIM1 (LHX1), expressed within the retina exclusively in horizontal cells (Liu et al., 2000; Poche et al., 2007) and PROX1, expressed in differentiating horizontal cells and populations of amacrine, bipolar and Müller glial cells (Dyer et al., 2003; Cid et al., 2010), as well as protein gene product 9.5 (PGP9.5; encoded by *Uchl1*), a deubiquitinating enzyme expressed in horizontal, amacrine and ganglion cells (Chen et al., 1994). At E17.5, a significant proportion of horizontal cells have yet to complete their migration to the outer retina and are intermingled

among the developing amacrine cell population (Poche et al., 2007; Huckfeldt et al., 2009). At this stage, 100% of LIM1-positive cells in both the inner and outer retina were immunoreactive for AP-2 $\beta$  (Fig. 2A, arrowheads). At all subsequent stages examined (P0, P7, and adult), we detected AP-2 $\beta$  expression in 100% of LIM1-positive cells (Fig. 2B,C,H and not shown). Expression analyses have shown that LIM1 appears to be expressed in all mouse horizontal cells (Liu et al., 2000); therefore, the complete overlap of AP-2 $\beta$  and LIM1 in developing and mature horizontal cells suggests that AP-2 $\beta$  is also a pan-horizontal cell marker in the mouse retina.

AP-2 $\alpha$  has been widely used as an amacrine cell marker; however studies in the chick retina have also revealed AP-2 $\alpha$  expression in developing horizontal cells (Edqvist and Hallbook, 2004; Edqvist et al., 2008). To investigate the possibility of AP-2 $\alpha$  expression in mouse horizontal cells, we first performed double immunostains for AP-2 $\alpha$  and AP-2 $\beta$  at birth, when strongly immunoreactive AP-2 $\beta$ -positive cells are present in the future horizontal cell layer. In general, we detected more extensive overlap between AP-2 $\alpha$ - and AP-2 $\beta$ -immunoreactive cells than previously appreciated in the neonatal retina (Fig. 2D), possibly due to the stage examined and the fact that we find our anti-AP-2 antibodies to work particularly well on P0 enucleated eyes. We then quantified the percent overlap of AP-2 $\alpha$ -positive and AP-2 $\beta$ -positive populations in the developing and adult retina, which showed that over 80% of AP-2 $\alpha$  and/or AP-2 $\beta$ -positive cells were co-labeled during development, while adult retinas exhibited  $77 \pm 1\%$  overlap (Fig. 2G). At all stages examined, the proportion of co-labeled cells was significantly greater than the proportion of singly stained cells (Fig. 2G). In the P0 outer retina, putative horizontal cells exhibiting a strong signal for AP-2 $\beta$  were also more weakly immunoreactive to anti-AP-2 $\alpha$  (Fig. 2D, arrowheads and inset), suggesting that AP-2 $\alpha$  is co-expressed with AP-2 $\beta$  in developing horizontal cells. Given that the anti-AP-2 $\alpha$  and anti-LIM1 antibodies are not compatible for double labeling, AP-2 $\alpha$  expression in developing horizontal cells was verified by colocalization with PROX1 at E16.5 (Fig. 2E, arrowheads) and with PGP9.5 in the P0 presumptive horizontal cell layer (Fig. 2F, arrowheads), where  $94 \pm 1.9\%$  of PGP9.5-positive cells expressed AP-2 $\alpha$  (Fig. 2H). However, by P7 AP-2 $\alpha$  protein was undetectable in horizontal cells (not shown), suggesting that it is only transiently expressed in this cell type during development.

### AP-2 $\gamma$ Is Absent From Horizontal Cells and Expressed in Postmitotic Amacrine Cells

Given our previous data demonstrating similar retinal expression patterns of *Tcfap2a*, *Tcfap2b* and *Tcfap2c* transcripts (Bassett et al., 2007), we examined AP-2 $\gamma$  protein expression in the developing and mature mouse retina. We used a mouse monoclonal anti-AP-2 $\gamma$  antibody (6E4) previously shown to specifically detect human and mouse AP-2 $\gamma$  protein (Gee et al., 2009; Jager et al., 2010; Kuckenberg et al., 2010). In contrast to AP-2 $\alpha$  and AP-2 $\beta$ , AP-2 $\gamma$  expression was not detected in the retina at E12.5 (not shown); however by E15.5, AP-2 $\gamma$ -immunoreactive cells were scattered in the inner and outer neuroblast layers (Fig. 3A). At P0, AP-2 $\gamma$ -positive cells were found in the neuroblast layer, a row of cells in the developing INL, and infrequently in the GCL (Fig. 3B). In the P7 and adult retina, AP-2 $\gamma$  was expressed in the inner INL and very few cells of the GCL (Fig. 3C,D). Although AP-2 $\gamma$  was clearly not expressed in mature horizontal cells, we determined whether it was transiently expressed in developing cells of this type, by co-immunolabeling with anti-PROX1 and anti-PGP9.5. We failed to detect co-expression of AP-2 $\gamma$  and PROX1 at E16.5 (Fig. 3E), and quantification of sections from five retinas co-labeled with AP-2 $\gamma$  and PGP9.5 at P0 showed that only  $0.92 \pm 0.2\%$  of PGP9.5-positive cells expressed AP-2 $\gamma$  (Fig. 3F and not shown), suggesting that AP-2 $\gamma$  is virtually absent from horizontal cells. We also failed to detect AP-2 $\gamma$  expression in retinal ganglion cells labeled by anti-POU4F1/2/3 (BRN3A/B/C), indicating that the AP-2 $\gamma$ -positive cells in the GCL are displaced amacrine cells (not shown).



To assess whether AP-2 $\gamma$  is expressed in mitotic or postmitotic retinal cells, we co-immunolabeled retinas for AP-2 $\gamma$  and PH3 at E15.5, and did not observe double-labeled cells (Fig. 4A). Similarly, quantification of cell staining in retinal sections showed that only  $1.51 \pm 0.36\%$  of AP-2 $\gamma$ -positive cells expressed Ki67 (Supp. Fig. S1A). Therefore, akin to AP-2 $\alpha$  and AP-2 $\beta$ , AP-2 $\gamma$  expression is not a feature of mitotic RPCs. Also like AP-2 $\alpha$  and AP-2 $\beta$ , AP-2 $\gamma$  expression was detected in both the GABAergic (GAT-1-positive) and glycinergic (GLYT1-positive) amacrine cell subsets in the mature mouse retina (Fig. 4B,C). Given the distribution of AP-2 $\gamma$ -immunoreactive cells in the neuroblast layer at birth (Fig. 3B,F), we considered the possibility that it is expressed in other developing INL or outer nuclear layer (ONL) cell types. However, in P0, P7 and P21 retinas, we did not detect co-expression of AP-2 $\gamma$  and markers of photoreceptor (OTX2, CRX), bipolar (VSX2) or Müller glial (SOX2) cells (not shown). Our immunofluorescent analyses therefore indicated that AP-2 $\gamma$  expression is restricted to amacrine cells. To confirm this, we double-immunolabeled retinas for AP-2 $\gamma$  and PAX6, which is initially expressed in all RPCs but persists only in differentiating and mature ganglion, amacrine, horizontal, and some Müller glial cells (de Melo et al., 2003; Roesch et al., 2008). At birth, strongly immunoreactive PAX6-positive cells in the neuroblast layer are presumptive amacrine or horizontal cells (Fig. 4D, arrowheads), while those in the INL are differentiating amacrine cells (Fig. 4D, bracket). As suspected, all AP-2 $\gamma$ -positive cells at this stage overlapped with brightly stained PAX6-positive cells, whether in the neuroblast layer (Fig. 4E, arrowheads) or INL (Fig. 4E, bracket). Quantification of P0 retinas revealed that  $38 \pm 0.9\%$  of PAX6-positive cells expressed AP-2 $\gamma$ , while  $81 \pm 1.8\%$  of these cells expressed AP-2 $\alpha$ , confirming that the AP-2 $\alpha/\beta$ -positive population is larger than the AP-2 $\gamma$ -positive one (Supp. Fig. S1B). Similarly, in P7 and adult retinas, AP-2 $\gamma$  continued to be confined to PAX6-positive cells and comprised a smaller proportion of the PAX6-positive population than AP-2 $\alpha$  (Fig. 4F,G and Supp. Fig. S1B). We compared the expression patterns of AP-2 $\alpha$ , AP-2 $\beta$ , and AP-2 $\gamma$  in amacrine cell subpopulations at birth. While all three family members were expressed in BHLHB5-positive GABAergic amacrine cells (Fig. 4H–J), only AP-2 $\alpha$  and AP-2 $\beta$  were detected in SOX2-positive cholinergic amacrine cells (Fig. 4K–M, arrowheads).

We also examined the extent to which the AP-2 $\gamma$  expression domain overlaps with that of AP-2 $\alpha/\beta$ . Due to antibody limitations (the validated anti-AP-2 $\alpha$  3B5 and anti-AP-2 $\gamma$  6E4 are both mouse monoclonal antibodies), we focused on co-immunolabeling with anti-AP-2 $\gamma$  and anti-AP-2 $\beta$ . Although we detected cells expressing both AP-2 $\gamma$  and AP-2 $\beta$  at E16.5, P0, P7, and adult stages (Fig. 5A–D, arrowheads), a significant proportion of immunopositive cells were singly labeled (Fig. 5A–E). At all stages, the AP-2 $\gamma$ -positive population was significantly smaller than the AP-2 $\beta$ -positive population (Fig. 5E). Postnatally, there was a significant progressive decrease in the percent of co-labeled cells. Approximately 20% of the AP-2 $\gamma$  and/or AP-2 $\beta$ -positive population was co-labeled at E16.5 and P0, while only  $3.4 \pm 0.7\%$  was co-labeled in the mature retina (Fig. 5E). Taken together, these expression studies show that AP-2 $\gamma$  is expressed in postmitotic developing and mature amacrine cells, in a pattern that only partially overlaps with the extensively colocalized AP-2 $\alpha/\beta$ -positive amacrine cell population.

### Loss of Horizontal Cells in *Tcfap2a/b*-Deficient Retinas

As mentioned, we were unable to detect retinal defects in mice with retina-specific deletion of *Tcfap2a*, and hypothesized that another AP-2 family member may compensate for the loss of AP-2 $\alpha$  (Bassett et al., 2007). Based on collective expression data showing that AP-2 $\alpha$  and AP-2 $\beta$  clearly have the most similar retinal expression patterns among the AP-2 family members, we sought to determine the effects of combined *Tcfap2a* and *Tcfap2b* deletion in the developing retina. To generate these mutants using available lines of mice, retina-specific *Tcfap2a* conditional KO mice were crossed onto a *Tcfap2b* germ-line KO

background to obtain *Tcfap2a*<sup>ki7lacZ/lox</sup>/*Tcfap2b*<sup>-/-</sup>/ $\alpha$ -Cre<sup>+/-</sup> mice, referred to herein as *Tcfap2a/b* mutants (Supp. Fig. S2). The  $\alpha$ -Cre transgenic mice used to delete the floxed *Tcfap2a* allele express Cre recombinase in the peripheral retina while leaving a central and dorsal gap (Marquardt et al., 2001; Baumer et al., 2002). As such, peripheral retina regions from horizontal sections at or near the optic nerve head are always shown. Also, retinas were stained with anti-AP-2 $\alpha$  and anti-AP-2 $\beta$  antibodies to confirm polymerase chain reaction (PCR) genotyping results (see example in Supp. Fig. S2).

