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Lhx2 Balances Progenitor Maintenance with Neurogenic Output and Promotes Competence State Progression in the Developing Retina

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The LIM-Homeodomain transcription factor *Lhx2* is an essential organizer of early eye development and is subsequently expressed in retinal progenitor cells (RPCs). To determine its requirement in RPCs, we performed a temporal series of conditional inactivations in mice with the early RPC driver *Pax6 α-Cre* and the tamoxifen-inducible *Hes1*^{CreERT2} driver. Deletion of *Lhx2* caused a significant reduction of the progenitor population and a corresponding increase in neurogenesis. Precursor fate choice correlated with the time of inactivation; early and late inactivation led to the overproduction of retinal ganglion cells (RGCs) and rod photoreceptors, respectively. In each case, however, the overproduction was selective, occurring at the expense of other cell types and indicating a role for *Lhx2* in generating cell type diversity. RPCs that persisted in the absence of *Lhx2* continued to generate RGC precursors beyond their normal production window, suggesting that *Lhx2* facilitates a transition in competence state. These results identify *Lhx2* as a key regulator of RPC properties that contribute to the ordered production of multiple cell types during retinal tissue formation.

Introduction

The ordered production of multiple cell types from a single pool of progenitor cells is a common strategy used in the developing vertebrate nervous system. However, the regulation of multipotent stem and progenitor cell populations is complex, requiring robust control and coordination to ensure reproducible outcomes. The retina has long served as a model for the study of ordered cell production, as the seven major cell types (retinal ganglion cells (RGC), cone photoreceptor, horizontal, amacrine, rod photoreceptor, bipolar, and Müller glia) arise in a successive yet overlapping sequence from a common pool of multipotent retinal progenitor cells (RPCs; Turner and Cepko, 1987; Holt et al., 1988; Turner et al., 1990; Rapaport et al., 2004; Wong and Rapaport, 2009). Still unresolved, however, is how RPCs control their developmental potential to generate precursors of each cell type at the correct time and in their correct proportions.

Two dominant models have emerged: one is based on competence changes and proposes that RPCs are equipotent, but transition irreversibly through a series of intrinsically defined states during which they are capable of generating specific cell types (Cepko et al., 1996). The other is based on progressive lineage restriction, arguing for the existence of RPC cohorts with unequal fate potential, identifiable on the basis of selective gene expression (Li et al., 2004; Vitorino et al., 2009; Brzezinski et al., 2011; Hafler et al., 2012). Both models portray RPCs as predictable, yet direct observations show their behavior to be stochastic (i.e., decisions regarding division mode and fate choice cannot be predicted on the basis of history or lineage) (Gomes et al., 2011; He et al., 2012). RPCs do not behave randomly, however, as all outcomes are not chosen with equal probability. As a result, mechanisms must exist to set and modulate the relative probability of competing outcomes.

Lhx2 is used in many different tissues, with context-dependent functions including the regulation of regional identity and the maintenance of stem cell character (Rhee et al., 2006; Dahl et al., 2008; Mangale et al., 2008). It is expressed early in the developing eye and required for regionalization, patterning, and lens formation (Yun et al., 2009); consequently, eye development arrests in *Lhx2*$^{-/-}$ mice (Porter et al., 1997) and the retinal domain is never specified. *Lhx2* is also expressed in RPCs during histogenesis, and given that it regulates fate decisions elsewhere, we predicted that *Lhx2* would contribute to regulation of neurogenesis in the retina. Using *Pax6 α-Cre* and the tamoxifen-inducible *Hes1*^{CreERT2}, we performed a temporal series of

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conditional inactivations and uncovered requirements for *Lhx2* in regulating RPC maintenance, output, and competence.

Materials and Methods

Animals. The *Lhx2* conditional allele was generated by Mangale et al., (2008), *Pax6 α-Cre* was generated by Marquardt et al. (2001), and the *Hes1*^{CreERT2} knock-in allele was generated by Kopinke et al. (2011). *R26R* (Soriano, 1999) and *Ai14 tdTomato* (Madisen et al., 2010) reporter mice were obtained from The Jackson Laboratory. *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/+}$ mice were used as a control for *Hes1*$^{CreERT2/+}$;*Lhx*$^{f/-}$. *α-Cre;Lhx2*$^{f/+}$ mice were used as controls for *α-Cre;Lhx2*$^{f/-}$ mice. The *R26R* and *Ai14 tdTomato* alleles were used as heterozygotes. Embryonic age determinations were based on plug date. Tissues were collected from mice of either sex. All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee and set forth in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and when possible, the number of animals needed per analysis was kept to a minimum.

Administration of tamoxifen and 5-ethynyl-2′-deoxyuridine. Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at a concentration of 10 mg/ml. For widespread activation of Cre recombinase in *Hes1*^{CreERT2} mice, 0.1 mg TM/g body weight was administered into pregnant dams by oral gavage at various embryonic stages with 22 G 1.5 inch feeding needle. The 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) was dissolved in filtered PBS at a concentration of 10 mM and administered via intraperitoneal injection. Pregnant dams were given a single injection of 30 μg/g body weight, 24 h before sacrifice.

Immunohistochemistry. Embryo heads or eyes were dissected in HBSS or PBS and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature or 2 h on ice. Following fixation, tissue was washed twice with PBS, put through a gradient of sucrose solutions, embedded in OCT (Sakura Finetek), and stored at −80°C. Frozen tissues were sectioned on a cryostat at a thickness of 12 μm. Primary antibodies used were as follows: anti-LHX2 (Edwin Monuki, University of California, Irvine; 1:50), anti-LHX2 (Santa Cruz Biotechnology; 1:1000), anti-Calretinin (Millipore Bioscience Research Reagents; 1:1000), anti-P27 (BD Bioscience; 1:100), anti-CCND1 (Lab Vision; 1:400), anti-PCNA (DAKO; 1:500), anti-BRN3 (Santa Cruz Biotechnology; 1:50), anti-SOX2 (Abcam; 1:400), anti-AQP4 (Santa Cruz Biotechnology; 1:300), anti-RXRγ (Santa Cruz Biotechnology; 1:200), anti-NR2E3 (Anand Swaroop, National Eye Institute; 1:100), anti-BHLHB5 (Santa Cruz Biotechnology; 1:1000), anti-GABA (Sigma; 1:1000), anti-VSX2 (Exα Biologicals; 1:300), anti-VSX1 (Clark et al., 2008; 1:500), anti-SOX9 (Millipore Bioscience Research Reagents; 1:400), anti-CRALBP (John Saari, University of Washington; 1:1000), anti-GFAP (Lipshaw; 1:1000), anti-βIII-Tubulin (Covance; 1:1000), anti-PTF1A (Helena Edlund, Umea University; 1:800), anti-PTF1A (Jane Johnson, University of Texas Southwestern; 1:8000), and anti-OTX2 (Millipore Bioscience Research Reagents; 1:15,000). Primary antibodies were followed with species-specific secondary antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 (Invitrogen), or donkey anti-goat IgG conjugated with tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka). Panels showing fluorescence-based protein detection are single-scan confocal images from a Fluoview 1000 confocal microscope (Olympus).

In situ *hybridization.* Embryo heads were dissected in HBSS and fixed overnight at 4°C in 4% PFA in PBS, washed twice with PBS, put through a gradient of sucrose solutions, embedded in OCT, and stored at −80°C. Frozen tissues were sectioned on a cryostat at 12 μm in the coronal plane, and *in situ* hybridization was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Probes used in this study were digoxigenin-labeled antisense probes against *Hes1*, *Hes5*, and *Atoh7*.

Quantification and statistical analyses. Retinal thickness was calculated manually in Photoshop as the average of three separate and equally spaced measures (individual lines oriented perpendicular to the apical membrane and spanning the entire width of the retina) of each image. For lineage tracing experiments, different cell types were identified based on the expression of precursor-specific markers. The percentage of *Lhx2*-inactivated RPCs that assumed any particular fate was calculated as the number of marker-positive, reporter-positive cells over total reporter-positive cells. As E12.5 inactivation produced embryos with an asymmetric phenotype along the dorsoventral axis (see Fig. 6), only the more affected sides of each retina, as determined by hierarchical clustering, were included in our quantification of neurogenic output in Figure 5. Birth-dating experiments were quantified using a similar method: the absolute number of Pou4f+EdU+ cells was counted on the more affected sides of each experimental retina and expressed as a count per unit area. Area was calculated in Photoshop. Hierarchical clustering in Figure 6*F* was performed using Ward's method, and distance joined to form each iterative cluster is depicted in both the scale of the dendrogram as well as the scree plot underneath. For determining significance in all comparisons we used an α level of 0.05 and a two-sided Aspin–Welch–Satterthwaite–Student's *t* test, assuming unequal variance. JMP 10.0 software was used for all calculations as well as generation of the dendrogram and scree plot. All data are shown as the mean ± SE.

