Ldb1 and Rnf12-dependent regulation of Lhx2 controls the relative balance between neurogenesis and gliogenesis in retina.

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Abstract

Precise control of the relative ratio of retinal neurons and glia generated during development is essential for visual function. We show that Lhx2, which encodes a LIM-homeodomain transcription factor essential for specification and differentiation of retinal Müller glia, also plays a critical role in the development of retinal neurons. Overexpression of Lhx2 with its transcriptional coactivator Ldb1, triggers cell cycle exit and inhibits both Notch signaling and retinal gliogenesis. Lhx2/Ldb1 overexpression also induced the formation of wide-field amacrine cells (wfACs). In contrast Rnf12, which encodes a negative regulator of LDB1, is necessary for the initiation of retinal gliogenesis. We also show that Lhx2-dependent neurogenesis and wfAC formation requires Ascl1 and Neurog2, and that Lhx2 is necessary for their expression, although overexpression of Lhx2/Ldb1 does not directly elevate expression of these proneural bHLH factors. Finally, we demonstrate that the relative level of the LHX2-LDB1 complex in the retina decreases in tandem with the onset of gliogenesis. These findings show
that control of Lhx2 function by Ldb1 and Rnf12 underpins the coordinated differentiation of neurons and Müller glia in postnatal retina.

**Significance statement**

Cofactor-dependent regulation of the LIM homeodomain factor Lhx2 controls the relative balance of neurogenesis and gliogenesis in developing retina.

**Introduction**

*Lhx2* is one of 12 genes that comprise the LIM class homeodomain (LIM-HD) family of transcription factors (TFs). *Lhx2* is dynamically expressed in multiple tissues, including discrete domains within the central nervous system (CNS) (Porter et al., 1997; Monuki et al., 2001). In the developing visual system, *Lhx2* activation is concurrent with patterning of the optic primordia and remains ubiquitous during formation of the optic vesicle and optic cup (Porter et al., 1997; Zuber et al., 2003). *Lhx2* is expressed in retinal progenitor cells (RPCs) throughout retinogenesis, ultimately becoming restricted to Müller glia (MG) and a subset of amacrine interneurons (de Melo et al., 2012; Balasubramanian et al., 2014).

Germline deletion of *Lhx2* results in complete anophthalmia (Porter et al., 1997). However, conditional neuroretinal knockout of *Lhx2* (*Lhx2ΔcKO*) during later retinogenic timepoints results in premature cell cycle exit, altered RPC competence, loss of neuroretinal-derived FGFs resulting in a secondary arrest in lens fiber development, and disrupted MG development (Gordon et al., 2013; de
Melo et al., 2016a; Gueta et al., 2016; Thein et al., 2016). The differentiation of neurons generated following Lhx2ΔcKO-induced cell cycle exit appears grossly normal, though neuronal diversity is limited by RPC competence at the stage when mitotic exit occurred (Gordon et al., 2013). Lhx2 functions similarly in progenitor cells in the cerebral cortex, where it is essential for maintaining proliferative competence and developmental multipotency (Chou and O’Leary, 2013).

