Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 January ; 50(1): 432–440. doi:10.1167/iovs.08-2270.

Characterization of a transient TCF/LEF-responsive progenitor population in the embryonic mouse retina

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Abstract

Purpose—High mobility group (HMG) transcription factors of the T-cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family are a class of intrinsic regulators that are dynamically expressed in the embryonic mouse retina. Activation of TCF/LEFs is a hallmark of the Wnt/ β -catenin pathway, however, the requirement for Wnt/ β -catenin and noncanonical Wnt signaling during mammalian retinal development remains unclear. Our goal was to characterize more fully a TCF/LEF-responsive retinal progenitor population in the mouse embryo, and to correlate this with Wnt/ β -catenin signaling.

Methods—TCF/LEF activation was analyzed in the TOPgal reporter mouse (TOP: TCF optimal promoter) at embryonic ages and compared to Axin2 mRNA expression, an endogenous readout of Wnt/ β -catenin signaling. Reporter expression was also examined in embryos with a retina-specific deletion of the β -catenin gene (Ctnnb1), using Six3-Cre transgenic mice. Finally, the extent to which TOPgal cells coexpress cell cycle proteins, basic helix-loop-helix (bHLH) transcription factors and other retinal cell type markers was tested by double-immunohistochemistry.

Results—TOPgal reporter activation occurs transiently in a subpopulation of embryonic retinal progenitor cells. Axin2 is not expressed in the central retina, and TOPgal reporter expression persists in the absence of β -catenin. Although a proportion of TOPgal-labeled cells are proliferative, most coexpress the cyclin-dependent kinase inhibitor p27/Kip1.

Conclusions—TOPgal cells give rise to the four earliest cell types: ganglion cells, amacrine, horizontal and photoreceptors. TCF/LEF activation in the central retina does not correlate with Wnt/ β -catenin signaling, pointing to an alternate role for this transcription factor family during retinal development.

Introduction

The neural retina develops from ventral forebrain neuroepithelium, and when mature, is comprised of seven major types of neurons and glia. These retinal cell types are generated in an evolutionary conserved order with ganglion cells formed first, then cones, horizontals,

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amacrines and rods, with bipolars and Müller glia generated last.^{1–5} Retinal progenitors are generally multipotent; however, over time they exhibit both lineage and competence restrictions such that fewer and fewer distinct cell types arise as development proceeds.^{6–11} The competence of retinal progenitors is regulated by both extracellular and intrinsic factors.^{11–14} The intrinsic factors have so far fallen into main two protein classes, either basic helix-loop-helix (bHLH) or homeobox transcription factors. In particular, the bHLH factors regulate the development of particular retinal cell types, as they do throughout the vertebrate nervous system.^{11,14,15} For example, one bHLH factor, Math5, is required for ganglion cell formation.^{16–20} while another, NeuroD, is necessary for amacrine, S cone and rod photoreceptor cell genesis.^{21–23} Interestingly, different bHLH factors are further segregated by their expression at distinct stages of the mitotic cell cycle, with Ngn2 and Mash1 expressed by many S phase retinal progenitors²⁴, and Math5 present in newly postmitotic, transitional cells.^{25,26} Because the expression patterns and functions of these intrinsic factors are insufficient to explain how all retinal cell types develop, their integration with other pathways is essential to understand retinal neurogenesis at the molecular level.

We and others observed activity of the HMG transcription factors TCF/LEF in retinal progenitors in the mouse embryo, consistent with endogenous TCF/LEF mRNA expression.^{27–29} TCF/LEFs (TCF1/TCF7, LEF1, TCF3/TCF711, TCF4/TCF712) are best known for mediating Wnt (wingless-type MMTV integration site family) signaling through interaction with the co-activator β -catenin.^{30–32} Upon Wnt binding to the Frizzled receptor, stabilized β -catenin translocates to the nucleus where it interacts with TCF/LEFs, to activate the transcription of target genes. Previous studies in *Xenopus* suggested that Wnt/ β -catenin signaling is critical for early retinal development, where it controls neural competence of retinal progenitor cells by regulating Sox2 function.³³ However, in the mammalian eye, the role of Wnt/ β -catenin signaling in retinal progenitors is less clear. Some aspects of TCF/LEF activity in the developing retina directly correlate with Wnt/ β -catenin signaling, for example, during ciliary body and iris formation.^{34–38} However, conditional deletion of β -catenin in the central embryonic retina only resulted in abnormal lamination, with no obvious defects in retinal progenitor proliferation or cell fate specification.^{39,40} In addition, direct modulation of LEF function in either chick or mouse retinal experiments did not affect progenitor proliferation or differentiation.^{38,39} Therefore, Wnt/ β -catenin signaling appears largely dispensable during embryonic retinal neurogenesis.