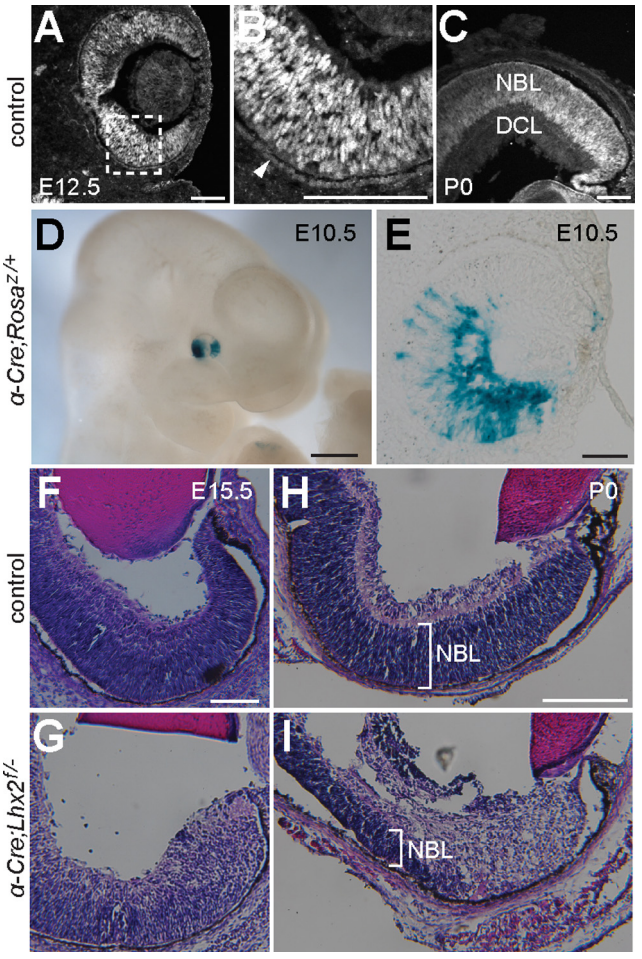

Figure 1. Lhx2 is expressed in RPCs during embryonic stages and inactivation with *α-Cre* results in profound mispatterning. ***A***, ***B***, Control sections stained for Lhx2 show expression in most if not all RPCs, as well as the retinal pigment epithelium (arrowhead; ***B*** shows boxed area in ***A***). ***C***, At later ages, expression is limited to the NBL and absent from the DCL. ***D***, ***E***, *α-Cre* drives recombination in the ventral and peripheral retina, evident by crossing *α-Cre* with *R26R* and staining with X-gal. ***F–I***, Histologic sections show that *α-Cre;Lhx2*$^{f/-}$ eyes are disorganized compared with controls. Scale bars: ***A–C***, ***E–I***, 100 μm; ***D***, 1 mm.

Results

Expression of *Lhx2* in the embryonic and postnatal retina

We found that during embryonic development Lhx2 was expressed in most if not all RPCs within the neuroblast layer (NBL) of the retina, as well as in the retinal pigment epithelium (Fig.

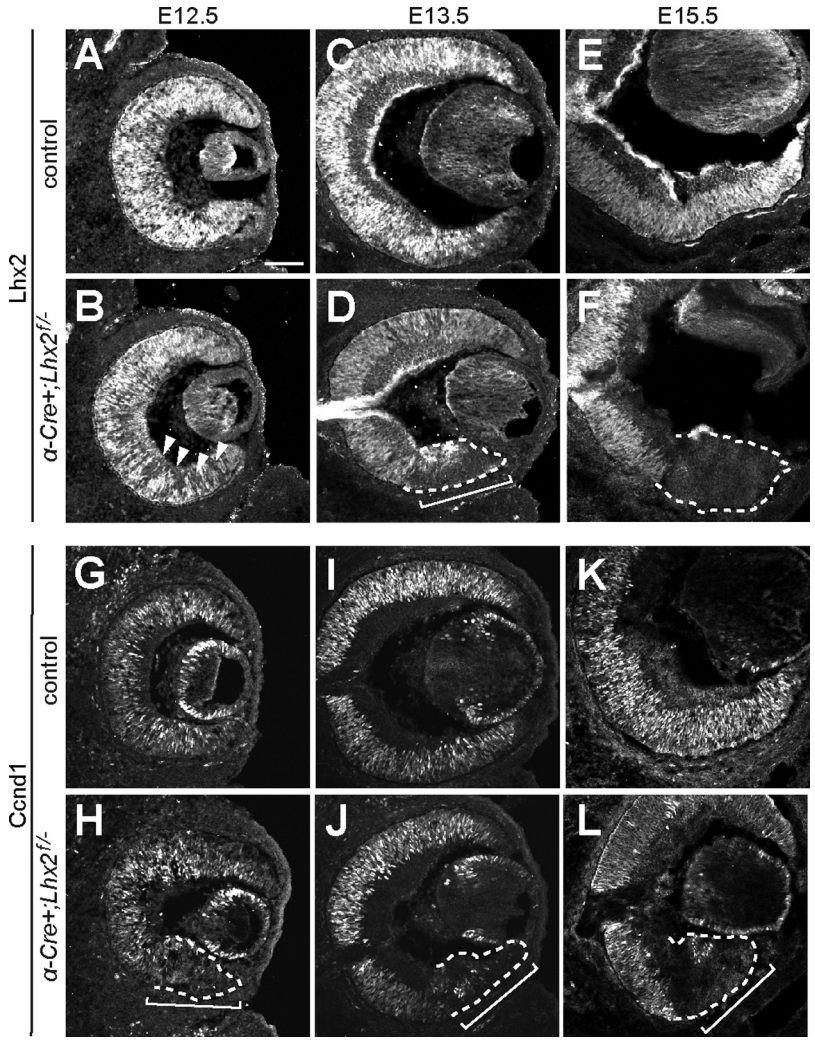

Figure 2. Loss of *Lhx2* results in the significant depletion of RPCs. ***A–L***, Sections from control and α-*Cre;Lhx2*$^{f/-}$ eyes stained with antibodies against Lhx2 (***A–F***) and Ccnd1 (***G–L***) show that loss of Lhx2 is first evident in patches (***B***, arrowheads) before becoming more obvious at later ages (dashed lines and brackets in ***D***, ***F***). Ccnd1 expression is lost in a similar manner (dashed lines and brackets in ***H***, ***J***, ***L***). Scale bar, 100 μm.

1*A*,*B*; arrowhead). Expression was lost upon differentiation, as Lhx2 was notably absent from the differentiated cell layer (DCL; Fig. 1*C*). Postnatally, expression was maintained in Müller glia and a subset of amacrine cells, indicated by colocalization with p27^{Kip1} and Calretinin, respectively (data not shown). Both embryonic and postnatal expression patterns described here correspond with previous findings in mice (Tétreault et al., 2009; de Melo et al., 2012) as well as other species (Seth et al., 2006; Viczian et al., 2006), suggesting a conserved role for *Lhx2*.