Histological inspection revealed subtle differences in *Tcfap2a/b* mutant retinal morphology that were apparent at E18.5 (Fig. 6). At E16.5, the emerging IPL was present, and no overt differences in the thickness or appearance of any retinal layers were noted (Fig. 6A,D). By E18.5 in control littermates, the GCL and INL were clearly separated by the inner plexiform layer (IPL), which at this stage contains amacrine and ganglion cell processes (Fig. 6B). In comparison, the inner retina of *Tcfap2a/b* mutants appeared slightly less organized, in that the general divisions between the INL, IPL and GCL were not as defined (Fig. 6E, arrowheads), a phenomenon that was also apparent at birth (Fig. 6, compare C with F, arrowheads). This disorganization was reflected in the staining pattern of STX1A. The band of strong STX1A expression representing amacrine cell neurites in the IPL was not as distinct in *Tcfap2a/b* mutants compared with controls (Fig. 6H,I,K,L, asterisks). In addition, the STX1A immunoreactivity in the outer retina was diminished in double mutants (Fig. 6G–L, arrowheads). By birth, the layer of STX1A-positive horizontal cells forming in the outer retina of control littermates was not detected in *Tcfap2a/b* mutants (Fig. 6I,L), suggesting that horizontal cell development might be severely affected. Because these mutants die at birth due to the *Tcfap2b* germ-line KO phenotype (Moser et al., 1997), in vivo analyses beyond embryonic and P0 stages could not be carried out.

We used several retinal markers to further investigate horizontal cell development in *Tcfap2a/b* mutant retinas. At E16.5, when the first significant LIM1 expression occurs in postmitotic horizontal cells (Poche et al., 2007), the number of LIM1-positive cells in double mutant retinas was drastically reduced compared with controls (Fig. 7, compare A with B). At all subsequent stages examined (E17.5, 18.5, and P0), we rarely observed LIM1-expressing cells in *Tcfap2a/b*-deficient regions of the retina (Fig. 7C,D and not shown), suggesting a near loss of horizontal cells. Interestingly, we did not detect the same loss of LIM1-positive cells in single mutant retinas lacking only *Tcfap2a* or only *Tcfap2b* (not shown). The LIM1 results were corroborated by immunolabeling for PROX1, another early horizontal cell marker that was similarly absent from the double mutant outer retina at E18.5 and P0 (not shown). Developing and mature mouse horizontal cells exhibit strong neurofilament (NF) immunoreactivity (Peichl and Gonzalez-Soriano, 1993; Chien and Liem, 1995), which we readily detected in P0 control retinas using NF light chain (NF-L) and NF medium chain (NF-M) antibodies (Fig. 7E and not shown). In contrast, the strong NF-L and NF-M signals indicative of horizontal cells were not present in the *Tcfap2a/b* mutants (Fig. 7F and not shown). Similarly, PGP9.5-positive cells were missing from the presumptive horizontal cell layer in *Tcfap2a/b* mutants (Fig. 7, compare G with H). Notably, we observed LIM1- and PGP9.5-positive horizontal cells in the *Tcfap2a/b* mutant central retina, where the  $\alpha$ -Cre activity (responsible for excising the *Tcfap2a*<sup>lox</sup> allele) diminishes (Supp. Fig. S3A, and not shown). We also took advantage of the fact that *Tcfap2a/b* mutants carry one copy of the *Tcfap2a* floxed (*Tcfap2a*<sup>lox</sup>), and one copy of the *Tcfap2a* IRES-*lacZ* knock-in null allele (*Tcfap2a*<sup>ki7lacZ</sup>), which has been shown to faithfully express  $\beta$ -galactosidase in a pattern identical to that of endogenous AP-2 $\alpha$  protein (Brewer et al., 2002). Given the extensive colocalization of AP-2 $\alpha$  and AP-2 $\beta$  expression in the developing retina, a large proportion of the *Tcfap2a*-null cells expressing  $\beta$ -galactosidase would also normally co-express functional AP-2 $\beta$  protein and are, therefore, double “*Tcfap2a/b* null” cells. Marking these *Tcfap2a/b* null cells by X-gal staining demonstrated a lack of X-gal-positive cells in

the outer retina of *Tcfap2a/b* mutants, showing that *Tcfap2a/b* null cells are missing from the presumptive horizontal cell layer, rather than present but failing to express horizontal cell markers (Supp. Fig. S3B). These results suggest that by birth, horizontal cells are virtually absent from retinas deficient in both *Tcfap2a* and *Tcfap2b*.

The apparent loss of an entire retinal cell type in *Tcfap2a/b* mutants prompted us to examine programmed cell death and proliferation during retinogenesis; however, from E16.5 to P0, we did not detect observable differences in the numbers of apoptotic (TUNEL [Terminal uridine deoxynucleotidyl transferase-mediated dUTP Nick End Labeling] -labeled) cells, or the staining patterns of the proliferative markers PH3, PCNA and Cyclin D1 (Supp. Fig. S4, and not shown). Taken together, our analyses demonstrate a redundant requirement for AP-2 $\alpha$  and AP-2 $\beta$  activity for horizontal cell development.

### Combined Deletion of *Tcfap2a* and *Tcfap2b* Causes Amacrine Cell Defects

Given the overlapping expression of AP-2 $\alpha$  and AP-2 $\beta$  in developing amacrine cells, we examined *Tcfap2a/b* mutant retinas for amacrine cell abnormalities. The pan-amacrine cell marker PAX6 showed comparable immunostaining patterns in *Tcfap2a/b* mutants and controls at E16.5, E18.5 and P0 (Fig. 8A,B and not shown). Although GABAergic amacrine cell birth peaks around mouse E14–E16, significant birth of glycinergic amacrine cells begins during late embryogenesis with a peak around P0, and is not detectable by glycinergic-specific marker expression until postnatal stages (Cherry et al., 2009; Voinescu et al., 2009). Given the death of *Tcfap2a/b* mutants at birth, our assessment of amacrine cell markers was limited to those expressed in the embryonic or neonatal retina, which restricted our subtype analysis to GABAergic populations. Transcription factor *Bhlhb5* (*Bhlhe22*) is the earliest subtype-specific amacrine marker, expressed in postmitotic GABAergic amacrine cells starting at E11.5 (Feng et al., 2006). The BHLHB5 expression pattern was initially similar in *Tcfap2a/b* mutants and controls at E15.5 (not shown); however, while BHLHB5-positive cells in control retinas were clearly concentrated in the developing INL by birth, this distinct band of cells was not observed in double mutant littermates (Fig. 8, compare C with D). We did not observe this altered BHLHB5 staining pattern in retinas deficient in *Tcfap2a* alone or *Tcfap2b* alone (not shown).

The cholinergic amacrine cells are one of the earliest born subsets of the GABAergic population, and form a nonrandom distribution in the late embryonic retina (Galli-Resta et al., 1997; Voinescu et al., 2009). *Bhlhb5* is not expressed in cholinergic amacrine cells (Feng et al., 2006); therefore, this subpopulation was examined with other markers. We used an antibody that detects the ISL1 and ISL2 proteins (Tsuchida et al., 1994), and labels retinal ganglion cells (RGCs) and cholinergic amacrine cells at embryonic and neonatal stages (Elshatory et al., 2007a). By E18.5, the ISL1-positive cholinergic amacrine mosaic was evident in the developing INL of control retinas (Fig. 8E, open arrowheads). In contrast, although immunolabeled cells were present in the INL of *Tcfap2a/b* mutants, they did not exhibit the same regular arrangement (Fig. 8F, asterisks). The transcription factor SOX2 is expressed in retinal progenitors, but is also a marker for cholinergic amacrine cells and Müller glia (Taranova et al., 2006; Cherry et al., 2009; Lin et al., 2009). Its lack of expression in RGCs allowed us to visualize the presumptive cholinergic amacrine distributions in both the INL and GCL. Similar to the results obtained with anti-ISL1/2, SOX2-immunoreactive cells were present in both controls and double mutants at E18.5 (Fig. 8G,H). However, the orderly distribution of labeled cells in the INL and GCL of controls (Fig. 8G, open arrowheads) was not detected in *Tcfap2a/b* mutants (Fig. 8H, asterisks). We asked whether the expression pattern of AP-2 $\gamma$  was altered in *Tcfap2a/b* mutant retinas. Based on immunostaining, there was no compensatory expansion of the AP-2 $\gamma$ -immunopositive population in *Tcfap2a/b* mutants, but rather an expression pattern reflective

of BHLHB5, in which the distinct INL band of AP-2 $\gamma$ -positive cells observed in controls (Fig. 8I, closed arrow) was lacking in double mutants (Fig. 8J, open arrow).

To investigate whether the loss of *Tcfap2a* and *Tcfap2b* impacted the development of cell types other than amacrine and horizontal cells, we analyzed the expression of additional retinal markers. We saw no differences between *Tcfap2a/b* mutant and control retinas immunolabeled at E17.5 and P0 for POU4F1/2/3 (BRN3A/B/C; ganglion cells), CRX and OTX2 (photoreceptors), and VSX2 (CHX10), which is expressed in progenitors and is maintained in the bipolar cell lineage (Supp. Fig. S5, and not shown). Thus, the current analyses show that, in addition to horizontal cells, AP-2 $\alpha$  and AP-2 $\beta$  are redundantly required for amacrine cell development.

## DISCUSSION

The AP-2 transcription factors have emerged as key regulators of eye morphogenesis in several species, including humans (Gestri et al., 2009; Dumitrescu et al., 2011; Milunsky et al., 2011). In this report, we have investigated the roles of multiple AP-2 family members in mouse retinal neurogenesis, and revealed their importance in the amacrine and horizontal cell lineages. We showed that AP-2 $\alpha$  and AP-2 $\beta$  exhibit highly overlapping expression patterns in postmitotic amacrine and horizontal cells. AP-2 $\gamma$  expression was detected in developing and mature amacrine cells, in a population that only partially overlapped with AP-2 $\alpha/\beta$ -expressing cells. Deletion of both *Tcfap2a* and *Tcfap2b* from the developing retina caused a striking loss of horizontal cells and defective amacrine cell development.