Here we characterize TCF/LEF-responsive retinal cells in greater depth during mouse embryonic development. Transgenic constructs with multimerized TCF/LEF binding sites upstream of a minimal promoter that drive expression of a reporter are commonly used as a readout of activated TCF/LEF-mediated transcription. At least four different mouse TCF/LEF reporter lines have been described.^{41–45} We analyzed the TOPgal reporter generated by Elaine Fuchs and colleagues⁴⁴, and demonstrate TCF/LEF activity within retinal progenitors that differentiate as ganglion cells, cone photoreceptors, amacrines or horizontal cells. Surprisingly, this reporter is active in the absence of β -catenin suggesting that TCF/LEF transcription factors work independent from the Wnt/ β -catenin pathway during retinal neurogenesis.

Material and Methods

Animals

TOPgal mice⁴⁴ were crossed with Ctnnb1 mice containing an allele of β -catenin with exons 2–6 of the gene flanked by loxP sites (termed floxed β -catenin in this paper).⁴⁶ A separate stock of mice carrying the Six3-Cre transgene (Tg(Six3-cre)⁶⁹Frty) was crossed with a β -catenin^{del} allele.^{46,47} For our experiments, Six3-Cre; β -catenin^{del/+} mice were crossed to those homozygous for both the TOPgal transgene and floxed β -catenin (β -catenin^{FL/FL}). In the resulting embryonic litters, Six3-Cre;TOPgal; β -catenin^{del/FL} embryos (termed β -catenin

mutant embryos) were compared to control littermates containing one wild type allele of β -catenin or no Six-Cre transgene. Genotyping for floxed β -catenin, β -catenin^{del} and the Six3-Cre was performed as described.^{46,47} TOPgal genotyping by PCR used the primers: 5' cgatgaatccagaaaagcgg 3' (forward); 5' gcttggtggagaggtatt 3' (reverse) and 35 cycles with an annealing temperature of 62°C within a standard protocol. The day of the observed plug was designated embryonic day 0.5. Animal experiments were performed according to the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Utah and Children's Hospital Research Foundation Institutional Animal Care and Use Committees.

Detection of X-Gal Activity

Tissue was fixed with 4% PFA for 10–20 min depending on the age. Cryostat sections (14–16 μ m) were incubated with X-Gal substrate for 8–15 hours, post-fixed and mounted in Fluoromount G (Southern Biotech). The pattern of β -galactosidase activity was confirmed by immunodetection of β -galactosidase protein expression (see below). Images were taken using Nomarski optics with an Olympus BX51 microscope and processed using Photoshop CSS.

In situ hybridization

Whole mount in situ hybridization on separate eyes was performed as previously described^{48,49} using digoxigenin-labeled antisense and sense riboprobes for Axin2⁵⁰ and an antisense probe for Math5.¹⁹