Loss of *Lhx2* depleted the RPC pool and increased neurogenesis

To bypass the essential roles of *Lhx2* during early eye development we conditionally inactivated a floxed allele of *Lhx2* (Mangale et al., 2008) using the *Pax6* α-*Cre* retinal driver (Marquardt et al., 2001), in which a retina-specific enhancer element (α) from the *Pax6* locus drives expression of Cre recombinase in the ventral peripheral retina from E10.5 onward (Kammandel et al., 1999; Marquardt et al., 2001; Fig. 1*D*,*E*). This onset of expression was appropriate for the study of neurogenic stages, as it occurs 1 d after the developmental arrest in *Lhx2*$^{-/-}$ mice and also coincides with the approximate beginning of neurogenesis. Initial inspection and subsequent H&E staining of α-*Cre;Lhx2*$^{f/-}$ eyes revealed aberrant morphology as early as E15.5 (Fig. 1*F*,*G*), with the laminar architecture of the retina disrupted and the NBL reduced in thickness by P0 (Fig. 1*H*,*I*). Loss of Lhx2 in α-*Cre;Lhx2*$^{f/-}$ eyes was observed in the ventral retina as early as E12.5 (Fig. 2*A*,*B*, arrowheads), and became more widespread by E13.5 and E15.5 (Fig. 2*C–F*, dashed lines and brackets). To assess the status of proliferative RPCs, we examined expression of *Cyclin D1* (Ccnd1), present in the majority of RPCs during neurogenesis (Barton and Levine, 2008; Das et al., 2009, 2012) (Fig. 2*G*,*I*,*K*). Ccnd1 expression was lost as early as E12.5 (Fig. 2*H*, dashed line and bracket) and almost completely absent by E13.5 (Fig. 2*J*, dashed line and bracket) and E15.5 (Fig. 2*L*, dashed line and bracket). These observations suggest that *Lhx2* is required for the maintenance and/or proliferation of RPCs during neurogenesis.

To determine whether RPCs were undergoing apoptosis, we examined the relative number of Tunel+ cells and found an increase at P0 but not E13.5 (data not shown). This suggested that *Lhx2*-inactivated RPCs were not immediately lost to cell death, but rather may have entered a quiescent state or undergone premature differentiation. As indicated by the enhanced expression of class-III β-Tubulin (Tubb3), a marker of postmitotic differentiating neurons, *Lhx2*-inactivated RPCs appeared to precociously differentiate (Fig. 3*A–F*). Within the ventral retina, Tubb3+ cells were observed ahead of the normal wave of neurogenesis at E12.5 (Fig. 3*B*, arrowheads) and present in increased numbers at later time points (Fig. 3*D*,*F*), consistent with the loss of Lhx2 in that region (Fig. 2*B*,*D*,*F*). Together, the depletion of RPCs and reciprocal increase in neurogenesis suggested that *Lhx2* normally plays a role in promoting the maintenance of RPCs.

Lhx2 inactivation at E10.5 resulted in selective overproduction of RGCs

To identify the fates of *Lhx2*-inactivated RPCs, we examined markers associated with precursor fate selection. An antibody against the RGC-specific markers *Pou4f1*, *2*, and *3* (hereafter referred to as *Pou4f*) showed a pattern similar to that of Tubb3 in α-*Cre;Lhx2*$^{f/-}$ eyes: Pou4f+ cells were observed ahead of the normal wave of neurogenesis at E12.5, again confined to the inactivated region of the ventral retina (Fig. 3*H*, arrowheads). At later time points (E15.5, P0) Pou4f+ cells were more abundant (Fig. 3*I–L*) and widely distributed while the NBL was much thinner (Fig. 3*K*,*L*, brackets), supporting the notion that ectopic RGCs were produced at the expense of the RPC pool. To determine whether *Lhx2* was acting upstream of genes important for RGC specification, we examined expression of *Atoh7* (formerly *Math5*) and *Hes1*. *Atoh7* is a proneural bHLH transcription factor, necessary yet not suf-

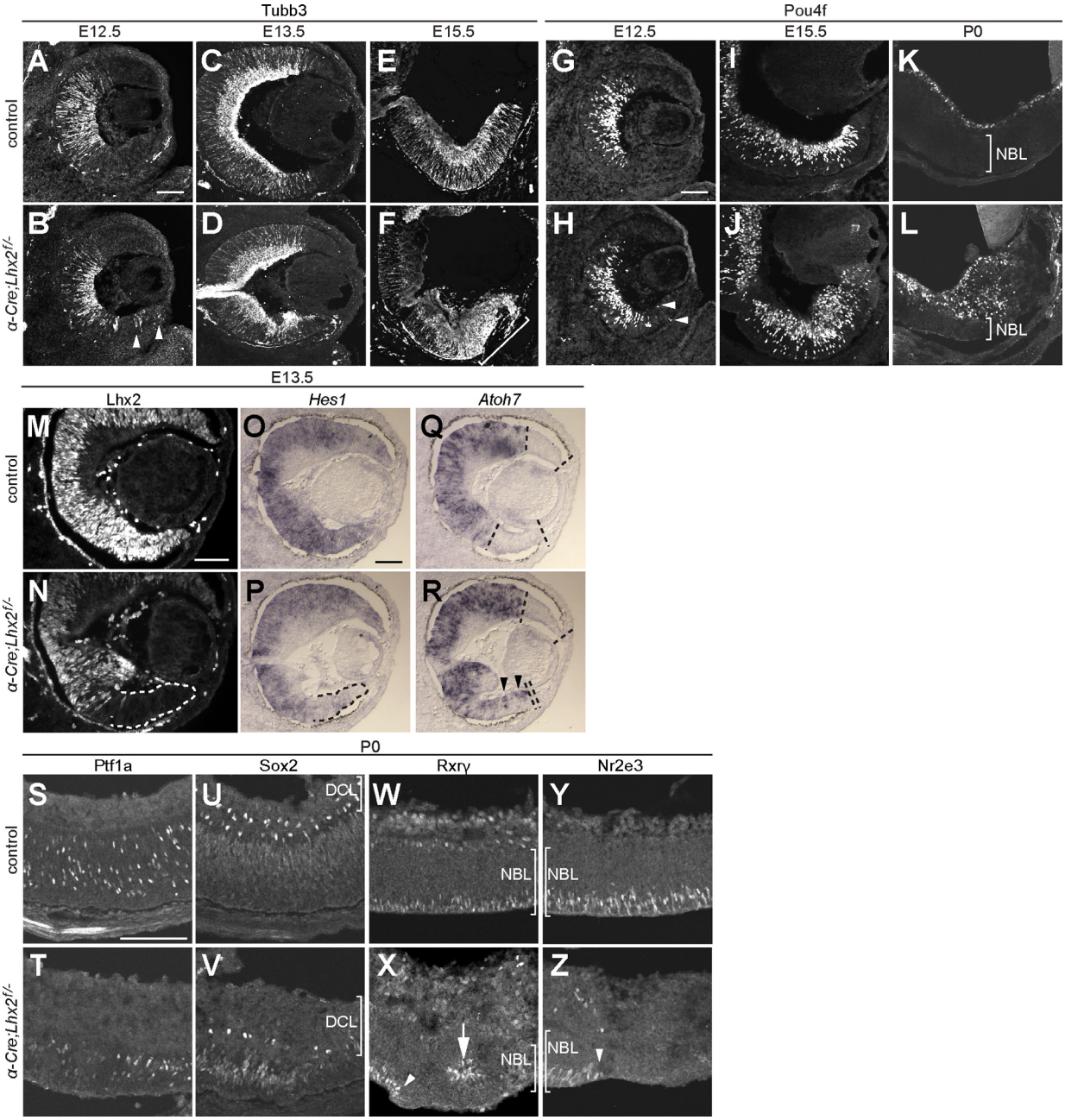

Figure 3. Inactivation of *Lhx2* with α-*Cre* results in the selective overproduction of RGCs. ***A–L***, Sections from control and α-*Cre;Lhx2*$^{f/-}$ eyes stained with antibodies against Tubb3 (differentiated cells, ***A–F***) and Pou4f (RGC precursors, ***G–L***) show that premature (arrowheads in ***B***, ***H***) and increased neurogenesis in the ventral retina is accounted for by RGC precursors and occurs at the expense of RPCs (brackets in ***K***, ***L***). ***M–R***, Immunostaining and *in situ* hybridization demonstrate that the loss of Lhx2 (dashed line in ***N***) is accompanied by loss of *Hes1* (dashed line in ***P***) and precocious *Atoh7* expression (arrowheads, dashed lines in ***Q***, ***R***). ***S–Z***, Immunostaining for Ptf1a (horizontal and amacrine cell precursors, ***S***, ***T***), Sox2 (amacrine cell precursors, ***U***, ***V***), Rxrγ (cone precursors, ***W***, ***X***), and Nr2e3 (rod precursors, ***Y***, ***Z***) demonstrates that RGC overproduction coincides with the underproduction of other early born and later born precursor types. ***X***, ***Z***, Arrowheads mark the presumptive boundary of recombination; arrow highlights cone precursors organized into a rosette. Scale bars: 100 μm.

ficient for RGC formation (Brown et al., 1998, 2001). *Hes1* is another bHLH transcription factor that acts upstream of *Atoh7* to inhibit differentiation, and *Hes1* mutants show precocious activation of *Atoh7* and increased RGC production (Lee et al., 2005; Riesenberg et al., 2009). We examined α-*Cre;Lhx2*$^{f/-}$ retinas at E13.5, when normal *Atoh7* expression has not yet reached the periphery, giving us the opportunity to observe precocious activation. α-*Cre;Lhx2*$^{f/-}$ eyes showed the expected changes: loss of Lhx2 (Fig. 3*M*,*N*, dashed line) and *Hes1* (Fig. 3*O*,*P*, dashed line) along with precocious *Atoh7* expression (Fig. 3*Q*,*R*, arrowheads). These results demonstrate that *Lhx2* acts at or above the level of *Hes1*, itself an important target of several signaling pathways known to promote the maintenance of progenitor and/or stem cell populations.