### Expression Patterns of AP-2 Proteins in Amacrine and Horizontal Cells

Previous studies in mouse and chick have indicated that four of the five known AP-2 family members (AP-2 $\alpha$ , AP-2 $\beta$ , AP-2 $\gamma$ , and AP-2 $\delta$ ) are expressed in the developing retina, and that three of these (AP-2 $\alpha$ , AP-2 $\beta$ , and AP-2 $\gamma$ ) are present in INL cell types while AP-2 $\delta$  is confined to RGCs (Bisgrove and Godbout, 1999; Zhao et al., 2003; Bassett et al., 2007; Trimarchi et al., 2007; Li et al., 2008). Here, we clarified the expression patterns of AP-2 $\alpha$  and AP-2 $\beta$  in horizontal cells of the mouse retina (Figs. 1, 2). While both family members were expressed in developing horizontal cells, AP-2 $\alpha$  expression diminished shortly after birth and only AP-2 $\beta$  protein was retained in mature horizontal cells, a finding that concurs with data from the chick retina (Bisgrove and Godbout, 1999; Edqvist et al., 2008). We also showed that, in the developing and adult retina, a considerably higher proportion of cells are co-labeled for AP-2 $\alpha$  and AP-2 $\beta$  compared with those singly labeled for AP-2 $\alpha$  or AP-2 $\beta$  (Fig. 2), thereby demonstrating their extensive overlap and supporting compensation by one protein when the other is deficient in the retina. Recently, the expression patterns of *TFAP2A* and *TFAP2B* in the human fetal and mature retina have been examined, and closely resemble those in chick and mouse retinas (Li et al., 2010). Thus, AP-2 $\alpha$  and AP-2 $\beta$  are key markers of amacrine and horizontal cells in the avian and mammalian retina, and, as the current study shows, they have overlapping roles in the development of both cell types.

Our spatiotemporal analysis of AP-2 $\gamma$  protein expression in the developing mouse retina showed that like AP-2 $\alpha$  and AP-2 $\beta$ , this family member is expressed in postmitotic amacrine cells of the GABAergic and glycinergic classes (Figs. 3, 4). While the vast majority of cells expressing any of these three AP-2 family members were Ki67-negative (Supp. Fig. S1A), Ki67 immunoreactivity was found in a very small percentage of AP-2-positive cells. However, low levels of Ki67 protein have been reported in quiescent cells (Bullwinkel et al., 2006), and our data could reflect the presence of Ki67, perhaps transiently, in postmitotic AP-2-positive neurons. Unlike AP-2 $\alpha$  and AP-2 $\beta$ , AP-2 $\gamma$  expression initiated later and was not a marker of developing horizontal cells (Fig. 3). We noted that cells expressing AP-2 $\gamma$  did not form an obvious band in the developing INL until

late embryogenesis, and a significant proportion of AP-2 $\gamma$ -immunopositive amacrine precursors were scattered in the outer neuroblast layer at early postnatal stages. This expression pattern, combined with the later onset of AP-2 $\gamma$  expression, suggests that it may be expressed in later-born amacrine cells compared with AP-2 $\alpha$  and AP-2 $\beta$ . This idea is supported by recent evidence showing that final amacrine cell soma position correlates with birthdate, in that the earliest born amacrine cells (such as the cholinergic subtype) reside in the GCL or inner INL, while later-born amacrine cells are found more toward the outer retina (Voinescu et al., 2009). In agreement with this finding, AP-2 $\gamma$  was not detected in the early-born cholinergic amacrines, whereas AP-2 $\alpha$  and AP-2 $\beta$  were expressed in this subtype (Fig. 4). Double immunolabeling with anti-AP-2 $\gamma$  and anti-AP-2 $\beta$  (Fig. 5) also emphasized the fact that the AP-2 $\gamma$ -positive amacrine cell population is partially distinct from the extensively colocalized AP-2 $\alpha/\beta$ -positive population, particularly in the adult retina where less than 4% of the AP-2 $\gamma$  and/or AP-2 $\beta$ -positive population overlaps (Fig. 5). This result is supported by a study in which gene expression profiles were obtained from individual developing retinal cells. Of the six amacrine cells profiled, all expressed *Tcfap2b*, five expressed *Tcfap2a*, and only one expressed *Tcfap2c* (Trimarchi et al., 2007).

### Requirement for *Tcfap2a/b* in Horizontal Cell Development

We showed here that like AP-2 $\alpha$ , AP-2 $\beta$  is expressed in postmitotic transition cells during retinogenesis (Fig. 1). The temporal and spatial expression patterns of AP-2 $\alpha$  and AP-2 $\beta$  are well correlated with amacrine and horizontal cell differentiation (Young, 1985). In contrast to amacrine cells, which are clearly present in *Tcfap2a/b* mutant retinas, horizontal cells are lost (Fig. 7). We failed to detect the expression of several horizontal cell markers, including the early markers LIM1 and PROX1. We suspect that the rare occurrence of a LIM1-positive horizontal cell in the peripheral retina of *Tcfap2a/b* mutants may be a result of incomplete Cre-mediated excision of *Tcfap2a*, which is supported by the fact that we detected horizontal cells in the *Tcfap2a/b* mutant central retina, where the  $\alpha$ -Cre activity diminishes (Supp. Fig. S3). We also detected LIM1-positive cells in the retinas of embryos with no intact *Tcfap2b* alleles and only one intact *Tcfap2a* allele, or no intact *Tcfap2a* alleles and only one intact *Tcfap2b* allele (not shown). Therefore a single functional copy of *Tcfap2a* or *Tcfap2b* appears to rescue horizontal cells to some extent. Taken together, these results suggest that *Tcfap2a* and *Tcfap2b* are redundantly required for the determination or maintenance of the entire horizontal cell population. The underlying cause for the absence of horizontal cells in *Tcfap2a/b* mutant retinas is not known. Considering that horizontal cells comprise only approximately 0.2% of all mouse retinal cells (Ajioka et al., 2007), it is difficult to determine whether these cells have adopted an alternative fate or were initially generated but quickly died by apoptosis. Given their expression in postmitotic cells, we favor the idea that AP-2 proteins are not involved in retinal cell fate determination, but rather in aspects of differentiation or survival. The specific roles for AP-2 $\alpha/\beta$  in horizontal cell development can be further clarified by experiments in which the *Tcfap2a/b*-null cells are traced and co-labeled with different retinal markers, as well as by gain-of-function studies.

### Impaired Amacrine Cell Development in *Tcfap2a/b*-Deficient Retinas

*Tcfap2a* and *Tcfap2b* are expressed in glycinergic and GABAergic amacrine cells and are also co-expressed with markers of many other smaller amacrine subpopulations including cholinergic (ISL1-positive, SOX2-positive), NR4A2-positive, CALB2-positive, and AII (DAB1-positive) amacrines (Bassett et al., 2007; Trimarchi et al., 2007; Cherry et al., 2009). Rather than being concentrated in the ventricular half of the embryonic retina like early amacrine determination factors such as FOXN4 or PTF1A (Li et al., 2004; Fujitani et al., 2006), AP-2 $\alpha$  and AP-2 $\beta$  are expressed in newly generated amacrine transition cells as they approach the presumptive INL, and both proteins are maintained in amacrine cells



throughout postnatal development and into adulthood. Before their death at P0, our assessment of different amacrine cell populations in *Tcfap2a/b* mutants was confined to the earlier born GABAergic types. We noted altered staining patterns of the early GABAergic marker BHLHB5, cholinergic (ISL1, SOX2) markers, and AP-2 $\gamma$  (Fig. 8). These altered staining patterns were indicative of abnormal amacrine cell positioning, suggesting that migration and consequent mosaic formation could be affected by loss of AP-2. The *Tcfap2a/b* mutants die around P0, and at this early time point we were unable to determine if BHLHB5-positive and AP-2 $\gamma$ -positive amacrine cell numbers were also significantly altered in the mutant compared with the control (data not shown). Ultimately, generation of a retinal-specific conditional *Tcfap2a/b*-deficient mouse model that survives postnatally will be required to investigate the final outcome of *Tcfap2a/b* deletion on the differentiation and numbers of amacrine cells, and to determine whether the loss of *Tcfap2a/b* permanently affects the morphology and/or positioning of amacrine cells, or possibly delays differentiation.

### AP-2 and Neural Development

Although all five AP-2 family members are expressed in specific regions of the embryonic CNS, their intrinsic roles in the development of neuroectoderm-derived tissues are not well studied. In vitro studies have shown that transfection of neuroectodermal cells with *Tcfap2a*, or neural crest progenitors with *Tcfap2b*, induces neuronal differentiation (Paggi et al., 2001; Hong et al., 2008). *Tcfap2a* has been shown to regulate the migration of gonadotropin-releasing hormone neurons (Orso et al., 2009), which undergo extensive movement from the nasal placode to the hypothalamus during development (Cariboni et al., 2007). The work presented here adds to a limited number of in vivo studies that have revealed cell autonomous roles for AP-2 in neurogenesis within the mammalian CNS (Hong et al., 2008; Feng et al., 2009; Pinto et al., 2009; Hesse et al., 2011; Schmidt et al., 2011). In neural tissues where the expression and function of AP-2 proteins have been examined in detail, they are not broadly distributed but rather exhibit cell type-specific expression in restricted subsets of neural progenitors or postmitotic developing neurons. These include AP-2 $\alpha$ , AP-2 $\beta$ , and AP-2 $\gamma$  expression in the retinal amacrine/horizontal lineage, AP-2 $\gamma$  expression in apical (radial glia) progenitors of the cerebral cortex (Pinto et al., 2009), and AP-2 $e$  expression in the output neurons (mitral and tufted cells) of the olfactory bulb (Feng et al., 2009). In regions of the brain and olfactory bulb, AP-2 proteins are required to drive the formation or differentiation of particular neuronal types, thereby conferring neuronal identity or downstream characteristics such as correct morphology or positioning. These include a requirement for AP-2 $\gamma$  in the specification of basal progenitors from apical progenitors (and consequent production of layer II/III pyramidal neurons) in the visual cortex (Pinto et al., 2009), and a role for AP-2 $e$  in the arrangement and dendrite outgrowth of olfactory bulb output neurons (Feng et al., 2009). Complementary to these studies, AP-2 $\alpha$  and AP-2 $\beta$  have also been implicated in the development of noradrenergic neurons within the mouse and zebrafish hindbrain (Holzschuh et al., 2003; Hong et al., 2008), and AP-2 $\delta$  is required for cell survival in the posterior midbrain (Hesse et al., 2011). Furthermore, functional overlap of AP-2 family members was recently reported in the developing peripheral nervous system, where AP-2 $\alpha$  and AP-2 $\beta$  have partially redundant roles in the survival of neural crest-derived sympathetic neurons (Schmidt et al., 2011). Given the early lethality associated with loss of the three AP-2 genes studied in this report (Schorle et al., 1996; Zhang et al., 1996; Moser et al., 1997; Auman et al., 2002; Werling and Schorle, 2002), tissue-specific KO mouse models will provide a means to examine the developmental roles of *Tcfap2* family members in particular regions of the nervous system in vivo, and it will be of interest to compare their functions in the retina with those in other neural tissues.