Immunohistochemistry and BrdU pulse labeling

Embryonic tissue was fixed for 45–60 min with 4% paraformaldehyde (PFA) in PBS. For BrdU pulse labeling, pregnant mice were injected as described in Le et al., 2006. Cryostat sections (10–12 μ m) were processed for antibody labeling as previously described.⁵¹ Primary antibodies used are: rat anti- β -galactosidase (1:750–1000, a gift from Tom Glaser), mouse anti-p27/Kip1 (1:100, BD Transduction), rabbit anti-Ki67 (1:1000, Vector), rabbit anti-phosphorylated histone H3 (1:1000, Upstate), mouse anti-PCNA (1:1000, DakoCytomation), rat anti-BrdU (1:200, Serotec), rabbit anti- β -catenin (1:4000, Sigma), rabbit anti-Hes1 (1:1000, Brown lab), rabbit anti-Ngn2 (1:1000, a gift from Masato Nakufuku), goat anti-Brn3b (1:50, Santa Cruz), rabbit anti-Otx2 (1:2500, Chemicon), rabbit anti-Olig2 (1:1000, a gift from Masato Nakufuku), rabbit anti-RXR γ (1:200, Santa Cruz), rabbit anti-Ptfla (1:800, a gift from Helena Edlund). Secondary antibodies were directly conjugated with Alexa Fluor 488, Alexa Fluor 568 or Alexa 594 (1:1000–2000, Molecular Probes) or indirectly with biotin (horse; 1:200, Vector or rodent; 1:200, Jackson) and streptavidin-conjugated Texas Red (1:200, Jackson Immunologicals). Nuclei were counterstained with 4,6-dimidino-2-phenylindole (DAPI). PCNA labeling was performed after antigen retrieval by immersion of slides in hot citric acid buffer for 30–40 min following anti- β -galactosidase detection. Anti-BrdU labeling was performed after antigen retrieval in 0.2M HCl/0.5% Triton X100 for 1 hour. At least four β -catenin mutant and control embryos were analyzed for β -catenin and β -galactosidase expression.

Cell Counting

Right and left eyes of three TOPgal transgenic animals were analyzed using two coronal, nonadjacent 10–12 μ m sections of the central retina. Images were taken using Olympus BX51 epifluorescence and Olympus Confocal microscope FV1000 and subsequently processed using ImageJ and Photoshop CS3; or using an Axioplan2 epifluorescent microscope equipped with Zeiss deconvolution, Axiovision software and processed with Photoshop 7. For each marker, the percentage of double-positive cells per total number of β -galactosidase-positive cells was

determined in each retinal section, from a minimum of three independent TOPgal embryos per age.

Results

The TOPgal reporter is transiently active in the embryonic mouse retina

In this study, we used TOPgal transgenic mice to define which retinal cells have TCF/LEF activity during embryonic development. This transgene contains three TCF/LEF consensus binding sites and the c-fos minimal promoter driving β -galactosidase expression.^{44,52} At E9.5, β -galactosidase expression is first observed in the dorsal optic vesicle, consistent with other TCF/LEF reporters (not shown and^{29,43,53}). At E10.5, the TOPgal reporter is also activated in the dorsal retinal pigment epithelium (RPE) of the optic cup, dorsal optic stalk and periocular mesenchyme, while no activation is observed in the retina (Fig. 1A). At E11.5, scattered cells in the neural retina initiate reporter expression and the number of these cells is obviously increased by E13.5 (Fig. 1B, C). Starting at E14, the spatial localization of the TOPgal (TOPgal +) expressing population changes; as these cells accumulate near the ventricular/proliferative zone of the retina, adjacent to the RPE where M-phase progenitors and photoreceptor precursors reside (Fig. 1D, shown here at E15.5). At E16.5, the number of TOPgal+ cells decreases substantially and is barely detectable by E17.5 (Fig. 1E, F). No TOPgal+ cells were present in the postnatal and adult central retina (not shown). These results indicate that activation of the TCF/LEF reporter occurs transiently in a subpopulation of embryonic retinal cells. Consistent with the activity of other TCF/LEF reporters, we also observed transient expression in the ciliary body, iris and RPE between birth and postnatal day 30 (not shown).^{5428,29}

TOPgal activity in the central mouse retina is independent of Wnt/ β -catenin signaling

We observed TOPgal reporter activity in the developing retina that is substantially different from TCF/LEF activity reported by other labs using different transgenic lines. To determine how well our TOPgal activity reports Wnt/ β -catenin signaling activity, we examined the mRNA expression of the scaffold protein Axin2, which is a universal readout and antagonist for Wnt/ β -catenin signaling. Axin2 mRNA is detectable at E13.5 in the RPE, periocular mesenchyme and presumptive ciliary body and iris, but, neither the antisense (Fig. 2A) nor sense (Fig. 2B) probes showed any expression in the central retina, where Math5 mRNA is easily observed (Fig. 2C).