In contrast to the change in Pou4f+ RGC precursors, precursors of other cell types were noticeably decreased in the ventral retina at P0. This included Ptf1a+ horizontal and amacrine cell precursors (Fig. 3*S*,*T*), the Sox2+ subset of amacrine cells in the DCL (Fig. 3*U*,*V*), Rxrγ+ cone photoreceptors in the NBL (Fig. 3*W*,*X*), and Nr2e3+ rod photoreceptors in the NBL (Fig. 3*Y*,*Z*). While underproduction of the later born rod

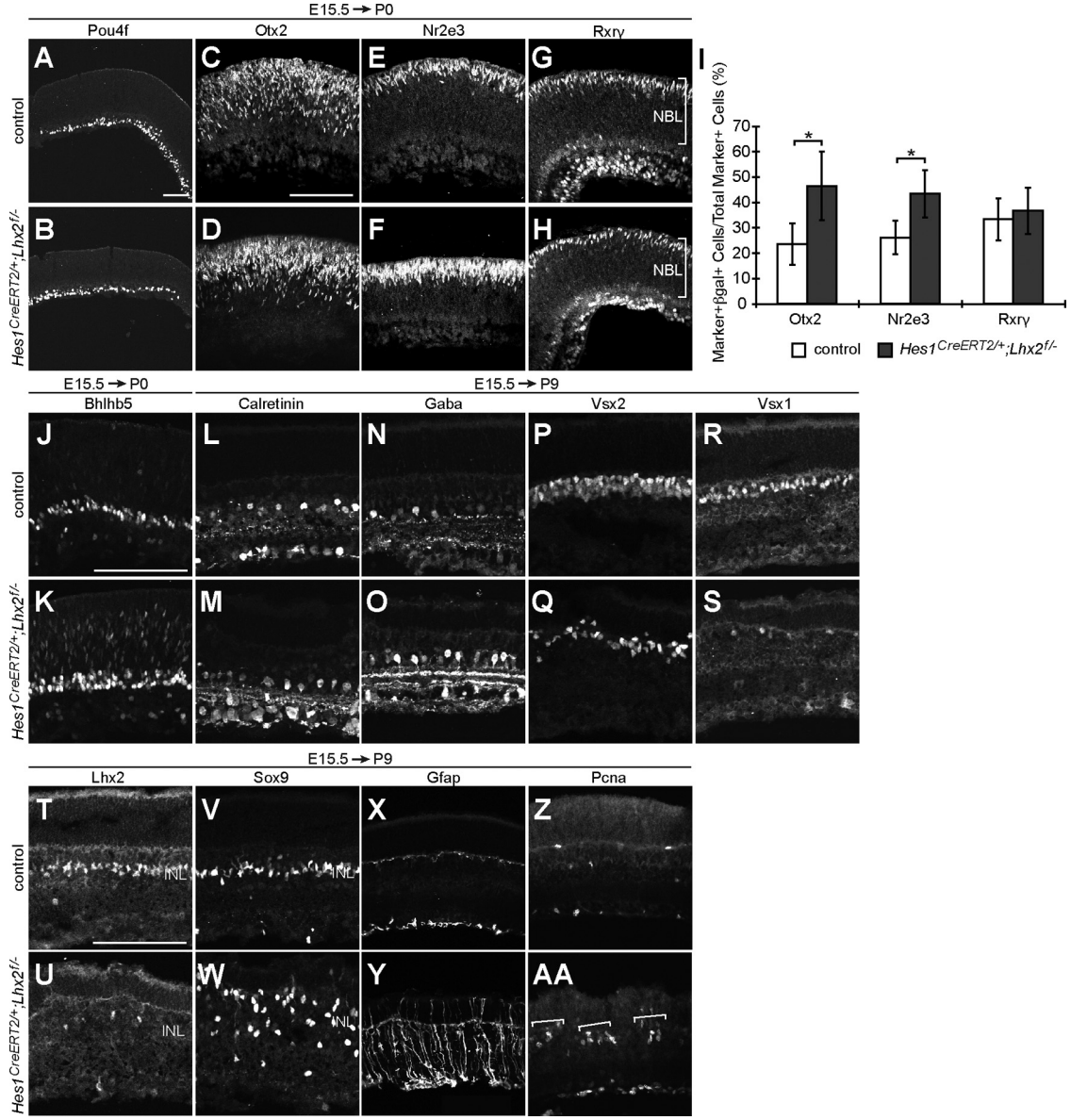

Figure 4. Inactivation of *Lhx2* with $Hes1^{CreERT2}$ at E15.5 results in the overproduction of rods. ***A–H***, Sections from control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes stained with antibodies against Pou4f (RGC precursors, ***A***, ***B***), Otx2 (photoreceptor precursors, ***C***, ***D***), Nr2e3 (rod precursors, ***E***, ***F***), and Rxrγ (cone precursors, ***G***, ***H***). ***I***, The number of RGC precursors is unchanged, while the number of photoreceptor precursors in the NBL is increased due to the selective overproduction of rods (*$p = 0.0007$ and *$p = 0.0004$, respectively; $n = 3$ mice for each genotype; $n > 200$ marker+ cells for each genotype). ***J–O***, Immunostaining for Bhlhb5 (***J***, ***K***), Calretinin (***L***, ***M***), and Gaba (***N***, ***O***) show that amacrine cell precursors are slightly increased. ***P–S***, Immunostaining for Vsx2 (***P***, ***Q***) and Vsx1 (***R***, ***S***) show that bipolar cells are decreased. ***T–W***, At P9, Lhx2, predominantly expressed in Müller glia, is largely absent in the $Hes1^{CreERT2/+};Lhx2^{f/-}$ retina (***T***, ***U***). Müller glia are still present, but disorganized, as indicated by Sox9 (***V***, ***W***). ***X–AA***, Immunostaining for Gfap (***X***, ***Y***) and Pcna (***Z***, ***AA***) show that the *Lhx2*-inactivated Müller glia are reactive and proliferative. Time points above each part indicate the time of inactivation and analysis, respectively. Scale bars:100 μm. INL, inner nuclear layer.

photoreceptor precursors could arise indirectly from RPC depletion, the underproduction of early born cell types demonstrates a selective effect on fate choice, as their production windows normally overlap with that of RGCs. These observations suggest that *Lhx2* has an important role in not only maintaining the RPC population, but also in promoting the generation of several early born precursor types by normally limiting RGC precursor production.