This study has provided insight into the roles of *Tcfap2a*, *Tcfap2b*, and *Tcfap2c* in retinogenesis, delineating their overlapping and unique expression patterns in developing horizontal and amacrine cell populations, and revealing a redundant requirement for *Tcfap2a* and *Tcfap2b* in horizontal and amacrine cell development. In future studies, it will be important to examine the outcome of *Tcfap2* gain-of-function in the retina, as well as *Tcfap2c* deletion from the retina, either alone or in combination with other AP-2 family member(s). In addition, once suitable mouse genetic resources become available, it will be important to extend the studies on AP-2 $\alpha$  and AP-2 $\beta$  redundancy into the postnatal period to analyze the morphology, positioning, and population sizes of a spectrum of amacrine cell subtypes, and also to assess visual function. Nevertheless, our current results demonstrate that the AP-2 transcription factors are required for specific aspects of retinal neurogenesis, broadening their roles as regulatory molecules with respect to both eye and neural development.

## EXPERIMENTAL PROCEDURES

### Generation of Mouse Lines

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) *Statement for the Use of Animals in Ophthalmic and Vision Research*. Mice were maintained on mixed C57B/16 and FVB/N backgrounds. To monitor genotypes by PCR, DNA was extracted from embryonic/neonatal tail samples, or ear clips of adult breeders using the DNeasy tissue kit (Qiagen).

***Tcfap2a/b* mutants**—The series of crosses used to generate “*Tcfap2a/b* mutants” are diagrammed in Supp. Fig. S2A. Two different *Tcfap2* alleles were used: A *Tcfap2a*<sup>ki7lacZ</sup> null allele [due to a germ-line IRES-*lacZ* knock-in insertion disrupting exon 7 (Brewer et al., 2002); MGI: 2183192/*Tcfap2a*<sup>tm1Hsv</sup>], and a *Tcfap2b*<sup>-</sup> null allele [due to a germ-line insertion of a PGK-*neo* cassette disrupting exon 4 (Moser et al., 1997); MGI: 2180473/*Tcfap2b*<sup>tm1Rbu</sup>]. *Tcfap2a*<sup>ki7lacZ/+</sup> mice were crossed with  $\alpha$ -*Cre*<sup>+/-</sup> transgenic mice (Marquardt et al., 2001) expressing Cre recombinase under control of the retina-specific “*Pax6*  $\alpha$  enhancer,” from the murine *Pax6* gene [MGI: 3052661; Tg(*Pax6*-cre,GFP)2Pgr]. *Tcfap2a*<sup>ki7lacZ/+</sup>/ $\alpha$ -*Cre*<sup>+/-</sup> mice were crossed with *Tcfap2b*<sup>+/-</sup> mice to generate mice that retained the  $\alpha$ -*Cre* transgene, and had only one functional copy of the *Tcfap2a* and *Tcfap2b* genes (*Tcfap2a*<sup>ki7lacZ/+</sup>/*Tcfap2b*<sup>+/-</sup>/ $\alpha$ -*Cre*<sup>+/-</sup>). This cross is expected (based on Mendelian genetics) to result in 12.5% of the offspring being *Tcfap2a*<sup>ki7lacZ/+</sup>/*Tcfap2b*<sup>+/-</sup>/ $\alpha$ -*Cre*<sup>+/-</sup>; however, unpublished observations in the West-Mays and Williams laboratories have shown that the low frequency of neural tube closure defects seen in *Tcfap2a* heterozygotes (Kohlbecker et al., 2002) is exacerbated in *Tcfap2a/Tcfap2b* double heterozygotes. Thus, we generated fewer *Tcfap2a*<sup>ki7lacZ/+</sup>/*Tcfap2b*<sup>+/-</sup>/ $\alpha$ -*Cre*<sup>+/-</sup> mice for the final cross (see below) than expected.

In a separate cross, *Tcfap2b*<sup>+/-</sup> mice were bred with mice homozygous for the *Tcfap2a*<sup>lox</sup> allele (Brewer et al., 2004), in which exons 5 and 6 are flanked by single *loxP* sites (MGI: 3038304/*Tcfap2a*<sup>tm2Will</sup>), to obtain *Tcfap2b*<sup>+/-</sup> mice that were also heterozygous for the *Tcfap2a*<sup>lox</sup> allele. These *Tcfap2b*<sup>+/-</sup>/*Tcfap2a*<sup>+lox</sup> mice were back-crossed to *Tcfap2a*<sup>lox/lox</sup> mice to regain homozygosity of the *Tcfap2a*<sup>lox</sup> allele, and the resulting desired progeny (*Tcfap2b*<sup>+/-</sup>/*Tcfap2a*<sup>lox/lox</sup>) were continually crossed with *Tcfap2a*<sup>lox/lox</sup> mice to maintain this line. In the final cross, mice from the *Tcfap2b*<sup>+/-</sup>/*Tcfap2a*<sup>lox/lox</sup> line were bred with the *Tcfap2a*<sup>ki7lacZ/+</sup>/*Tcfap2b*<sup>+/-</sup>/ $\alpha$ -*Cre*<sup>+/-</sup> mice, resulting in double  $\alpha/\beta$  mutants that are heterozygous for the *Tcfap2a*<sup>ki7lacZ</sup> null allele in all tissues, have the second copy of *Tcfap2a* conditionally deleted only from the developing retina, and lack both functional copies of *Tcfap2b* in all tissues (*Tcfap2a*<sup>ki7lacZ/lox</sup>/*Tcfap2b*<sup>+/-</sup>/ $\alpha$ -*Cre*<sup>+/-</sup>, referred to for

simplicity as *Tcfap2a/b* mutants). This final cross in the breeding scheme is expected (based on Mendelian genetics) to result in 6.25% (1/16th) of the offspring being double  $\alpha/\beta$  mutants, therefore numerous breedings were required. At birth, however, we found that double  $\alpha/\beta$  mutants were often stillborn or died immediately, making the P0 stage particularly difficult to obtain. Littermates used as controls either contained two functional copies of *Tcfap2a* and two functional copies of *Tcfap2b* in the retina, or were missing one functional copy of either *Tcfap2a* or *Tcfap2b* if the former was not available (Table 1).

## Histology

Dissected embryo heads or eyes were either fixed in 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON) overnight at room temperature, processed and embedded in paraffin (Paraplast tissue embedding media, Fisher Scientific, Waltham, MA), or fixed in 4% PFA overnight at 4°C, cryoprotected overnight in 30% sucrose/PBS, and embedded in Tissue-Tek OCT. Horizontal sections were cut 4  $\mu$ m in thickness (paraffin) or 8  $\mu$ m in thickness (frozen) and used for hematoxylin and eosin (H&E) staining, immunofluorescent analysis, or the Terminal uridine deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay. We did not obtain large sample sizes of *Tcfap2a/b* mutants at each stage examined, due to the breeding scheme and viability issues caused by reduced *Tcfap2a/b* dosage (described above). The numbers of *Tcfap2a/b* mutant eyes examined at each stage were as follows: Two at E15.5; four at E16.5; two at E17.5; five at E18.5; two at P0. Results were consistent between the limited samples.

## Immunofluorescence, TUNEL Assay, and Quantification of Immunolabeled Cells

Indirect immunofluorescence was performed using the primary antibodies listed below. To detect AP-2 $\beta$ , we primarily used a rabbit polyclonal anti-AP-2 $\beta$  at 1:80 (Cell Signaling Technology, Inc., Danvers, MA); however if co-staining with a primary antibody made in rabbit, we used a mouse polyclonal anti-AP-2 $\beta$  at 1:400 (Abnova Corporation, Taipei City, Taiwan). Note that these two AP-2 $\beta$  antibodies gave identical staining patterns. Additional antibodies were as follows: Mouse monoclonal anti-AP-2 $\alpha$  (clone 3B5, supernatant) used undiluted (developed by Dr. Trevor Williams, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); mouse monoclonal anti-AP-2 $\gamma$  (clone 6E4/4) at 1:800 (Sigma-Aldrich, Oakville, ON); goat polyclonal anti-Bhlhb5 (E-17) at 1:400 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); goat polyclonal anti-Brn3 (C-13) at 1:200 (Santa Cruz Biotechnology); rabbit polyclonal anti-Crx (H-120) at 1:100 (Santa Cruz Biotechnology); mouse monoclonal anti-CyclinD1 at 1:100 (Santa Cruz Biotechnology); goat polyclonal anti-glycine transporter 1 (GlyT1) at 1:5,000 (Millipore-Chemicon, Billerica, MA); rabbit polyclonal anti- $\gamma$ -aminobutyric acid (GABA) transporter 1 (GAT-1) at 1:250 (Abcam, Cambridge, MA); rabbit polyclonal anti-Islet1/2 (K5) at 1:500 (Dr. Thomas Jessell lab, Columbia University, New York, NY); mouse monoclonal anti-Ki67 (clone B56) at 1:100 (BD Biosciences, San Jose, CA); rabbit polyclonal anti-Ki67 at 1:500 (Novocastra, Newcastle, UK); mouse monoclonal anti-Lim1/2 (clone 4F2) at 1:50 (developed by Dr. Thomas Jessell and Susan Brenner-Morton, Developmental Studies Hybridoma Bank, University of Iowa); mouse monoclonal anti-Neurofilament 160 (clone NN18) at 1:300 (Sigma-Aldrich); mouse monoclonal anti-Neurofilament 68 (clone NR4) at 1:300 (Sigma-Aldrich); rabbit polyclonal anti-Otx2 at 1:100 (Abcam); rabbit polyclonal anti-Pax6 at 1:200 (Covance, Princeton, NJ); mouse monoclonal anti-PCNA (clone PC10) at 1:800 (Dako Canada, Mississauga, ON); rabbit polyclonal antiphospho-histone H3 (PH3) at 1:100 (Millipore-Upstate); rabbit polyclonal anti-PGP9.5 at 1:500 (Ultrasclone Ltd., Isle of Wight, UK); rabbit polyclonal anti-Prox1 at 1:500 (AngioBio, Del Mar, CA); rabbit polyclonal anti-Sox2 at 1:1,000 (Millipore-Chemicon); mouse monoclonal anti-syntaxin-1 (clone HPC-1) at 1:2,000 (Sigma-Aldrich); sheep polyclonal anti-Vsx2/Chx10 at 1:100 (Exalpha Biologicals, Inc., Watertown, MA). Fluorescent secondary antibodies were Alexa

Flour 488 or 568 (Invitrogen – Molecular Probes, Burlington, ON) used 1:200 for 1 hr at room temperature. For all primary antibodies, paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95% and 70% ethanol, followed by water), treated for antigen retrieval with 10 mM sodium citrate buffer pH 6.0, boiling for 20 min, blocked with 5% normal serum from the host of the secondary antibody and incubated with primary antibodies overnight at 4°C. Frozen sections were rinsed in PBS, treated for antigen retrieval and blocked as above, and incubated with primary antibodies overnight at 4°C. For all co-immunostains, both primaries and both secondaries were mixed and incubated simultaneously. The TUNEL assay was performed using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Millipore-Chemicon), according to the manufacturer's instructions for fluorescent staining of paraffin-embedded tissue or cryosections. Following immunofluorescence or the TUNEL assay, stains were mounted with Vectashield mounting medium containing 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON) or Pro-Long Gold antifade reagent with DAPI (Invitrogen – Molecular Probes). All stains were visualized with a microscope (Leica, Deerfield, IL) equipped with a fluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA). To quantify immunolabeled cells, a 320 µm length of retinal section equidistant from the optic nerve head was scored on a minimum of 3 different sections from 3 animals at each stage counted, and expressed as the mean ±SEM. Data were tested for significance by two-way ANOVA or Student's *t*-test where appropriate (SPSS 19.0 Software). Least significant difference post hoc comparisons were made when statistical significance ( $P < 0.05$ ) was found between observations.