Lhx2 is essential for multiple aspects of retinal gliogenesis, with early Lhx2 loss of function resulting in RPC dropout prior to the onset of gliogenesis. Lhx2ΔcKO at later timepoints yields disrupted Müller differentiation, leading to morphological abnormalities and a loss of MG-specific gene expression (de Melo et al., 2016a; de Melo et al., 2016b). Lhx2ΔcKO in fully differentiated mature MG causes cell-autonomous initiation of hypertrophic Müller gliosis in the absence of injury (de Melo et al., 2012). The effect of Lhx2ΔcKO on both RPC maintenance and gliogenesis may be mediated in part by Lhx2-dependent activation of genes in the Notch signaling pathway. Lhx2 is a direct transcriptional regulator of multiple Notch pathway genes in both the retina (de Melo et al., 2016a) and cerebral cortex (Chou and O’Leary, 2013). Notch signaling regulates the maintenance of multipotent RPCs through the downstream activation of the Hes family members Hes1 and Hes5, before ultimately promoting gliogenesis through the repression of proneural bHLH genes (Artavanis-Tsakonas et al., 1999; Mizeracka et al., 2013; Huang et al., 2014).
The molecular mechanisms that control the pleiotropic and context-dependent functions of Lhx2 are unclear. However, several different transcriptional co-factors function as either co-activators or co-repressors with LHX2 proteins. LIM-HD transcriptional activator function is dependent on the formation of protein complexes with LIM domain-binding (LDB) cofactors (Matthews et al., 2008). Targeted loss of function of Ldb genes phenocopies targeted disruption of LIM-HD genes (Becker et al., 2002). Knocking out Ldb1 with Ldb2 in RPCs phenocopies Lhx2ΔcKO (Gueta et al., 2016), as does misexpression of a dominant negative (DN) form of Ldb1 in hippocampal progenitors (Subramanian et al., 2011). Expression of Rnf12, which encodes a RING finger LIM domain-interacting nuclear ubiquitin ligase, has been shown to result in the degradation of LDB proteins complexed with LIM-HD TFs, and thereby negatively regulates the transcriptional activity of LIM-HD TFs (Ostendorff et al., 2002; Hirata et al., 2003). However, Rnf12 has not been studied in the context of neuronal development.

In this study, we investigate the role played by Lhx2-interacting transcriptional coregulators during mammalian postnatal retinal development. We find that misexpression of Lhx2, in combination with Ldb1, in the neonatal mouse retina results in increased development of rod photoreceptors at the expense of MG and bipolar interneurons. Misexpression of Lhx2 also drives a dramatic shift in amacrine cell (AC) morphology from narrow field diffuse patterns to wide field stratified patterns. We show that Lhx2 directly regulates expression of multiple bHLH factors, and that the effects observed following misexpression
are dependent on *Ascl1* and *Neurog2*, respectively. In contrast, we show that co-expression of *Rnf12* with *Lhx2* is both necessary and sufficient for Müller gliogenesis. These results identify a unique molecular switching mechanism that regulates the balance of retinal neurogenesis and gliogenesis through direct interaction with *Lhx2*.

**Results**

**Overexpression of *Lhx2* blocks Müller gliogenesis, and drives formation of rod photoreceptors and wide field amacrine cells (wfACs)**

To examine the effect of misexpression of *Lhx2* on retinal development, we electroporated postnatal day (P)0 mice with control (pCAGIG) and *Lhx2*-expressing (pCAGIG-Lhx2) DNA constructs (Fig. 1a-j). *Lhx2* electroporation promoted the generation of rod photoreceptors at the expense of both MG and bipolar interneurons (Fig. 1c, d). Less than 1% of *Lhx2*-electroporated cells expressed either of the two MG markers P27^Kip1^ or GLUL, compared to nearly 5% of controls (Fig. 1d), and there was a significant reduction in cells with radial morphology (Fig. 1b, h-j). We also observed altered morphology among electroporated ACs (Fig. 1a, b arrows; Supplementary Fig. 1a-c). Narrow-field, diffusely arborizing ACs were generated in control electroporations (Fig. 1a), while *Lhx2*-electroporated RPCs generated wfACs with distinct inner plexiform layer stratification into sublamina s1, s3, and s5 (Fig. 1b, Supplementary Fig. 1a arrow). Co-labeling of generated wfACs with AC subtype markers revealed that these cells did not express markers associated with any distinct AC subtypes,
and only co-labeled with the pan-AC marker PAX6 (Supplementary Fig. 1d-m arrows; Supplementary Fig. 5; retina in panel d also imaged at lower 40X mag in Supplementary Fig. 5b).