Lhx2 inactivation at E15.5 resulted in overproduction of later born cell types, without affecting RGCs

To determine whether *Lhx2* regulates the production of diverse fates during later stages of retinal neurogenesis, we used $Hes1^{CreERT2}$, a knock-in allele in which tamoxifen-dependent CreERT2 is expressed under the regulatory control of the *Hes1* gene locus (Yun et al., 2009; Kopinke et al., 2011). Confirming the results obtained with α-*Cre*, administration of tamoxifen at E10.5 resulted in RPC depletion and RGC overproduction; however, the phenotype was observed throughout the retina, consistent with the broad domain of recombinase activity (data not shown). We next shifted the time of inactivation to E15.5, when the peak of RGC production has passed and rod and amacrine cell precursors are the predominant cells produced. In examining $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes at P0, the profile of Pou4f+ RGCs was not altered (Fig. 4*A*,*B*). Instead, the number of Otx2+ photoreceptor precursors had increased (Fig. 4*C*,*D*). This was due pri-

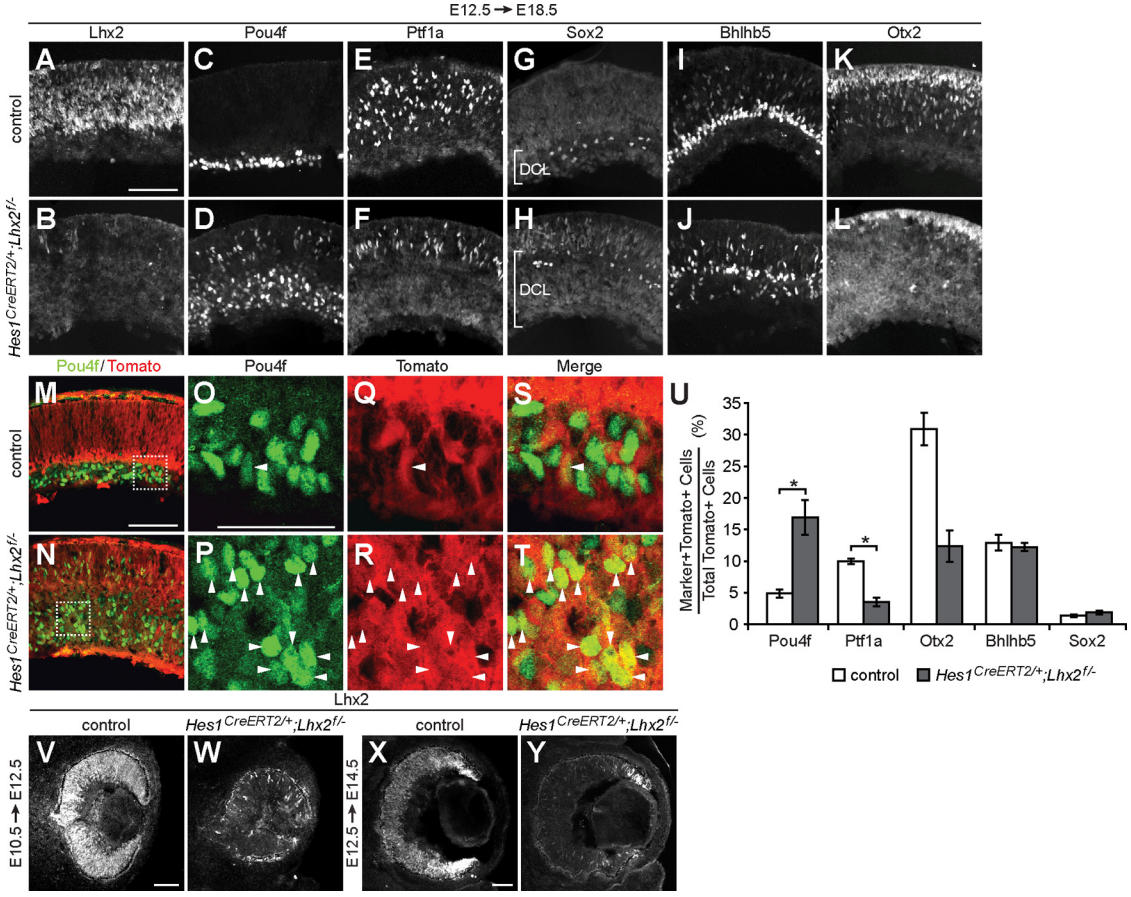

Figure 5. Inactivation of *Lhx2* at E12.5 does not result in overproduction of other early born cell types. ***A–L***, Sections from control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes stained with antibodies against Lhx2, Pou4f (RGC precursors, ***C***, ***D***), Ptf1a (horizontal and amacrine cell precursors, ***E***, ***F***), Sox2 (amacrine cell precursors, ***G***, ***H***), Bhlhb5 (amacrine cell precursors, ***I***, ***J***), and Otx2 (photoreceptor precursors, ***K***, ***L***) show that, similar to inactivation at E10.5, RGC precursors are selectively overproduced while other early born cell types are unchanged or decreased in number. ***M–T***, The progeny of *Lhx2*-inactivated RPCs are more likely to adopt the RGC fate and subsequently express the precursor marker Pou4f (arrowheads, ***O–T***). Tomato expression marks the recombined population in both control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes (***O***, ***Q***, and ***S*** show the boxed area in ***M***; ***P***, ***R***, and ***T*** show the boxed area in ***N***). ***U***, The percentage of the recombined population expressing Pou4f+ is significantly increased while the percentages expressing Ptf1a+ and Otx2+ are decreased (*$p = 0.0429$, *$p = 0.0021$, and $p = 0.066$, respectively; $n = 3$ mice for each genotype; $n > 1000$ Tomato+ cells for each experiment). ***V–Y***, Lhx2 inactivation proceeds with similar kinetics whether initiated at E10.5 (***V***, ***W***) or E12.5 (***X***, ***Y***). In each case, only a few Lhx2+ cells remain after 2 d. Error bars indicate SEM. Scale bars: 100 μm.

marily to enhanced rod precursor production, as revealed by the increased expression of Nr2e3 (Fig. 4*E*,*F*), and minimal effect, if any, on cone precursor production, as revealed by Rxrγ expression (Fig. 4*G*,*H*). We confirmed these observations by using the *R26R* recombination reporter (Soriano, 1999) to mark the progeny of *Lhx2*-inactivated RPCs. Indeed, inactivated RPCs contributed a significantly higher percentage of cells to the Otx2+ and Nr2e3+ precursor populations in $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes (Fig. 4*I*). The relative abundance of cells expressing amacrine cell markers at both P0 and P9 was generally unchanged or modestly increased (Fig. 4*J–O*). Bipolar cells, however, were severely reduced in number as indicated by Vsx1 and Vsx2 (formerly Chx10; Fig. 4*P–S*). We expected a similar reduction in the number of Müller glia, yet while expression of Lhx2 in the inner nuclear layer was expectedly absent due to inactivation (Fig. 4*T*,*U*), their numbers remained relatively unchanged at P9 as assessed with Sox9 (Fig. 4*V*,*W*). Instead, Müller glia were proliferative and reactive as indicated by Pcna (Fig. 4*Z*,*AA*), Gfap (Fig. 4*X*,*Y*), and the displacement of Sox9+ cells (Fig. 4*V*,*W*). These effects on Müller glia are similar to the recently reported inactivation of *Lhx2* in adult Müller glia, with the exception that evidence of proliferation was not observed (de Melo et al., 2012). This difference could be due to a nonautonomous response by the Müller glia to the alterations in retinal histogenesis caused by *Lhx2* inactivation in E15.5 RPCs. Regarding neural cell types, however, inactivation at E10.5 and E15.5 together demonstrate that *Lhx2* regulates diversity and ensures that the major cell types of the retina are generated in their correct proportions.

RGC overproduction predominates after *Lhx2* inactivation at E12.5

To test further the temporal dependence of cell-type production in response to *Lhx2* inactivation, we shifted the tamoxifen treatment time to E12.5 and determined the neurogenic output at E18.5, a time chosen to allow adequate recombination and precursor generation, yet also to avoid the complicating increase in cell death observed at P0 in α-$Cre;Lhx2^{f/-}$ eyes. Since production of horizontal, cone, and amacrine cell precursors is underway at E12.5, we predicted that *Lhx2* inactivation at this stage would result in the overproduction of one or more of these early born precursors, all previously underproduced after inactivation at E10.5. However, Pou4f+ RGC precursors were overproduced (Fig. 5*C*,*D*) and these other early born cell types were again present in similar or decreased amounts (Fig. 5*E–L*). We quantified these changes by using the $Rosa^{tdTomato}$ (Tomato) recombination reporter (Madisen et al., 2010) to mark the progeny of *Lhx2*-inactivated RPCs and count the number of these Tomato+ cells that went on to express different precursor markers. Thus, we