### X-gal Staining

Dissected E18.5 control and *Tcfap2a/b* mutant heads were fixed in 4% PFA for 1 hr at 4°C, rinsed several times in PBS, cryoprotected overnight in 30% sucrose/PBS, and embedded in Tissue-Tek OCT. Horizontal sections were cut 14µm in thickness. X-Gal dilution buffer (5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate and 2 mM magnesium chloride in 1× PBS) was warmed to 37°C, and used to dilute (1:40) the X-Gal stock solution (40 mg/ml X-Gal in *N,N*-dimethylformamide) to create a 1 mg/ml X-Gal working solution. After rinsing cryosections in PBS to remove OCT, the warmed X-gal working solution was applied directly to the slides, which were incubated overnight at 37°C. Slides were rinsed in PBS and dehydrated in an ethanol series (70%, 95%, 100%), then placed in xylene before mounting with Permount (Fisher Scientific, Pittsburgh, PA).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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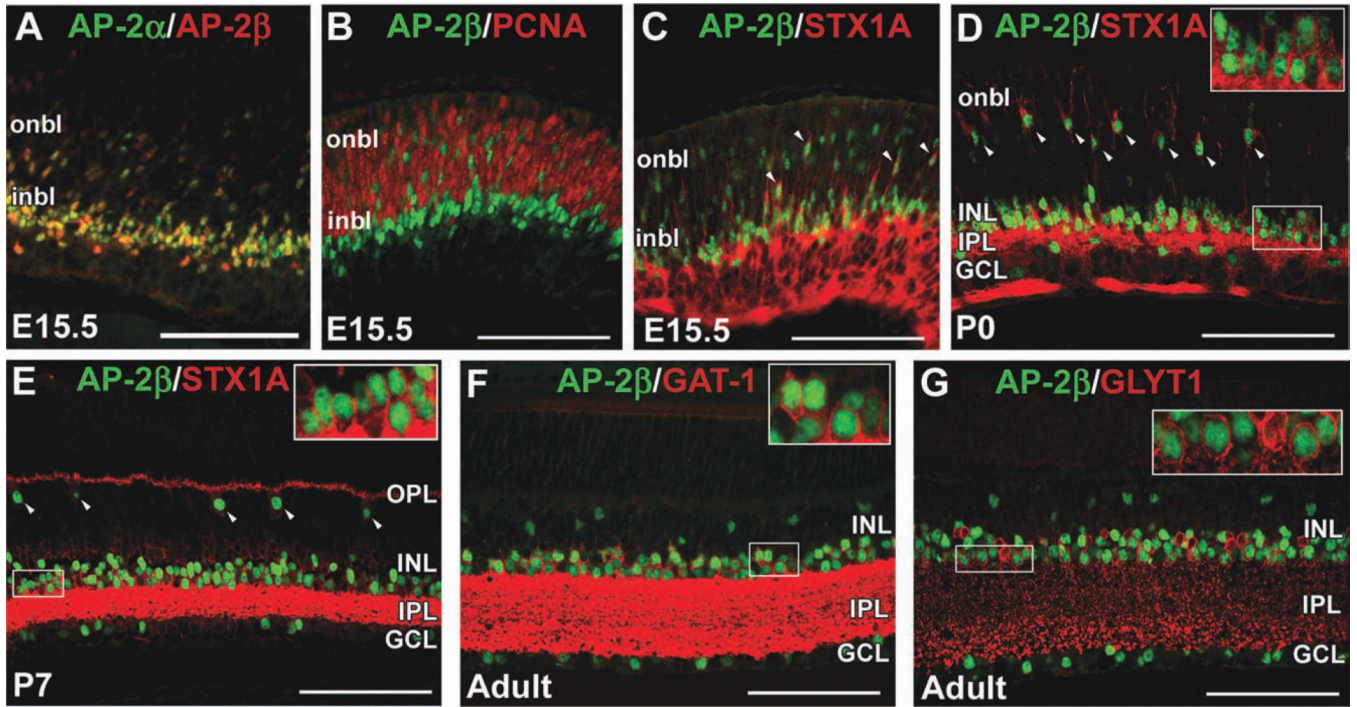
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**Key findings**

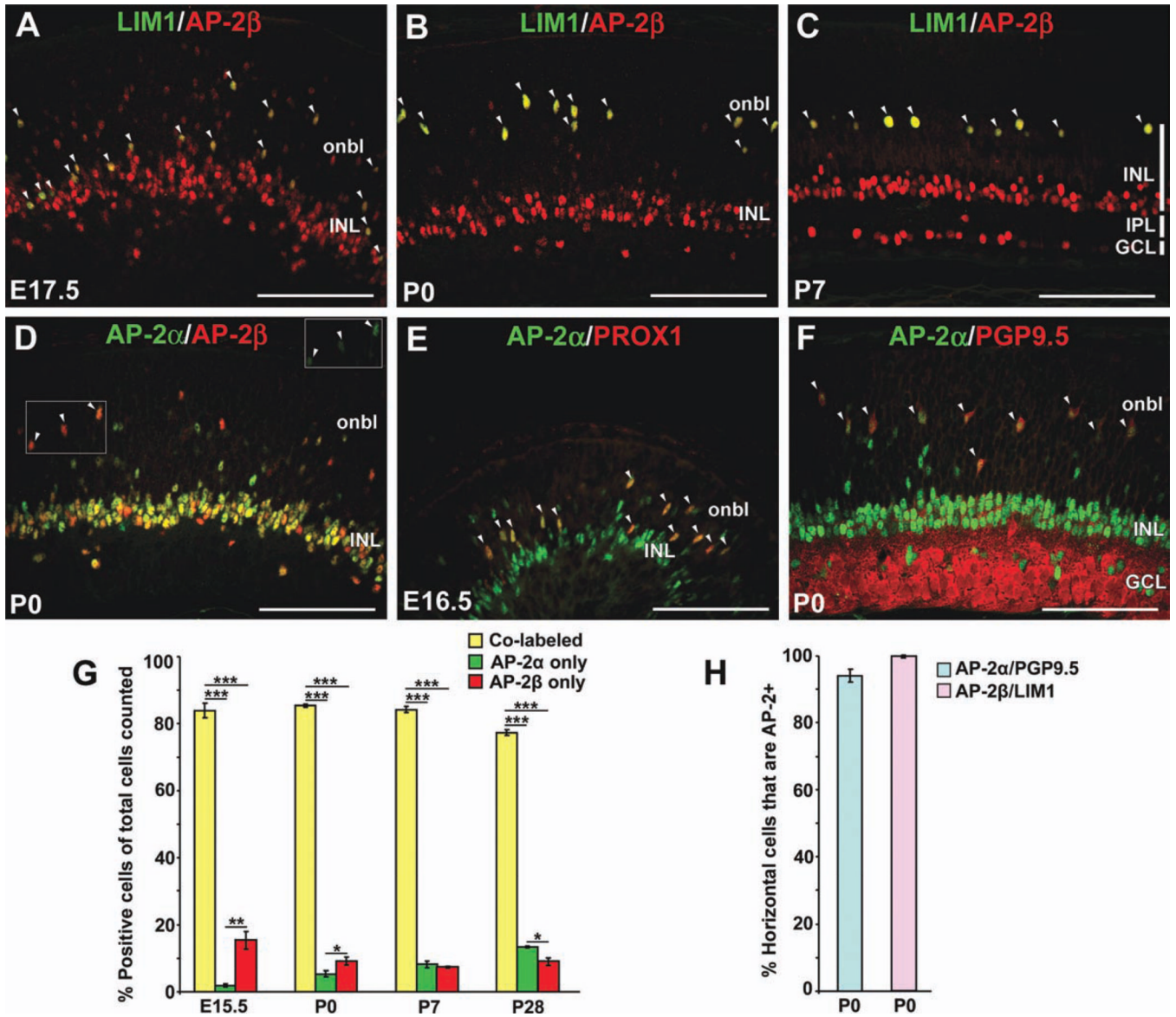
- Three AP-2 (*Tcfap2*) transcription factor family members, AP-2 $\alpha$ , AP-2 $\beta$  and AP-2 $\gamma$ , are expressed in the amacrine/horizontal cell lineages in the developing mouse retina.
- AP-2 $\alpha$  and AP-2 $\beta$  are co-expressed in postmitotic horizontal and amacrine cells, while AP-2 $\gamma$  expression is restricted to a subset of postmitotic amacrine cells that is partially distinct from the AP-2 $\alpha$ / $\beta$ -immunopositive population.
- *Tcfap2a/b*-deficient retinas exhibit horizontal and amacrine cell defects that are not detected upon deletion of either family member alone, revealing critical redundant roles for AP-2 $\alpha$  and AP-2 $\beta$  in horizontal and amacrine cell development.



**Fig. 1. AP-2 $\beta$  is expressed in postmitotic amacrine and horizontal cells in the developing mouse retina**

**A:** Co-immunostain with anti-AP-2 $\alpha$  (green) and anti-AP-2 $\beta$  (red) on horizontal section of E15.5 wild-type eye. **B–G:** Co-immunostain with anti-AP-2 $\beta$  (green) and either anti-PCNA (B), anti-STX1A (C–E), anti-GAT-1 (F), or anti-GLYT1 (G) antibodies (red) on horizontal sections of wild-type eyes, at the stages indicated. Arrowheads in C–E point to cells co-labeled by anti-AP-2 $\beta$  and anti-STX1A. Boxed areas in E–G are magnified in inset. i/onbl, inner/outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer. Scale bars = 100  $\mu$ m.