**Overexpression of Lhx2 promotes cell cycle exit and downregulation of Notch signaling**

Because Lhx2 electroporation resulted in a loss of MG and bipolar interneurons, both populations being among the last cell types generated in the retina, we tested whether Lhx2 overexpression affected the timing of RPC cell cycle exit (Fig. 1k-m). Electroporation of Lhx2 resulted in premature cell cycle dropout and progenitor depletion by P2 (Fig. 1m). The number of cells co-labeled with the RPC marker VSX2 was reduced from 44% in controls to 15% in cells overexpressing Lhx2 (Fig. 1m). Similarly, the number of electroporated cells co-labeled with the proliferation marker KI67 was reduced from 45% in controls to 22% with Lhx2 (Fig. 1m).

Since Lhx2 electroporation promoted rod photoreceptor production at the expense of bipolar cells and MG, a process that requires the inhibition of Notch signaling in newly post-mitotic retinal precursors (Mizeracka et al., 2013), we tested whether Notch signaling was suppressed in Lhx2 electroporated cells. P0 retinas were co-electroporated with a pCAG-DsRed cell reporter, pCBFRE-GFP Notch signaling reporter and either pCAG control or pCAG-Lhx2 construct (Fig. 1n-p). Analysis at P1 and P2 revealed significant decreases in Notch reporter labeling in cells electroporated with Lhx2 compared to controls (95% vs. 63% at
P1, p<0.05, N=5; 87% vs. 75% at P2, p<0.05, N=5) (Fig. 1p). Taken together, these results show that electroporation of Lhx2 results in rapid cell cycle dropout and downregulation of Notch signaling.

**Lhx2 regulates neurogenesis and neuronal differentiation in part by regulation of proneural bHLH gene expression**

The phenotype resulting from misexpression of Lhx2 closely mirrors that of Lhx2 loss of function (de Melo et al., 2016a). To determine why Lhx2 misexpression might phenocopy Lhx2 loss of function, we first analyzed the expression of multiple proneural bHLH genes as well as Hes6 in an Lhx2 ΔcKO model using the *Pdgfra-Cre; Lhx2\textsuperscript{lox/lox}* mouse line. In these animals Lhx2 is deleted from late-stage RPCs, resulting in a loss of MG and consequent photoreceptor degeneration (de Melo et al., 2016a) (Supplementary Fig. 2). RNA-Seq data obtained from *Pdgfra-Cre; Lhx2\textsuperscript{lox/lox}* mice (de Melo et al., 2016a), showed substantially reduced expression, relative to controls, of multiple bHLH genes, including Neurod1, Neurod4, Neurog2, Ascl1, Hes6 and Olig2 (Table ST1). We performed in situ hybridization to validate these results, and found that expression of each of these genes was reduced in *Pdgfra-Cre; Lhx2\textsuperscript{lox/lox}* mice (Fig. 2a-f; arrows). These data suggest that Lhx2 is necessary not only for expression of gliogenic bHLH factors in RPCs, as described previously (de Melo et al., 2016a), but also for multiple proneural bHLH factors. We tested whether Lhx2 might directly regulate expression of these genes by conducting ChIP-qPCR, examining evolutionarily conserved candidate *cis*-regulatory sequences
located upstream of genes that contained consensus LHX2 binding sites. We found that LHX2 selectively bound to cis-regulatory sequences associated with the proneural bHLH genes *Neurod1*, *Neurod4* and *Neurog2* (Fig. 2g, h). Analysis of the normalized ratio of LHX2 binding at P2 vs. P8 revealed increased occupancy at P2, correlating closely with the period of active neurogenesis (Fig. 2h). Intriguingly, the wfAC phenotype generated following *Lhx2* electroporation closely resembles phenotypes resulting from overexpression of the *NeuroD* family member *Neurod2* (Cherry et al., 2000).