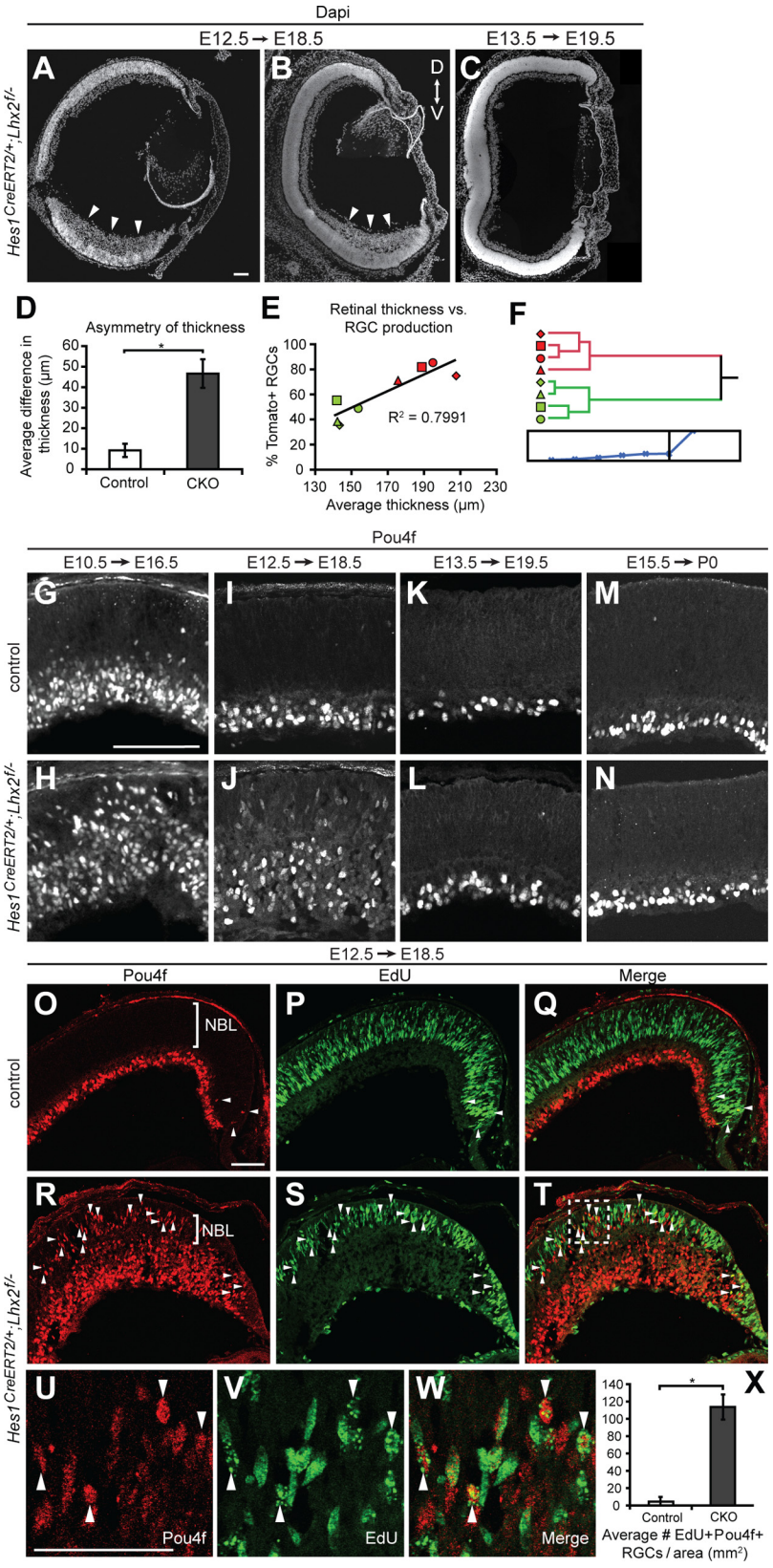

Figure 6. *Lhx2* affects the production of early born cell types during a critical window, and if inactivated during that window, prevents a transition in competence state. ***A–C***, *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/-}$ sections stained with DAPI display an asymmetric phenotype after E12.5 (arrowheads), but not E13.5, inactivation. ***D***, ***E***, This difference in retinal thickness is significant when quantified (*$p = 0.0059$; $n \geq 3$ for each genotype) and correlates with increased RGC production (different sides of the same retina are indicated with similar shapes). ***F***, Hierarchical clustering also demonstrates the existence of two distinct groups (indicated in color; joining distance at each step is represented on the *x*-axis of the dendrogram and the *y*-axis of the scree plot underneath). ***G–N***, Immunostaining for Pou4f shows that RGC precursor production after inactivation at E12.5 and E13.5 resembles earlier (E10.5) and later (E15.5) phenotypes, respectively. ***O–X***, Immunostaining for Pou4f and detection of EdU shows that RGC precursor production is largely complete at E18.5 in control eyes. In *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/-}$ eyes there is a significant number of newly specified RGCs precursors (arrowheads, EdU+Pou4f+) evident in the NBL (***U–W*** show the boxed area in ***T***; *$p = 0.0108$; $n = 3$ mice for each genotype). Error bars indicate SEM. Scale bars: 100 μm.

determined the relative change in production of different precursors in this population. This revealed a significant increase in the percentage of Pou4f+ RGCs (Fig. 5*M–T*) and reductions in the percentages of Ptf1a+ horizontal/amacrine precursors and Otx2+ photoreceptor precursors. Both Bhlhb5+ and Sox2+ amacrine cell precursors were produced in similar proportion (Fig. 5*U*). To address the possibility that the similarities in neurogenic output between the E10.5 and E12.5 inactivations were due to a delay in Lhx2 downregulation in the E10.5 experiment, we examined Lhx2 expression 48 h after tamoxifen exposure at E10.5 or E12.5. In each case, Lhx2 downregulation was exhaustive (Fig. 5*V–Y*), demonstrating that the similarities in the phenotypes at the two ages were not due to different kinetics of Lhx2 downregulation. These results suggest that RPCs are similar in potential at both E10.5 and E12.5, arguing for a small number of broadly defined competence states and a role for *Lhx2* in ensuring that the different fates available at any given time are produced in the correct proportions.

Lhx2 regulates the RGC competence window

Unexpectedly, *Lhx2* inactivation at E12.5 produced an asymmetric phenotype, obvious through differences in total retinal thickness (Fig. 6*A*,*B*,*D*) that correlated with RGC overproduction (Fig. 6*E*). Analysis of eyes in which axial orientation was maintained revealed that the severely affected region was always located ventrally (Fig. 6*B*). This asymmetry was not due to differential effects on Lhx2 expression since downregulation was largely complete along the extent of the dorsoventral axis within 48 h of tamoxifen exposure (Fig. 5*X*,*Y*). When the two sides of *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/-}$ retinas (separated by the optic nerve head) were treated as individual samples and graphed according to thickness and RGC production, they consistently segregated into two distinct groups (Fig. 6*E*), confirmed by hierarchical clustering analysis (Fig. 6*F*).

Since neurogenesis in the mouse retina initiates dorsally and lags behind in the ventral retina for the first few days (Hufnagel et al., 2010), we suspected that the asymmetric phenotype reflected a temporal shift in which RPCs move away from a dependence on *Lhx2* to limit RGC production. Indeed, the asymmetric phenotype was not observed when tamoxifen treatment was done at E13.5 (Fig. 6*C*), and similar to inactivation at E15.5, the production of RGCs was modestly altered (Fig. 6*G–N*). Since the production of early born cell types including RGCs continues past E13.5, our data reveals that the dependence of RPCs on *Lhx2* for controlling the generation of early born precursors ends before their production windows close.

While these observations suggest that *Lhx2* limits RGC production during an early window of retinal neurogenesis, ectopic RGCs were consistently observed in the NBL at E18.5 in mice treated with tamoxifen at E10.5 or E12.5. Since RGC precursors are initially specified in the NBL, this suggested that RGCs were being generated after the close of their normal production window. To test this, a short-term birth-dating assay was performed: tamoxifen was administered at E12.5 and EdU was administered at E17.5 to mark dividing cells. Retinas were harvested at E18.5 and stained for EdU and Pou4f; double-labeled cells were presumably RGC precursors specified between E17.5 and E18.5. Control retinas never showed more than a few double-labeled cells, confined to the extreme periphery and consistent with the near completion of RGC production (Fig. 6*O–Q*, arrowheads). In contrast, a significant number of newly generated RGC precursors were located throughout the *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/-}$ retinas, both centrally and peripherally (Fig. 6*R–X*, arrowheads). Together, these observations reveal a critical period, from approximately E10.5 to E12.5, in which *Lhx2* is required to constrain RGC production. But, if *Lhx2* is inactivated during this critical period, RPCs continue producing RGCs and are less efficient at progressing to the next phase of histogenesis. This latter finding suggests that *Lhx2* contributes to the progression in competence state that restricts the production of early generated cell types and allows for the production of later generated cell types.