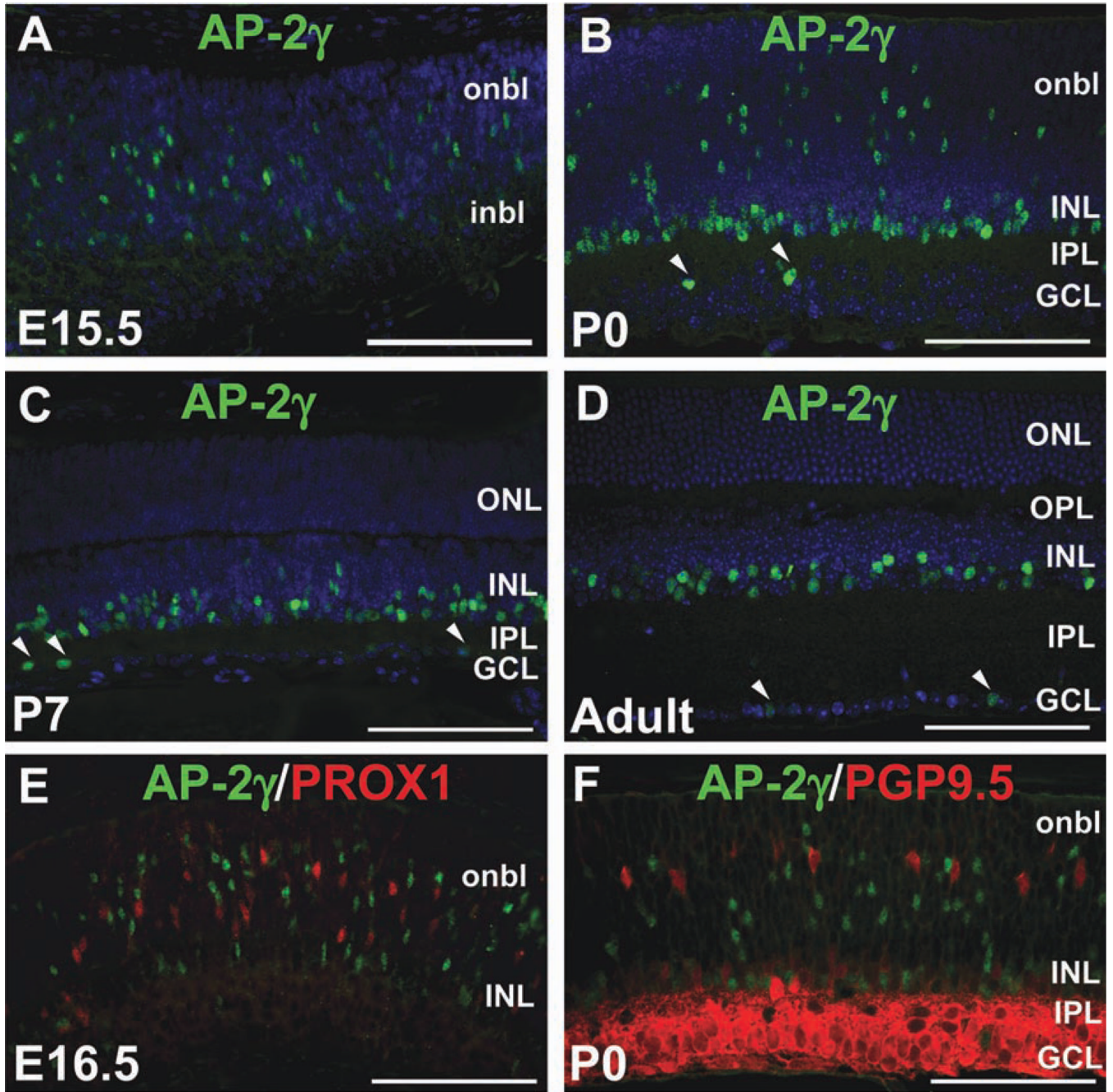




**Fig. 2. Both AP-2 $\alpha$  and AP-2 $\beta$  are expressed in developing mouse horizontal cells, although AP-2 $\alpha$  expression is transient**

**A–F:** Co-immunolabeling with anti-LIM1 (green) and anti-AP-2 $\beta$  (red; A–C); anti-AP-2 $\alpha$  (green) and anti-AP-2 $\beta$  (red; D); anti-AP-2 $\alpha$  (green) and anti-PROX1 (red; E); or anti-AP-2 $\alpha$  (green) and anti-PGP9.5 (red; F) on horizontal sections of wild-type eyes, at the stages indicated. All LIM1-positive horizontal cells are AP-2 $\beta$ -positive (A–C, arrowheads). Arrowheads in D denote cells that are strongly AP-2 $\beta$ -immunoreactive and also more weakly AP-2 $\alpha$ -immunoreactive (inset). Arrowheads in E and F point to PROX1-positive or PGP9.5-positive horizontal cells that co-express AP-2 $\alpha$ . **G:** Quantification of the proportions of co-labeled versus singly labeled cells in the total AP-2 population examined (AP-2 $\alpha$  and/or AP-2 $\beta$ -positive), at the stages indicated. Note that the relative proportions of co-labeled versus singly labeled cells were not significantly affected by the stage examined. **H:** Quantification of the proportion of PGP9.5-positive horizontal cells expressing AP-2 $\alpha$  and proportion of LIM1-positive cells expressing AP-2 $\beta$  at P0. Bars represent the mean  $\pm$ SEM of counts from 3 animals. onbl, outer neuroblast layer; INL, inner nuclear layer;

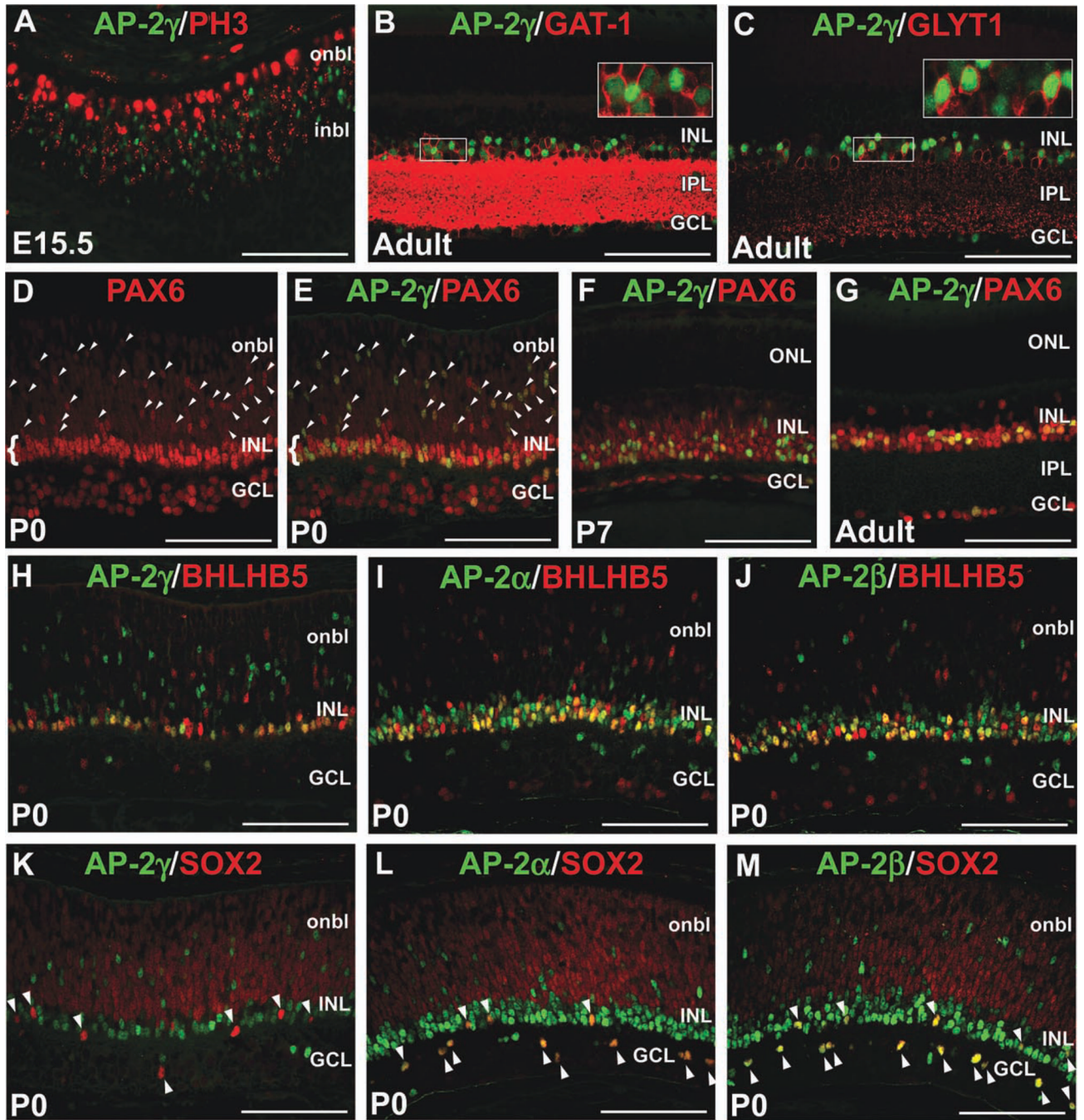
GCL, ganglion cell layer; IPL, inner plexiform layer. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .  
Scale bars = 100  $\mu\text{m}$ .



**Fig. 3. AP-2 $\gamma$  is not a horizontal cell marker**

**A–D:** Immunofluorescence using anti-AP-2 $\gamma$  clone 6E4 (green) on horizontal sections of wild-type mouse heads or eyes counterstained with DAPI (4,6-diamino-2-phenylindole (DAPI; blue), at the stages indicated. Arrowheads in B–D indicate AP-2 $\gamma$ -immunoreactive cells in the GCL. **E,F:** Co-immunostain with anti-AP-2 $\gamma$  (green) and either anti-PROX1 (E) or anti-PGP9.5 (F) antibodies (red) on horizontal sections of wild-type eyes, at the stages indicated. i/onbl, inner/outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer. Scale bars = 100  $\mu$ m.