*Hes5* encodes an E-box-selective bHLH protein that inhibits retinal neurogenesis and promotes MG specification (Hojo et al., 2000). *Lhx2* function is required for the gliogenic effects of *Hes5* in the retina (de Melo et al., 2016a). Since *Lhx2* appears to be necessary for expression of proneural bHLHs, yet is essential for *Hes5*-dependent gliogenesis, we next tested whether simultaneous misexpression of *Lhx2* and *Hes5* could promote MG specification. Electroporation of pCAGIG-Hes5 potently promoted the formation of MG (Fig. 2i, k). However, co-electroporation with pCAGIG-Lhx2 blocked the gliogenic effects of *Hes5*, and disrupted MG morphogenesis (Fig. 2j, k). The fraction of cells that expressed P27Kip1 was similar to that of vector controls, while the fraction expressing GLUL was identical to that observed in retinas electroporated with *Lhx2* alone (Fig. 2k; Fig. 1d). These data indicated that *Lhx2* expression is sufficient to override the gliogenic activity of *Hes5*, and that *Hes5* cannot suppress the neurogenic properties of *Lhx2*. 
**Lhx2 overexpression blocks gliogenesis through an Ascl1-dependent mechanism, while promoting wfAC formation through Neurog2**

The previously described data shows that Lhx2 is necessary for proneural bHLH expression. Furthermore, electroporation of Lhx2 disrupts MG development, blocks Hes5-mediated MG formation, and suppresses Notch signaling in RPCs. This indicates that, although Lhx2 is required for both Notch pathway gene expression and Notch-mediated Müller gliogenesis (de Melo et al., 2016a), misexpression of Lhx2 in RPCs can inhibit Notch signaling and promote retinal neurogenesis, similar to the effects of overexpressing Lhx2 in the hippocampus (Subramanian et al., 2011). One mechanism by which this might occur is through Lhx2-dependent regulation of the expression of Ascl1 and Neurog2 in RPCs, which depending on context can negatively (Hufnagel et al.; 2010; Vasconcelos et al., 2016; Ware et al., 2016) or positively (Nelson et al., 2009) regulate Notch signaling in neural progenitors.

We first tested whether shRNA knockdown of Ascl1 concurrent with Lhx2 electroporation could reverse the inhibition of gliogenesis. We observed that co-electroporation of Ascl1 shRNA constructs with pCAGIG-Lhx2 partially rescued MG differentiation, as indicated by the restoration of P27\textsuperscript{Kip1} positive cells that display radial morphology characteristic of MG (Fig. 2l-n). Interestingly, GLUL expression remained suppressed, indicating that Ascl1 knockdown cannot fully rescue terminal glial differentiation. In light of the partial rescue of gliogenesis, we investigated whether Ascl1 knockdown could also reverse Lhx2-dependent inhibition of Notch signaling. We observed that Ascl1 knockdown, much like
misexpression of \(Lhx2\), led to a modest reduction in Notch reporter expression (Supplementary Fig. 3a-c, e), consistent with studies showing a dual role for \(Ascl1\) in both promoting Notch signaling as well as activating expression of proneural bHLH factors in retinal progenitors (Nelson et al., 2009). However, simultaneous overexpression of \(Lhx2\) with knockdown of \(Ascl1\) leads to a dramatic reduction in Notch reporter expression (Supplementary Fig. 3d, e). This is in stark contrast with the observed partial rescue of glial development (Fig. 2l-n), and indicates that rescue of Notch signaling is not the mechanism by which partial recovery of MG development occurs.