Not all RPCs require *Lhx2* to prevent premature differentiation

Conditional inactivation of *Lhx2* consistently led to a depletion of RPCs, yet neu-

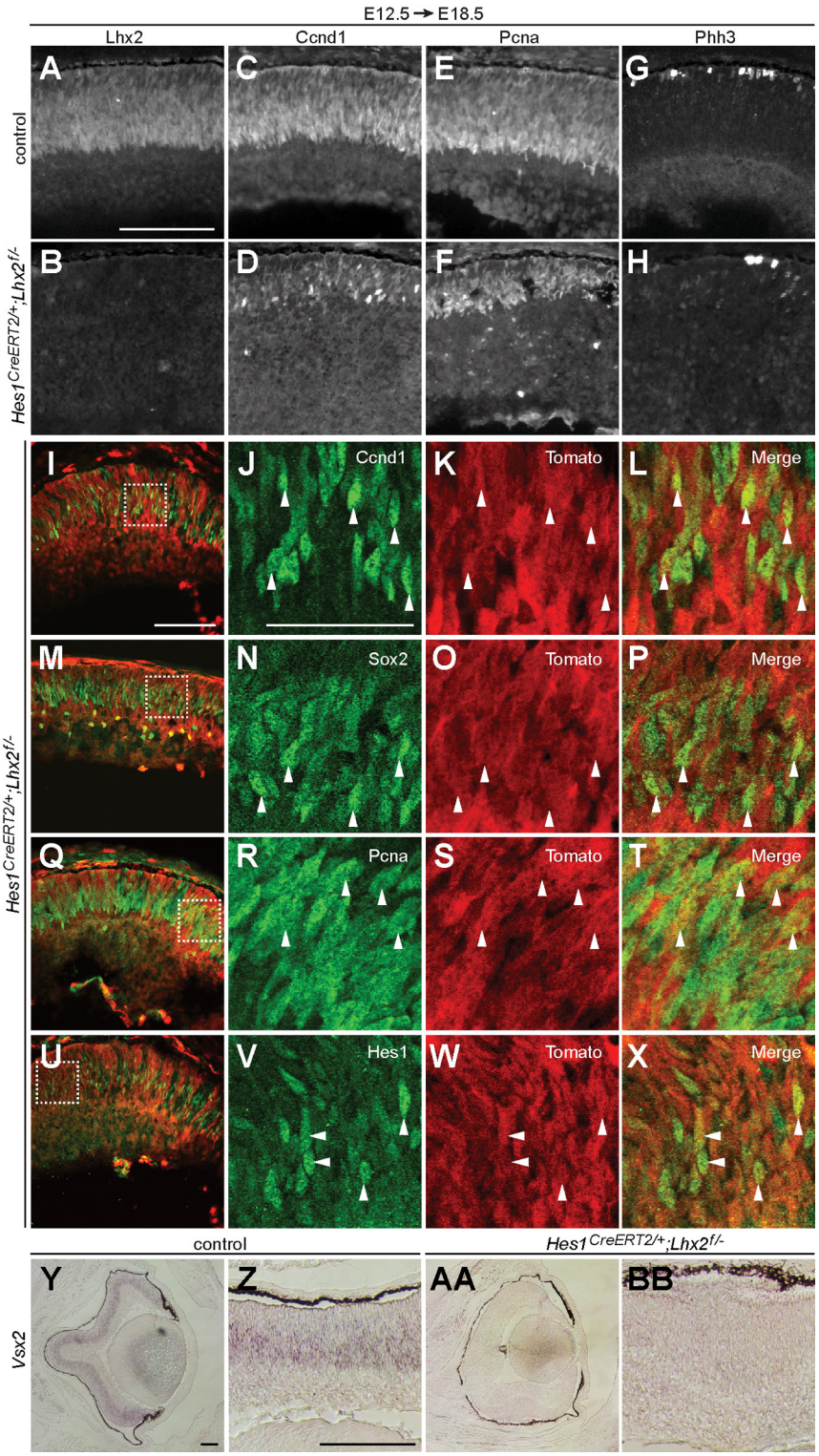

Figure 7. Many progenitors continue to proliferate despite loss of *Lhx2*. ***A–H***, Sections from control and *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/-}$ eyes stained with antibodies against Lhx2 (***A***, ***B***), Ccnd1 (***C***, ***D***), Pcna (***E***, ***F***), and Phh3 (***G***, ***H***) show that while Lhx2 expression is almost completely lost, proliferation markers are still expressed. ***I–X***, Immunostaining for Ccnd1 (***I–L***), Sox2 (***M–P***), Pcna (***Q–T***), and Hes1 (***U–X***) show that *Lhx2*-inactivated cells marked by Tomato expression continue to express both proliferative and progenitor markers (***J–L***, ***N–P***, ***R–T***, and ***V–X*** show boxed areas in ***I***, ***M***, ***Q***, and ***U***, respectively; arrowheads denote examples). ***Y–BB***, RPCs that do remain are still affected, as *Vsx2* expression is completely lost. Scale bars: 100 μm.

rogenesis continued after inactivation (Fig. 6*O–X*), suggesting that some RPCs persisted. It is possible that these RPCs escaped recombination. Alternatively, *Lhx2* may not be used in the same manner in all RPCs. To identify the more likely explanation, we inactivated *Lhx2* at both E10.5 (data not shown) and E12.5 (Fig. 7), in each case examining the RPC population 6 d later. While we observed near-complete loss of Lhx2 (Fig. 7*A*,*B*), reductions in Ccnd1, Pcna, and Phh3 were less severe (Fig. 7*C–H*). This disparity suggested that a cohort of RPCs continued to proliferate despite their loss of *Lhx2*. To directly demonstrate this, we used the Tomato reporter to track recombined RPCs. As expected, several Tomato+ cells in the NBL also expressed markers of proliferation (Ccnd1 and Pcna; Fig. 7*I–L*,*Q–T*) or progenitor status (Sox2 and Hes1; Fig. 7*M–P*, *U–X*). This demonstrates that a subset of RPCs do not require *Lhx2* to maintain their status as proliferating progenitors. Two pieces of evidence suggest, however, that the RPCs remaining after *Lhx2* inactivation were indeed altered. First, expression of the RPC gene *Vsx2* was downregulated throughout the retina (Fig. 7*Y–BB*), consistent with its strong dependence on *Lhx2* activity for its expression in RPCs (Yun et al., 2009). Second, the persistent RPCs produced RGCs after the normal RGC production window closed (Fig. 6*O–X*). Together, these findings argue for the coexistence of at least two cohorts of RPCs that differ in regard to their dependence on *Lhx2* for their maintenance but not for their neurogenic output.

Discussion

The vertebrate retina is a complex and ordered tissue with many constituent cell types, all of which arise from a single population of multipotent progenitor cells. The actions of individual RPCs display surprisingly little order and predictability, yet order is clearly present at the population level. How is this achieved? Though RPCs behave stochastically, this likely occurs within a framework where the number, type, and probability of different outcomes are regulated. Here we have used the inducible *Hes1*^{CreERT2} allele to demonstrate a complex role for *Lhx2* in regulating this framework by promoting RPC maintenance, regulating the likelihood of competing or alternative fate choices, and allowing for a shift in competence state.