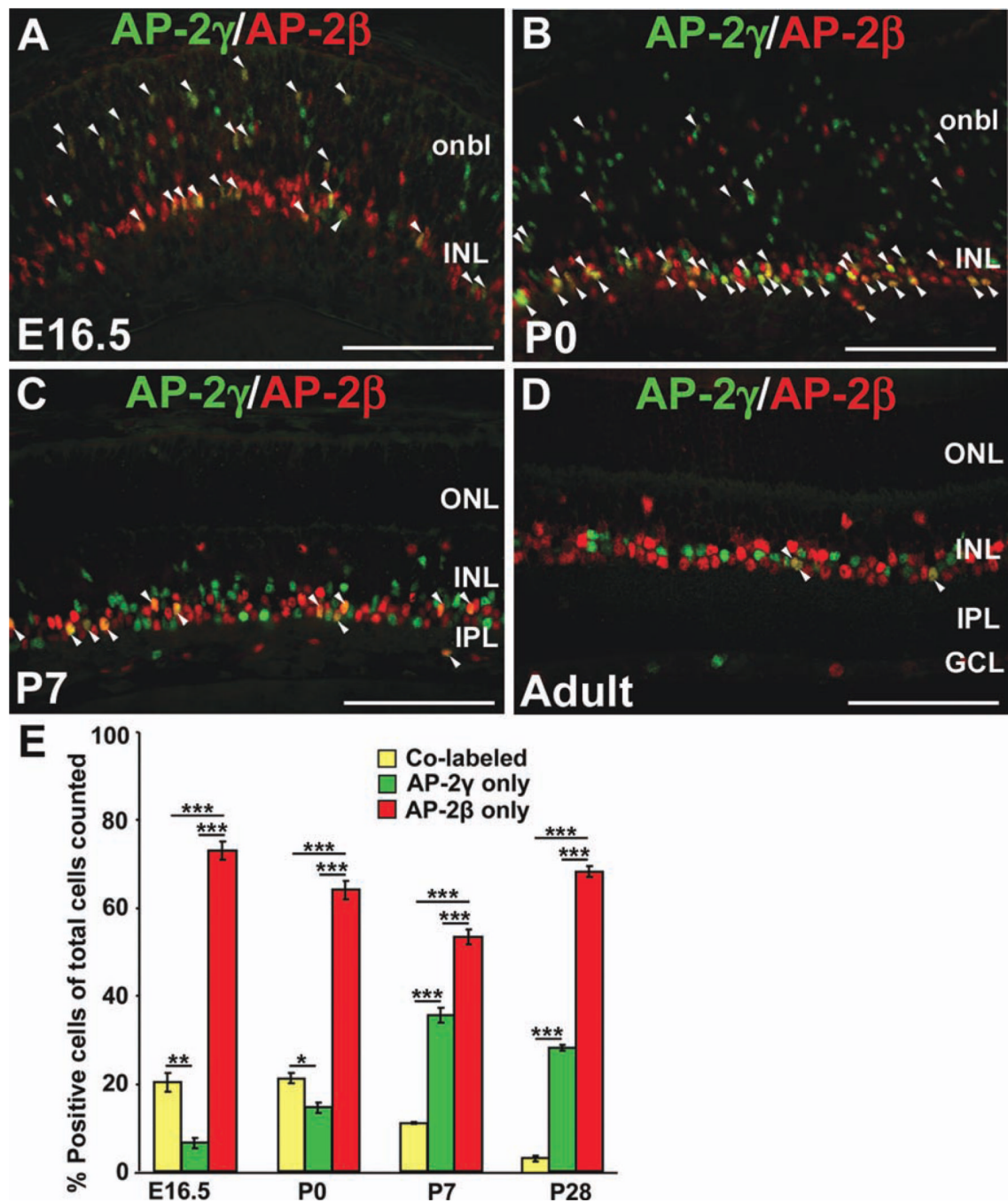


**Fig. 4. AP-2γ is expressed in postmitotic amacrine cells**

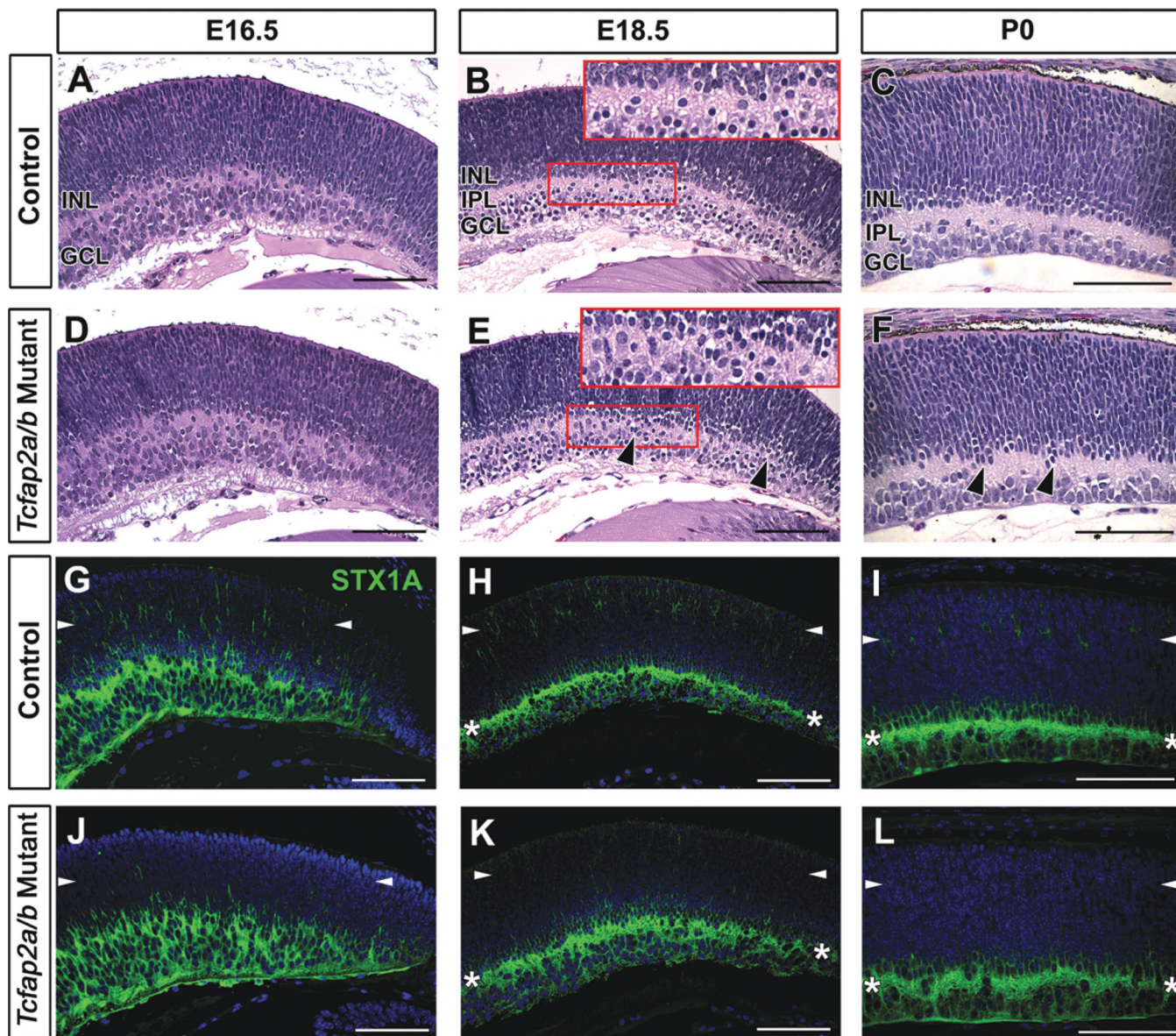
**A–C:** Co-immunostain with anti-AP-2γ (green) and either anti-PH3 (A), anti-GAT-1 (B), or anti-GLYT1 (C) antibodies (red) on horizontal sections of wild-type eyes, at the stages indicated. Boxed areas in B and C are magnified in inset. **D–G:** Co-immunostain with anti-AP-2γ (green) and anti-PAX6 (red) on horizontal sections of wild-type eyes at the stages indicated. D shows PAX6 alone while E shows double merge of the same image. Arrowheads in D and E denote strongly immunoreactive PAX6-positive cells in the neuroblast layer. Brackets in D and E indicate PAX6-positive amacrine cells in the INL. **H–M:** Co-immunostains of different AP-2 family members indicated (green) with BHLHB5 or

SOX2 (red) at P0. Arrowheads in K–M point to SOX2-positive cholinergic amacrine cells, which co-express AP-2 $\alpha$  and AP-2 $\beta$  (L,M) but not AP-2 $\gamma$  (K). i/onbl, inner/outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars = 100  $\mu$ m.