The role of \(Neurog2\) is less well understood in the retina, due to its functional redundancy with \(Ascl1\) (Hufnagel et al., 2010). Electroporation of pCAGIG-Neurog2 at P0 was neurogenic, resulting in the specification of PAX6+ narrow field ACs with diffuse dendritic morphology (Fig. 3a). The population of ACs increased from 8.8% with pCAGIG to 19.8% with pCAGIG-Neurog2 (Fig. 3f). Co-electroporation of pCAGIG-Neurog2 with pCAGIG-Lhx2 yielded AC numbers that were not significantly different from controls (9.9%) (Fig. 3f). The ACs generated were wfACs with stratified dendritic morphology in layers S1, S3, and S5 of the IPL identical to that seen following electroporation of pCAGIG-Lhx2 (Fig. 3b-e). A small fraction of these ACs were co-labeled with calretinin (CALB2) (Fig. 3b-e, i). The range of field coverage varied but was typically very large, with arbors often extending to the retinal periphery (Fig. 3c-e). We tested whether the neurogenic and wfAC phenotypes promoted by \(Lhx2\) required \(Neurog2\) by co-electroporating pCAGIG-Lhx2 with a \(Neurog2\) shRNA construct. We found that
knockdown of Neurog2 expression completely blocked the formation of wfACs as shown by the disappearance of the distinct stratified arborization pattern but continued presence of ACs (Fig. 3g, h arrows). However, in contrast to knockdown of Ascl1, Neurog2 knockdown did not rescue the disruption of MG development that resulted from Lhx2 overexpression (Fig. 3h).

The neurogenic role of Lhx2 is mediated by interaction with Ldb1, but the Lhx2 cofactor Rnf12 activates Lhx2-dependent gliogenesis

The LIM domain binding protein LDB1 directly interacts with LHX2 (Bach et al., 1999). Recent studies of LDB function in early-stage RPCs have shown that loss of function of either Ldb1 or Ldb2 does not affect RPC proliferation or gliogenesis, but loss of function of both Ldb1 and Ldb2 genes phenocopies the loss of function of Lhx2 (Gueta et al., 2016). We found that Ldb1 mRNA expression is broadly expressed in the developing retina, being readily detectable in RPCs in the retinal neuroblastic layer (NBL), and in differentiated neurons (Supplementary Fig. 4). Expression becomes localized primarily to ganglion cell layer (GCL) and inner nuclear layer (INL) cells in the mature retina (Supplementary Fig. 4f”, g”). Co-expression of Ldb1 and Lhx2 mRNA is observed in RPCs in the NBL from E14-P2, and in INL cells from P5 to P21 (Supplementary Fig. 4). Because of this overlap of expression of Ldb1 and Lhx2, we tested whether electroporation of Ldb1 could modify the developmental effects induced by Lhx2 overexpression.
Electroporation of pCAGIG-Ldb1 resulted in a significant decrease in the production of MG, from 4.7% P27^Kip1^+ve and 4.7% GLUL+ve in control electroporated cells, to 2.1% and 2.0%, respectively (Fig. 4a, b, i). The reduction was less pronounced than that observed following electroporation with pCAGIG-Lhx2, and unlike Lhx2 electroporation no notable changes in AC morphology were observed (Fig. 4a, b; Supplementary Fig. 5). Outside of the reduction in MG, no significant changes in the patterns or morphology of electroporated cells could be distinguished between pCAGIG and pCAGIG-Ldb1 (Fig. 4a, b; Supplementary Fig. 5). Co-electroporation of pCAGIG-Lhx2 with pCAGIG-Ldb1 produced a phenotype identical to that observed in Lhx2 electroporations -- a significant loss of MG and production of wfACs (Fig. 4c, d, i; Supplementary Fig. 5).

We also investigated the effects of loss of LDB function by overexpressing a dominant-negative (DN) construct of Ldb1, which has previously been shown to phenocopy loss of Lhx2 function in hippocampal progenitors (Subramanian et al., 2011). We observed that overexpression of DN-LDB1 in P0 retina phenocopies the previously described loss of function of Lhx2 (de Melo et al., 2016a), resulting in a loss of MG, but not in the fraction of BCs or photoreceptors (Supplementary Fig. 6). These results confirmed that LDB factors are indeed necessary for Lhx2-mediated regulation of RPC maintenance and gliogenesis.
Since developmental outcomes mediated by LIM-HD factors, including LHX2, are co-regulated by Rnf12 (Ostendorff et al., 2002; Hiratani et al., 2003), we tested whether Rnf12 expression could alter Lhx2 function in the retina. Analysis of Rnf12 mRNA expression in the developing retina revealed relatively low expression in RPCs at from E14-E18 timepoints (Supplementary Fig. 4a'-c') compared to the enriched expression observed at post-natal timepoints (Supplementary Fig. 4d'-f'). Postnatal expression revealed a distinct upregulation and enrichment of RNA expression in subsets of cells in the NBL from P0 to P2 and in the medial INL at P5, consistent with the spatial and temporal onset of Müller gliogenesis (Supplementary Fig. 4d'-f'), as well as with previous studies which reported increased expression of Rnf12 during differentiation of MG precursors (Nelson et al., 2011).