At every age examined, the conditional inactivation of *Lhx2* resulted in a significant depletion of the RPC population and an increase in neurogenesis. This suggested that *Lhx2* normally serves to increase the probability of self-renewal, resulting in maintenance of the RPC population. However, this depletion was never complete: many *Lhx2*-inactivated RPCs continued to proliferate and express progenitor markers well after inactivation. This suggested the presence of more than one cohort of RPCs, differing in regard to their requirement for *Lhx2*. Such a scenario may be explained by *Lhx2*-dependent and *Lhx2*-independent cohorts of RPCs that coexist but develop in parallel. Another possibility is that *Lhx2* has multiple roles in RPCs at different times during their normal developmental progression. We favor the latter possibility, as our results show that RPCs that do not immediately exit the cell cycle are still affected in terms of gene expression and neurogenic output. This is also consistent with our previous work examining *Ccnd1*$^{-/-}$ mutant mice, which demonstrated a significant yet incomplete depletion of RPCs and selective overproduction of RGCs (Das et al., 2009). Both *Ccnd1*$^{-/-}$ and *Lhx2* conditional inactivation phenotypes suggest the presence of distinct steps in RPC progression. Thus, we propose the following model (Fig. 8): at any given time, a subset of RPCs are specified to differentiate on the basis of stochastic differences and/or signals received. These RPCs then become limited in proliferative capacity and biased toward the generation of certain cell types, reflecting their competence at the time. The specification of these "biased" RPCs is an iterative process, such that as they exit the cell cycle,

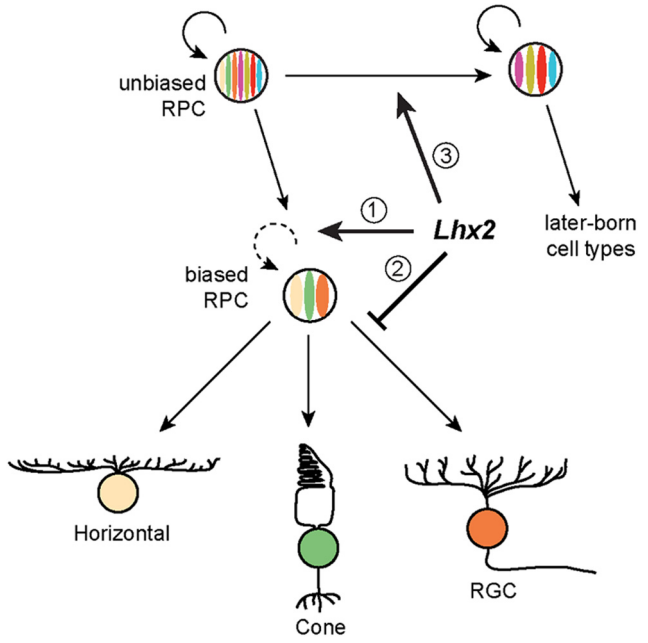

Figure 8. Model of *Lhx2* function in RPCs. While all RPCs are presumably competent to undergo differentiation, some RPCs do so on the basis of stochastic differences and/or signals received, and become biased toward both neurogenic divisions as well as the production of certain fates (a reflection of their competence at the time). We hypothesize that *Lhx2* acts within this set of biased RPCs to not only increase the probability of proliferative divisions (arrow 1), but to ensure that different precursors are produced in the correct proportions by limiting the production of RGC precursors (arrow 2). This process is likely to be reiterated later in retinal development. In addition, *Lhx2* impacts unbiased RPCs by regulating competence progression during a limited temporal window (before ~E13.5; arrow 3), and possibly proliferation through its regulation of *Vsx2* and other factors yet to be identified. Colors in RPCs depict their potential to generate fated precursors.

they are replaced from an upper pool of "unbiased" RPCs. Our findings suggest that RPCs that require *Lhx2* or *Ccnd1* for their immediate maintenance may represent cohorts of this biased RPC population (Fig. 8, arrow 1). There is precedence for such a model of progenitor progression both in the retina (Brzezinski et al., 2011; Hafler et al., 2012) and elsewhere, as biased RPCs in our model are analogous to both intermediate neural progenitors generated from stem-like radial glia in the cortex (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) as well as ganglion mother cells generated from *Drosophila* neuroblasts (Isshiki et al., 2001).

Inactivation of *Lhx2* during both early and late phases of neurogenesis resulted in the selective overproduction of certain cell types, occurring at the expense of others. This suggested that RPCs are competent to generate more than one cell type at a time, an idea supported by the observation of discordant two-cell clones in many different lineage studies (Turner and Cepko, 1987; Wong and Rapaport, 2009; Brzezinski et al., 2011; Hafler et al., 2012). However, RPCs do not behave randomly when choosing between fates—some are chosen more often than others. Mechanisms must exist to provide bias, and our results suggest that *Lhx2* is a key player in such a mechanism(s) (Fig. 8, arrow 2). Accordingly, both negative and positive feedback signals affect the generation of specific cell types and promote diversity (Reh and Tully, 1986; Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Belliveau and Cepko, 1999; Kim et al., 2005; Wang et al., 2005). Notch signaling introduces differences between otherwise equivalent cells, and unique components of the pathway (ligands, receptors, effectors) regulate the generation of specific cell types (Jadhav et al., 2006; Yaron et al., 2006; Riesenberg et al., 2009). Asymmetric inheritance of the Notch antagonist *Numb* acts generally to promote asymmetric terminal divisions, with

daughter cells assuming different fates (Kechad et al., 2012). *Lhx2* may regulate diversity through interaction with one or more of these pathways, as loss of *Lhx2* reduces the relative probability of assuming different fates.

In addition to demonstrating that *Lhx2* regulates the generation of distinct cell types during early and late phases of neurogenesis, temporal analysis allowed us to locate the approximate time at which this change in regulation occurs. The different phenotypes seen after earlier (E10.5, E12.5) versus later (E13.5, E15.5) inactivations are indicative of a normal shift in competence among the RPC population. Early loss of *Lhx2* affects competence by preventing this transition, yet later inactivation does not. And while certain cell types are selectively overproduced in both cases, the other cell types are not completely lost. Thus, *Lhx2* regulates the shift between competence states rather than actually conferring and/or limiting competence (Fig. 8, arrow 3). How *Lhx2* does this is not yet clear. One possibility is that it regulates the expression of *Ikaros*, a *Hunchback* ortholog that confers competence to RPCs to generate early born cell types (Elliott et al., 2008). Other possibilities are that *Lhx2* interacts with the Dicer or Sonic Hedgehog (Shh) pathways. The *Dicer* and *Shh* conditional mutants (Wang et al., 2005; Georgi and Reh, 2010) produced phenotypes similar to *Lhx2* inactivations initiated at E12.5 and earlier. As *Shh* also operates as a negative feedback signal for controlling RGC precursor production (Zhang and Yang, 2001; Wang et al., 2005; Sakagami et al., 2009), *Lhx2* may regulate neurogenic output and competence progression through interaction with a single pathway.

Lhx2 acts as a selector gene necessary for autonomous specification of regional identity in the cortex (Bulchand et al., 2001; Mangale et al., 2008; Chou et al., 2009), and similar roles may exist in the developing amygdala and pituitary (Remedios et al., 2004; Zhao et al., 2010). Correspondingly, *Lhx2* participates in eye-field formation (Zuber et al., 2003; Tétreault et al., 2009; Hägglund et al., 2011) and is required for regionalization of the optic vesicle through cell-autonomous regulation of gene expression (Yun et al., 2009). At a cellular level, the function of *Lhx2* is context dependent. In the olfactory epithelium, hippocampus, and thalamus *Lhx2* promotes neurogenesis as well as the maturation and axonal outgrowth of postmitotic neurons (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Lakhina et al., 2007; Saha et al., 2007; Peukert et al., 2011; Sanuki et al., 2011; Subramanian et al., 2011; Berghard et al., 2012; Marcos-Mondéjar et al., 2012). In the cortex, hematopoietic lineage, and hair follicle, *Lhx2* promotes progenitor and stem-cell maintenance, preventing premature differentiation (Rhee et al., 2006; Dahl et al., 2008; Kitajima et al., 2011; Chou and O'Leary, 2013). In addition to cellular context, several of these functions are limited to a critical period and thus very dependent on timing (Mangale et al., 2008; Chou et al., 2009; Subramanian et al., 2011).

In the present study we have demonstrated that *Lhx2* is required for the maintenance of RPCs during retinal neurogenesis, consistent with a role in stem and/or progenitor cell maintenance and self-renewal. Importantly, the ability to induce inactivation at multiple time points has allowed us to gain additional insight into the function of *Lhx2*, and our results are significant in several different regards: First, we have reported a *selective* role for *Lhx2* in progenitor maintenance, indicative of heterogeneity within the progenitor population. Second, we have reported a function for *Lhx2* in regulating neuronal *diversity* rather than the general promotion or prevention of neurogenesis. Last, we have reported a function for *Lhx2* in regulating competence progression, a fundamental property of progenitors that allows for ordered cell production in multiple tissues. In line with *Lhx2*'s requirements in promoting early eye development, these results position *Lhx2* as an intrinsic factor essential for coordinating multiple aspects of retinal development.

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