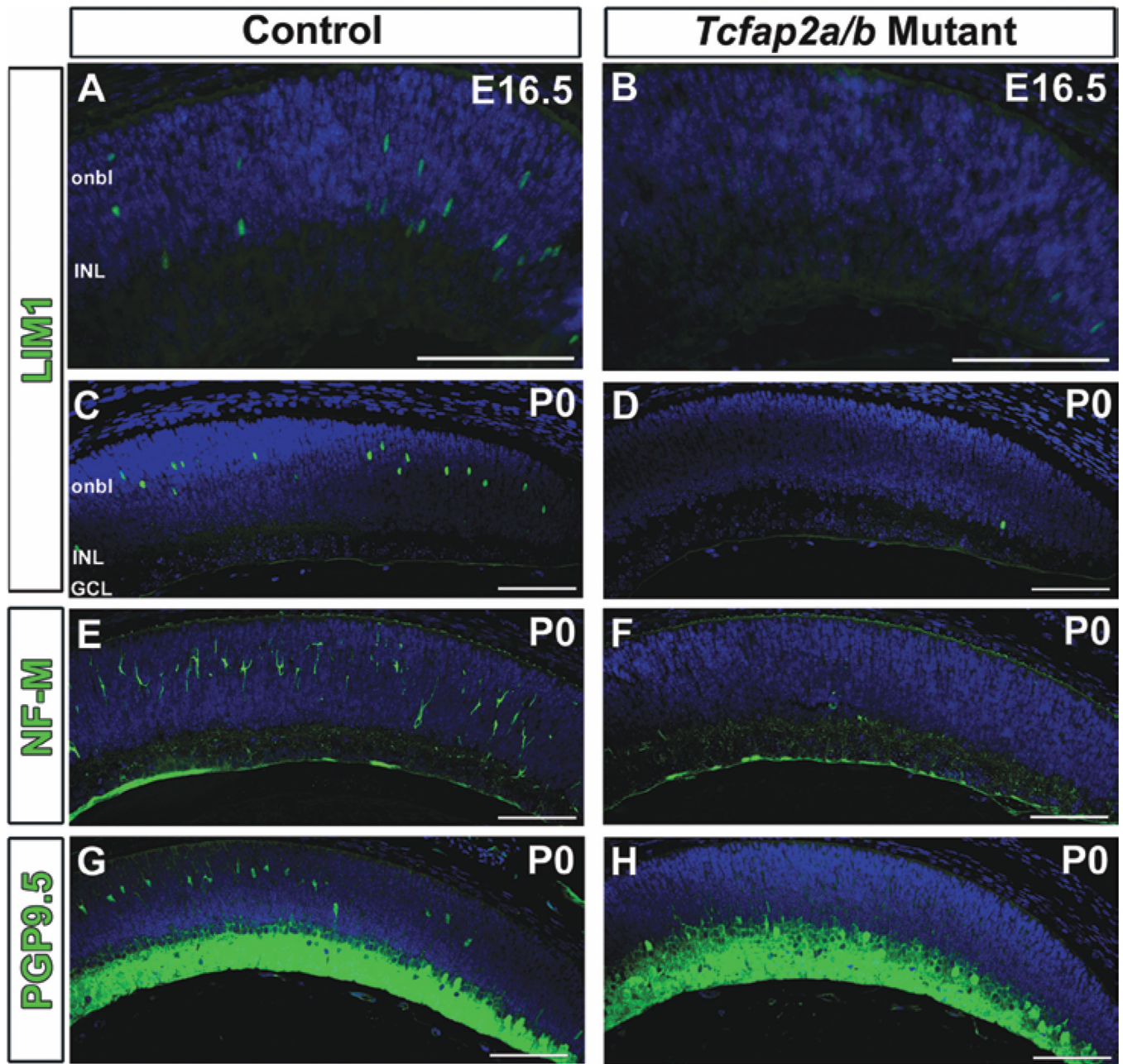


**Fig. 5. The AP-2 $\gamma$ -immunopositive amacrine cell population only partially overlaps with the AP-2 $\beta$ -immunopositive amacrine cell population**  
**A–D:** Co-immunolabeling with anti-AP-2 $\gamma$  (green) and anti-AP-2 $\beta$  (red) on horizontal sections of wild-type eye, at the stages indicated. Arrowheads denote double-labeled cells.  
**E:** Quantification of the proportions of co-labeled versus singly labeled cells in the total AP-2 population examined (AP-2 $\gamma$  and/or AP-2 $\beta$ -positive), at the stages indicated. Bars represent the mean  $\pm$ SEM of counts from 3 animals. \*\*\* $P$  < 0.001; \*\* $P$  < 0.01; \* $P$  < 0.05. onbl, outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars = 100  $\mu$ m.

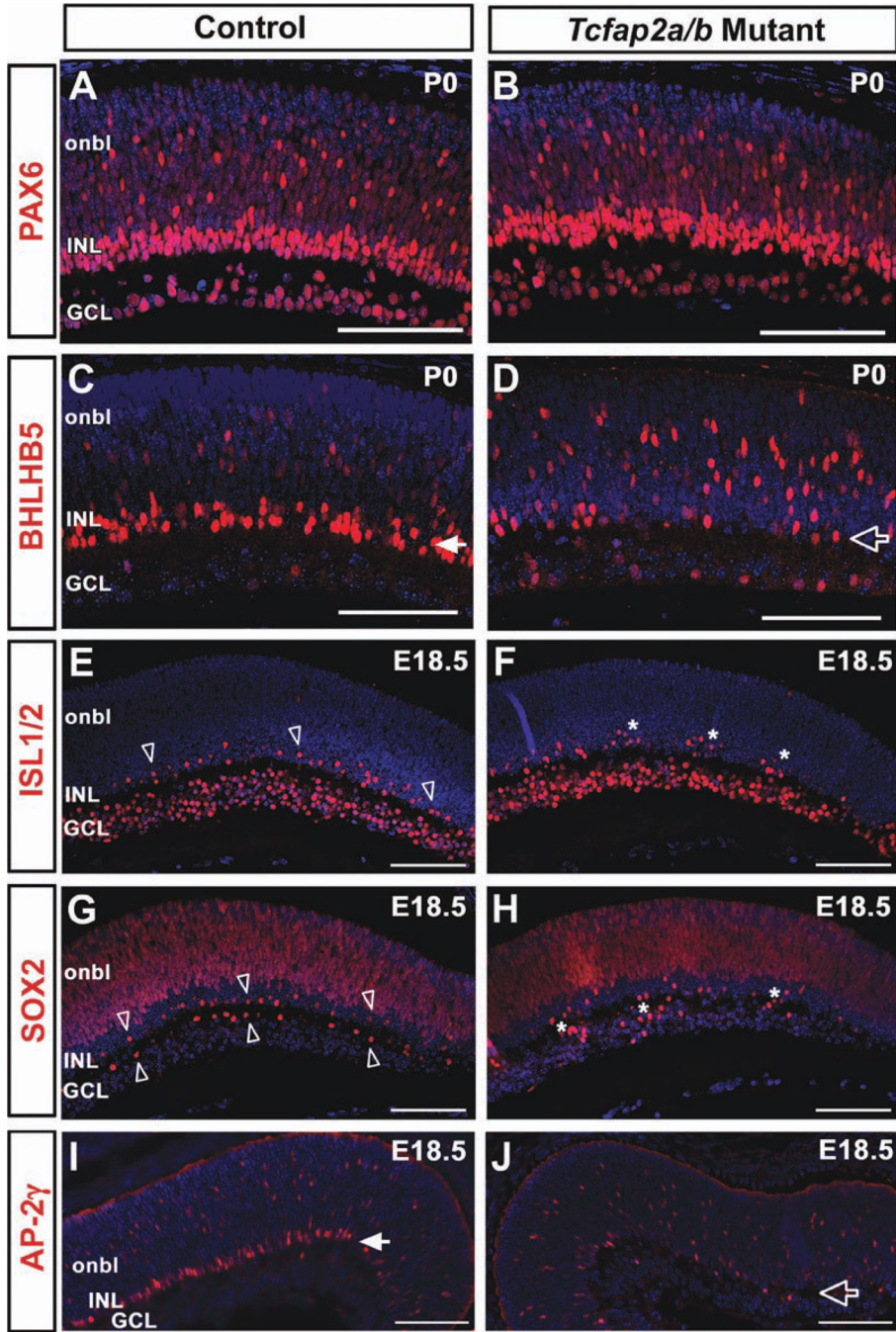


**Fig. 6.** Subtle disorganization and diminished syntaxin immunoreactivity in the retina in *Tcfap2a/b* mutants. **A–F:** Horizontal H&E-stained sections of control (A–C) and *Tcfap2a/b* mutant (D–F) peripheral retinas, at the stages indicated. Black arrowheads (E,F) show regions of mutant retinas that appear less organized than controls. Red boxes in B and E are magnified in insets. **G–L:** Horizontal sections of control (G–I) and *Tcfap2a/b* mutant (J–L) peripheral retinas immunolabeled with anti-STX1A (green). Asterisks (H,I,K,L) indicate the band of STX1A-labeled amacrine cell processes in the IPL. The STX1A immunoreactivity detected in the outer retina of control mice (G–I, arrowheads) was diminished in *Tcfap2a/b* mutants (J–L, arrowheads). INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bars = 100  $\mu$ m.





**Fig. 7.** Deletion of *Tcfap2a* and *Tcfap2b* leads to loss of retinal horizontal cells. **A–H:** Horizontal sections of control (A,C,E,G) and *Tcfap2a/b* mutant (B,D,F,H) peripheral retinas immunolabeled (green) with anti-LIM1 (A–D), anti-neurofilament medium chain (NF-M; E,F) or anti-PGP9.5 (G,H) antibodies and counterstained with DAPI (4,6-diamino-2-phenylindole; blue) at E16.5 (A,B) or P0 (C–H). onbl, outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars = 100  $\mu$ m.



**Fig. 8.** Altered immunostaining patterns of amacrine cell markers in *Tcfap2a/b* mutant retinas. **A–J:** Horizontal sections of control (**A,C,E,G,I**) and *Tcfap2a/b* mutant (**B,D,F,H,J**) peripheral retinas immunolabeled (red) with anti-PAX6 (**A,B**), anti-BHLHB5 (**C,D**), anti-ISL1/2 (**E,F**), anti-SOX2 (**G,H**) or anti-AP-2 $\gamma$  (**I,J**) and counterstained with DAPI (4,6-diamino-2-phenylindole; blue), at the stages indicated. Closed arrows in **C** and **I** denote the band of BHLHB5-positive or AP-2 $\gamma$ -positive cells, respectively, clearly forming in the control INL, which was not observed in mutants (open arrows in **D,J**). Open arrowheads in **E** and **G** point to the developing cholinergic amacrine mosaic in control retinas, whereas asterisks in **F** and

H indicate cholinergic cells that are failing to exhibit regular spacing in mutant retinas. onbl, outer neuroblast layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars = 100  $\mu\text{m}$ .



TABLE 1

## Genotyping

Alleles	Primers	Conditions	Products
<i>Tcfap2a</i> <sup>ki7lacZ</sup> versus <i>Tcfap2a</i> <sup>+</sup>	Alpha 6/7 5'-GAA AGG TGT AGG CAG AAG TTT GTC AGG GC -3' Alpha 3' KO 5'-CGT GTG GCT GTT GGG GTT GTT GCT GAG GTA C -3' IRESUP 5'-GCT AGA CTA GTC TAG CTA GAG CGG CCC GGG -3'	45 s at 95°C, 1 min at 67°C, 1 min 10 sec at 72°C for 33 cycles	<i>Tcfap2a</i> <sup>ki7lacZ</sup> 300 bp <i>Tcfap2a</i> <sup>+</sup> 500 bp
<i>Tcfap2b</i> versus <i>Tcfap2b</i> <sup>+</sup>	4 Exon DW 5'-CCT CCC AAA TCT GTG ACT TCT-3' PGK-PolyA DW 5'-CTG CTC TTT ACT GAA GGC TCT TT-3' 4 Exon Rev 5'-TTC TGA GGA CGC CGC CCA GG-3'	45 s at 95°C, 45 s at 58°C, 1 min at 72°C for 37 cycles	<i>Tcfap2b</i> 380 bp <i>Tcfap2b</i> <sup>+</sup> 221 bp
$\alpha$ - <i>Cre</i> transgene	Cre1 5'-GCT GGT TAG CAC CGC AGG TGT AGA G-3' Cre3 5'-CGC CAT CTT CCA GCA GGC GCA CC-3'	45 s at 95°C, 1 min at 67°C, 1 min 10 sec at 72°C for 33 cycles	Presence of $\alpha$ - <i>Cre</i> transgene 420 bp
<i>Tcfap2a</i> <sup>lox</sup> versus <i>Tcfap2a</i> <sup>+</sup>	Alflox4 5'-CCC AAA GTG CCT GGG CTG AAT TGA C-3' Alfscsq 5'-GAA TCT AGC TTG GAG GCT TAT GTC-3'	45 s at 95°C, 45 s at 65°C, 1 min at 72°C for 39 cycl	<i>Tcfap2a</i> <sup>lox</sup> 560 bp <i>Tcfap2a</i> <sup>+</sup> 490 bp