The absence of Axin2 expression in the central retina suggests that either Wnt/ β -catenin signaling may not be active, or Axin2 expression may not reflect such signaling in this tissue. To examine this question further, we examined embryonic retinas in which the β -catenin gene was conditionally inactivated by Six3-Cre.^{40,47} We confirmed that Six3-Cre-mediated recombination at E14.5 is detectable in the optic stalk, in the central retina and in some cells of the peripheral retina (S.F and M.L.V.; not shown). Normally, β -catenin protein is present throughout the retina, including within TOPgal+ cells (Fig. 3A–F).⁴⁰ In β -catenin mutant embryos, we observed variable β -catenin deletion in large patches in the central retina, along with lamination defects consistent with other studies (Fig. 3H, K).^{40,55} Surprisingly, we also observed the persistence of TOPgal+ cells in regions lacking β -catenin expression (Fig. 3D–I). This TCF/LEF activity, suggests that TCF/LEF may act independently of Wnt/ β -catenin signaling during embryonic retinal development.

The TOPgal expression is present in both proliferating and postmitotic retinal progenitors

To define TOPgal+ retinal cells more fully, we next examined the cell cycle status of these cells by performing antibody double-labeling for β -galactosidase and cell cycle markers (Fig. 4 and Table 1). To understand if TCF/LEF activity is confined to a particular stage of the cell

cycle, we quantified the proportion of TOPgal+ cells that coexpress proliferating cell nuclear antigen (PCNA), Ki67, BrdU, phosphohistone H3 and the cyclin-dependent kinase inhibitor p27/Kip1. The markers PCNA and Ki67 are expressed in all phases of the cell cycle⁵⁶. Our analysis shows that 53% (E13.5) and 56% (E15.5) of the TOPgal+ population coexpress PCNA (Fig. 4A–C; Table 1). Likewise, we observed 69% (E13.5) and 63% (E15.5) coexpression of TOPgal with Ki67 (Fig. 4D–F; Table 1). Next we found that there is essentially no overlap of TOPgal expression with BrdU (Table 1) and only 7% of TOPgal+ cells coexpress the M phase marker phosphohistone H3 (Fig. 4G–I; Table 1).⁵⁷ However, more than 76% of TOPgal+ cells coexpress the CKI p27/Kip1 at E13.5 and E15.5 (Fig. J–L, Table 1). These observations indicate that TCF/LEF-responsive cells are largely nonproliferative, with the greatest degree of overlap occurring with p27/Kip1 expression. This suggests that TCF/LEF is most active when retinal progenitors are transitioning out of the cell cycle to differentiate.

TOPgal reporter expression is present in multiple early retinal cell types

Next we wished to correlate TOPgal+ cells with markers of neuronal specification and differentiation. First we compared β -galactosidase expression to that of Math5 and NeuroD, two bHLH factors known to be expressed by a number of retinal cells at E13.5 and E15.5.^{19, 22,58} However, only rare TOPgal+ cells were found to coexpress either Math5 or NeuroD in TOPgal retinal sections (not shown). Next we tested the bHLH factor Neurogenin2 (Ngn2), whose lineage contributes to all seven retinal cell types, but in which an obvious role in cell type specification has not yet been determined.²⁴ We found that at E13.5, but not at E15.5, a subset of TOPgal cells was also Ngn2+. The double positive cohort represented 26% of the TOPgal+ population (Fig 5A–C; Table 1). Then, expression of the bHLH factor Olig2 was tested for the extent to which it overlaps with the TOPgal-expressing cells. In the mouse retina, Olig2 initiates expression at E13 in undifferentiated progenitors and is present postnatally in ganglion cells, amacrine, horizontal, bipolar neurons and Müller glia.^{59,60} Interestingly, we found substantial coexpression of β -galactosidase and Olig2 at both E13.5 and E15.5, 64% and 57% respectively (Fig. 5D–F; Table 1). Finally, no overlap of the TOPgal reporter was seen with the bHLH factor Hes1 (Fig. 5G–I), which is expressed in proliferating progenitors and acts as a transcriptional repressor to regulate the timing of neuronal differentiation.^{61–63} Thus, when combined with the high numbers of TOPgal-p27Kip1 coexpressing cells our data suggest that TCF/LEF activity may act within retinal progenitors as they exit the cell cycle.