Electroporation of pCAGIG-Rnf12 at P0 led to a significant increase in the production of MG from 4.7% and 4.6% (P27^Kip1+ve and GLUL+ve respectively) in controls, to 7.72% and 8.3 % (Fig. 4e arrows, f arrows, j). Furthermore, co-electroporation of Rnf12 with Lhx2 rescued the reduction in gliogenesis observed following electroporation of Lhx2 alone (Fig. 4g arrows, h arrows, j). We also observed that co-electroporation of Rnf12 with Lhx2 reversed the observed changes in amacrine cell morphology that resulted from Lhx2 electroporation, preventing the formation of wfACs (Fig. 4g, h; Supplementary Fig. 5f). Electroporation of Rnf12 alone or with Lhx2 inhibited the formation of ACs broadly (Supplemental Fig. 5e-g). We next tested whether Rnf12 was required for glial development. Electroporation with shRNA constructs targeting Rnf12 at
P0 resulted in a loss of MG as determined by P27\(^{Kip1}\) and GLUL immunostaining (Fig. 4k arrows, l arrows, m-o). The relative loss of MG was nearly identical to that reported following \(Lhx2\) loss of function (de Melo et al., 2016a). To determine if \(Rnf12\) requires \(Lhx2\) in order to promote MG differentiation, we co-electroporated pCAGIG-Rnf12 with pCAG-Cre into \(Lhx2^{+/+}\) and \(Lhx2^{lox/lox}\) retinas at P0. Concurrent loss of function of \(Lhx2\) blocked the \(Rnf12\)-dependent increase in gliogenesis (Fig. 4p-r). In these mice, the proportion of P27\(^{Kip1}\) and GLUL+ve electroporated cells (1.3% and 1.1% respectively) was nearly identical to that reported following \(Lhx2\) loss of function (de Melo et al., 2016a). Taken together, these results suggest that \(Rnf12\) acts through an \(Lhx2\)-dependent mechanism in late-stage RPCs to induce gliogenesis.

**Misexpression of \(Lhx2/Ldb1\) or \(Rnf12\) does not result in altered mRNA levels of proneural or proglial bHLH factors**

We have demonstrated that \(Lhx2\) is necessary for expression of multiple proneural bHLH factors in RPCs (Fig. 2a-f, Supplementary Table 1). \(LHX2\) directly binds to putative \(cis\)-regulatory elements associated with these genes (Fig. 2g,h), and the effects of overexpression of \(Lhx2\) are disrupted by knockdown of \(Ascl1\) and \(Neurog2\) (Fig. 2l-n, Fig. 3g, h). We therefore investigated whether overexpression of \(Lhx2\) and \(Ldb1\) directly resulted in increased mRNA levels of these genes, relative to pCAGIG alone or \(Rnf12\) overexpression. Surprisingly, when conducting qRT-PCR analysis of cells isolated 36 hrs after electroporation, we did not observe significant changes in
mRNA levels for any of the bHLH factors tested, despite the fact that robust expression of vector-derived \textit{Lhx2}, \textit{Ldb1} and/or \textit{Rnf12} are all detected at this stage (Supplementary Fig. 7).

**Lower relative levels of LHX2-LDB1 protein complexes are seen as gliogenesis is initiated.**

We have previously shown that LHX2 interacts with LDB1 in developing retina at E15.5 and P0.5 (Gueta et al., 2016). The findings in this study, suggest that upregulation of \textit{Rnf12} may lead to the relative fraction of LHX2 bound to LDB1 decreasing during gliogenesis due to RNF12-dependent degradation of LDB1.

To test this hypothesis, we performed immunoprecipitation analysis of LHX2 and LDB1 at three different timepoints. We analyzed LHX2 protein complexes at E16, when only neurons are born; P2, when gliogenesis is beginning; and P5, when gliogenesis peaks (Young 1985). Both LHX2 and LDB1 were expressed and directly interacted with one another at all three stages (Fig. 5a). However, when immunoprecipitation was performed with antibodies to LHX2, levels of recovered LDB1 levels showed a substantial reduction at P2 relative to E16, and even more pronounced reductions at P5 (Fig. 5b), when normalized to total levels of immunoprecipitated LHX2.
Discussion

The molecular mechanisms that control CNS gliogenesis are still poorly understood. Work from many groups has shown that a combination of genes encoding extrinsic and intrinsic signals control gliogenesis. Extrinsic signals include the Notch/Delta pathway, while several transcription factors – including Sox9, Nfia, Hes5 and Zbtb20 – have been shown to be either necessary or sufficient to induce gliogenesis (Hojo et al., 2000; Kang et al., 2012; Nagao et al., 2016).

Here, we shed light on the mechanism by which context-specific functions of Lhx2 are regulated during retinal development. In this study, we confirm and extend previous work that demonstrated an essential role for Ldb1-Lhx2 function in both RPC proliferation and retinal gliogenesis. We show that ectopic Lhx2 expression potently suppresses Notch signaling, resulting in early RPC cell cycle exit and blocking retinal gliogenesis, while at the same time promoting formation of wfACs. The formation of wfACs was further enhanced by ectopically co-expressing Lhx2 with Neurog2, resulting in more expansive lateral arborization and co-expression of the AC marker CALB2. Conversely, co-electroporation of Lhx2 with a Neurog2 shRNA blocked wfAC formation. These results indicate that concurrent activation of pro-neural Neurog2 function may be required for the complete instructive wfAC effects of Lhx2. We further demonstrate that these effects are dependent on Ascl1 and Neurog2, respectively. We also show that Rnf12, which specifically ubiquitinates LDB proteins and targets them for proteolysis, is both necessary and sufficient to promote Müller glial development,
and does so in a strictly \textit{Lhx2}-dependent manner. Upregulation of \textit{Rnf12} expression correlated with a reduction in retinal levels of the LHX2-LDB1 complex, concurrent with the onset and progression of gliogenesis.

These findings highlight the importance of LIM cofactor-mediated \textit{Lhx2}-dependent transcriptional activation in controlling cell fate specification in the CNS. This does not, however, exclude a parallel function of \textit{Rnf12} in promoting \textit{Lhx2}-dependent transcriptional repression. While \textit{Lhx2}-dependent transcriptional activation is dependent on LDB proteins, \textit{Lhx2}-dependent transcriptional repression involves recruitment of histone modifying enzymes, including the HDAC and NuRD protein complexes (Bach et al., 1999; Muralidharan et al., 2017). RNF12 itself can directly bind both LHX2 and SIN3A, leading to recruitment of HDAC proteins (Bach et al., 1999). It is thus possible that \textit{Rnf12} may promote gliogenesis by both attenuating \textit{Lhx2-Ldb1}-dependent activation of proneural genes, and by triggering \textit{Lhx2}-dependent repression of these genes.