TCF/LEF activity is present in neurons or neuronal precursors generated during the embryonic period

Our observations suggest that TOPgal+ cells largely represent the postmitotic transitional cells of separate neuronal lineages. Therefore, we predicted that TOPgal reporter expression would be present in nascent ganglion cells, photoreceptors, amacrine and horizontal cells. The paired-type homeobox transcription factor Otx2 controls maturation of photoreceptor and bipolar cells in the rodent retina.^{64–67} Otx2 is coexpressed in many TOPgal+ cells: 59% at E13.5 and 67% at E15.5 (Fig. 6A–C; Table 1). To determine independently that TCF/LEF activity is present in photoreceptor precursors, we looked in the outer retina for coexpression of β -galactosidase with RXR γ , a nuclear hormone receptor essential for fine-tuning the differentiation of cone subpopulations (arrowheads in Fig. 6D–F).^{68–70} In the embryonic retina, RXR γ also labels ganglion cells and we observed β -galactosidase- RXR γ double positive inner retinal cells as well (arrows in Fig. 6D–F). To quantify the percentage of TOPgal + ganglion cells, we compared β -galactosidase and Brn3b coexpression^{71,72} and found 30% of E12.5 TOPgal+ cells also express Brn3b (Table 1 and data not shown). Finally, to examine early amacrine and horizontal neurons, we compared β -galactosidase expression to that of Ptf1a, a bHLH factor expressed specifically by amacrine and horizontal precursors.^{73,74} Between 20% (E13.5) and 23% (E15.5) of the TOPgal+ cells co-express Ptf1a indicating that the TOPgal+ cells also contribute to amacrine and horizontal cell fates (Fig. 6G–I, Table 1).

Discussion

Here we define more fully the transient TCF/LEF-responsive population of retinal progenitors that contribute to the four earliest retinal cell types: ganglion cells, amacrine, horizontal and cone photoreceptors. We conclude that TCF/LEF reporter activity does not require Wnt/ β -catenin signaling, because a) Axin2 is not expressed in the central retina, and b) TOPgal expression persists in the absence of functional β -catenin. Our results point to a possible regulatory role for TCF/LEFs during early mammalian retinal neurogenesis, which correlates with the cell cycle exit of these early-generated cell types.

TCF/LEFs encode different protein isoforms and, depending on the interaction with other cofactors, they can activate or repress target gene transcription.^{30–32,75} For reviews, see TCF/LEFs promote a variety of processes when activated by the Wnt/ β -catenin pathway, such as embryonic patterning, regeneration, stem cell renewal or differentiation in both embryonic and adult tissues, and deregulated activity can lead to tumor formation. For reviews, see^{79,80} Substantial progress has been made in elucidating the interaction of β -catenin with TCF/LEFs, however, previous reports show that other coregulators can interact with TCF/LEFs to promote transcription in the absence of β -catenin. LEF1 can transactivate the T-cell receptor α enhancer in a complex with the co-activators ALY and AML-1, which does not require the β -catenin interaction domain of LEF-1.^{81,82} Similarly, a LEF1 version lacking the β -catenin binding domain activates transcription of the *Xenopus* homeobox gene *twin* by interacting with effectors of the TGF β /Activin signals, Smad2, 3 and 4.⁸³ Our findings also suggest that not all TCF/LEF functions are dependent on Wnt/ β -catenin signaling. In the developing retina, LEF1 and TCF3 expression is present in the central retina between E12.5 and E14.5 while TCF1 and TCF4 show weak expression at E14.5. However, targeted inactivation of TCF1, LEF1 and TCF4 do not appear to cause eye defects, which may be due to functional redundancy^{84–90}. Mutations in both TCF1 and LEF1 result in severe developmental defects and lethality around E10, which has so far prevented further analyses of retinal neurogenesis.⁸⁵ Thus, loss of function-studies where multiple TCF/LEF genes are simultaneously deleted during retinal development are required to elucidate the role of TCF/LEFs in this tissue.

Surprisingly, our results strongly suggest that Wnt/ β -catenin signaling is not active in the embryonic retina in mouse, despite TCF/LEF reporter activation. TOPgal activation in the embryonic mouse retina is not artifactual, since endogenous TCF/LEF and TOPgal reporter expression overlap and an independently generated reporter shows a very similar expression pattern in the embryonic mouse retina (TCF/LEF line).^{28,29} For example, this TCF/LEF reporter is expressed in apical embryonic retinal cells that express CRX, a transcription factor expressed in photoreceptor precursors, consistent with TOPgal-Otx2 coexpression in our study. However, ectopic activation of Wnt/ β -catenin suppresses CRX expression indicating that this pathway must be inactive in committed precursors to ensure proper development of photoreceptors.³⁷ Data presented here agree with this notion. Why the previously characterized TCF/LEF reporter shows persistent activity in embryonic and adult ganglion and amacrine cells is unclear.³⁷ Since we observed TOPgal activity in embryonic ganglion cells and putative amacrine precursors, it is possible that the activity levels and perdurance of these different reporters vary, since not all transgenic constructs are equivalent and undoubtedly reside in different insertion sites throughout the mouse genome.

To provide context for the TCF/LEF reporter activity during retinal development, we compared TOPgal expression to that of transcription factors promoting retinal cell fates, mitotic cell cycle and differentiation markers for the four main embryonic retinal cell types. Together these data show that TCF/LEF reporter activity is not confined to a single retinal lineage. Among the bHLH factors examined, very little TOPgal expression was seen in Math5+ or NeuroD+ cells, but more extensive in early Ngn2+ cells. However, the highest coincidence occurred in Olig2

+ retinal cells. Intriguingly, there is extensive TOPgal activity in both Olig2+ and Otx2+ cohorts, which are complementary. Because very few Math5-TOPgal double positive cells were observed, yet a subset of Brn3b+ cells express TOPgal, the onset of TCF-LEF reporter activity is consistent with retinal progenitor progression towards differentiation. In support of this idea, we found many more TOPgal+ cells express p27/Kip1 than any of the other cell cycle markers examined. Therefore, we propose that TCF/LEF activity assists in some aspect of retinal progenitor transition into a postmitotic precursor. The integrated expression of TCF/LEF proteins with bHLH and homeobox factors appears to be one means by which the complexity of retinal cell type specification may occur. Future studies will address the combinatorial functions of these pathways.

Acknowledgements

We are grateful to Elaine Fuchs, Yasuhide Furuta, Gabrielle Kardon and Charles Murtaugh for providing TOPGAL, Six3-Cre, floxed β -catenin and β -catenin^{del} mice, respectively. We thank Ben Atkins, Alyssa van Bibber, Annie Chen, Amy Eggers, Kim Howes, Ashley Riesenberger, the Levine lab and specifically Gaurav Das for technical help. We are grateful to Masato Nakafuku for the gift of Ngn2 and Olig2 antibodies and Valerie Wallace for sharing experimental data before publication. We thank Ed Levine and Rich Dorsky for helpful comments and critical reading of the manuscript.

Supported by NIH grants, EY14954 (SF, MLV) and EY13612 (NLB), NIH Core Grant, EYO14800, as well as an unrestricted grant from Research to Prevent Blindness, Inc., to the Department of Ophthalmology, University of Utah (SF).

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Fig. 1. Transient TCF/LEF activation in the embryonic retina of the TOPgal reporter mouse
 Transverse sections were stained with X-gal substrate to detect β -galactosidase activity (dorsal is up). (A) At E10.5, TCF/LEF is activated in the dorsal RPE (arrow) and dorsal portion of the optic stalk (asterisk). Arrowhead: Expression of the reporter in periocular mesenchyme separating the ventral lens vesicle and surface ectoderm. (B, C) Expression is detectable in a few scattered cells in the central retina at E11.5 and E13.5 (arrows). (D) At E15.5, the TOPgal reporter is activated in a higher number of retinal progenitors that localize close to the apical surface of the retina (arrow). Expression is also observed in a few cells encapsulating the optic nerve (arrowhead). (E) The number of TOPgal+ cells in the retina decreases substantially at E16.5. Activation of the reporter also occurs in the hyaloid vasculature (arrowhead). (F) At E17.5, only a few cells show TCF/LEF activation (arrows). Asterisks: shows localization of the optic stalk.

Fig. 2. Expression of Axin2 in the embryonic mouse eye at E13.5

Transverse sections of E13.5 TOPgal transgenic whole eyes labeled by whole mount in situ hybridization. (A) Axin2 mRNA expression is detectable in the peripheral retina (arrow), RPE (black arrowhead) and periocular mesenchyme (asterisks). No expression is observed in the central retina (white arrowhead). (B) Axin2 sense control showing no specific labeling of ocular tissues. (C) By comparison, simultaneous in situ hybridization for Math5 mRNA shows strong expression in the central retina (white arrowhead), and not in the RPE (black arrowhead) or peripheral retina (arrow).

Fig. 3. TOPgal reporter activity independent of β -catenin expression

Transverse sections of E14.5 TOPgal mouse heads were double-labeled for β -gal (green; A, D, G, J) and β -catenin (red; B, E, H, K). The merge of both is presented in (C, F, I, L). In control retinas, β -catenin is expressed throughout the retina and overlaps with β -gal protein of TOPgal + expressing cells (Fig. 3A–C). (D–F) Magnification of boxed area shown in (C). Disruption of β -catenin using Six3-Cre results in widespread deletion of β -catenin (H, I). (J–L) Magnification of boxed area shown in (I). In conditional mutants, β -gal expressing cells do persist in regions devoid of β -catenin (J–L; arrowheads). Arrows: TOPgal+ cells in regions with β -catenin expression (asterisks). Dotted line: RPE. Scale bars = 50 μ m.

Fig. 4. TCF/LEF activation occurs in retinal progenitors in different stages of the cell cycle
 Transverse sections of TOPgal mouse heads were double-labeled for β -gal (green; A, D, G, J) and cell cycle proteins (red; B, E, H, K). Overlap of both markers is shown in (C, F, I, L). Immunohistochemistry was performed at E15.5 (A–I) and E13.5 (J–L). TOPgal+ cells show a high level of co-expression with PCNA (A–C), Ki67 (D–F), phosphohistone H3 (PHH3; G–I) and p27^{KIP1} (J–L). Arrowheads: double-labeled cells. Arrows: single-labeled, β -gal+ cells. Scale bars = 50 μ m.

Fig. 5. Overlap of TOPgal reporter expression with transcription factors expressed in retinal progenitor cells at E13.5

Transverse sections were double-labeled for β -gal (green; A, D, G) and transcription factors (red; B, E, H), merge shown in (C, F, I). Retinal progenitor cells with TCF/LEF activation co-express Ngn2 (A–C) and Olig2 (D–F) but not Hes1 (G–I) suggesting that TOPgal+ cells may start to differentiate soon. Arrowheads: double-labeled cells. Arrows: single-labeled TOPgal+ cells. Scale bar = 50 μ m.

Fig. 6. TCF/LEF is active in embryonic cone photoreceptors, ganglion, amacrine and horizontal cells

Transverse sections of E15.5 TOPgal embryonic retina co-labeled for β -gal (green; A, D, G) and different cell-type-specific proteins (red; B, E, H), merge shown in (C, F, I). Substantial overlap is observed with Otx2 (A–C), RXR γ (D–F) and Ptf1a (G–I). Arrowheads: double-labeled cells in the outer retina. Arrows: double-labeled cells in the presumptive ganglion cell layer. Scale bar = 50 μ m.

Table 1Percentage of β -galactosidase-positive cells co-expressing the following markers

	E13.5	E15.5
PCNA	53.4% $\pm$ 6.66*	55.83% $\pm$ 1.26
Ki67	69.3% $\pm$ 3.6	62.9% $\pm$ 3.3
BrdU	10.5% $\pm$ 1	8% $\pm$ 2
PHH3	6.97% $\pm$ 1.74	7.32% $\pm$ 1.94
P27^{Kip1}	75.7% $\pm$ 2.3	78.8% $\pm$ 13
Ng2	26.1% $\pm$ 4.98	No overlap
Olig2	63.0% $\pm$ 9.73	56.7% $\pm$ 6.5
Otx2	58.77% $\pm$ 2.51	67.53% $\pm$ 6.44
Ptf1a	19.63% $\pm$ 5.46	23.26% $\pm$ 1.10
Brn3b(E12.5)	30.3% $\pm$ 3.05	Not determined

* standard deviation