The precise identity of these genes, however, remains unclear. We and others have shown that LHX2 regulates expression of multiple Notch pathway genes (de Melo et al., 2016a), and that this is of central importance in actively regulating the balance of neurogenesis and gliogenesis in RPCs. In this study we also show that \textit{Lhx2} regulates expression of Notch-regulated bHLH factors -- such as \textit{Ascl1}, \textit{Neurog2} and \textit{Hes6}, as well as multiple Notch-independent proneural bHLH factors such as members of the \textit{NeuroD} family (Morrow et al., 1999; Jadhav et al., 2006). \textit{NeuroD} family genes in turn induce wfAC formation
when overexpressed (Cherry et al., 2011). We also demonstrate that neurogenesis and wfAC formation induced by misexpression of Lhx2 are both blocked by knockdown of Ascl1 and Neurog2, respectively. While this suggested that overexpression of Lhx2 might inhibit Notch signaling, while enhancing neurogenesis and wfAC formation by stimulating transcription of proneural bHLH factors, this proved not to be the case. It appears that misexpression of Lhx2 may regulate these processes through presently uncharacterized target genes and/or by regulating the activity or function of proneural bHLH factors through as yet unknown mechanisms. Identifying precisely how Lhx2 regulates neurogenesis, Notch signaling and wfAC formation will be an important topic for future studies.

In both the retina and other CNS regions, Lhx2 acts as a selector gene that simultaneously activates and represses different sets of tissue and/or state-specific genes. In early-stage RPCs, Lhx2 simultaneously activates expression of RPC-specific genes, while suppressing genes that are specific to anterodorsal hypothalamus and thalamic eminence (Roy et al., 2013). Likewise, in early cortical progenitors, Lhx2 activates cortical plate-specific genes, while repressing expression of genes enriched within the cortical hem (Mangale et al., 2008). The dynamic regulation of Rnf12 and Ldb activity, which plays a central role in control of retinal cell fate, may also be important for these selector functions of Lhx2.
**Materials and Methods:**

**Animals**
Timed pregnant CD-1 mice used for *in situ* hybridization, electroporation and ChIP were purchased from Charles River Laboratories. Mice were housed in a climate-controlled pathogen free facility, on a 12 hour-12 hour light/dark cycle (08:00 lights on-20:00 lights off). *Pdgfra-Cre* (stock #013148) mice were purchased from the Jackson Laboratory while *Lhx2*<sup>lox/lox</sup> mice were obtained from Dr. Edwin Monuki, University of California, Irvine. *Lhx2*<sup>lox/lox</sup>; *Pdgfra-Cre* and *Lhx2*<sup>+/+</sup>; *Pdgfra-Cre* mice were bred and maintained in the lab as previously described (de Melo et al., 2016a). All experimental procedures were preapproved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

**Cell counts**
All counts were performed blinded on whole retinal sections or dissociated retinas as previously described (de Melo et al. 2012; de Melo et al. 2016a). Differences between the two means were assessed using an unpaired two-tailed Student’s t-test.

**Chromatin immunoprecipitation (ChIP)**
CD-1 mice were sacrificed at postnatal day (P)2 and P8 according to Johns Hopkins IACUC animal policies. ChIP was performed as previously described (de Melo et al., 2016a). Whole dissected retinas were dissociated in a
collagenase I suspension, cross-linked in 1% formaldehyde, quenched in 125 mM glycine and the extracted nuclei were sheared to produce 100 to 500 bp fragments by means of probe sonication. Chromatin was immunoprecipitated by using goat anti-Lhx2 antibody (Santa Cruz Biotechnology) or the related isotype control (Abcam), retained on agarose beads (Invitrogen), washed and purified by organic extraction. Candidate target genes demonstrating altered expression levels in Lhx2 conditional knockout retinas by RNA-Seq were screened for LHX2 consensus binding sites within annotated regulatory regions by querying the JASPAR repository database (Mathelier et al., 2016), and was based on GSE48068 (Folgueras et al., 2013). Computationally inferred Lhx2 binding sites and proximal negative control regions were analyzed in ChIP-enriched fractions and isotype controls by SYBR–qPCR (Agilent Technologies).

**Electroporation**

For *in vivo* electroporation experiments, retinas were electroporated at P0 as previously described, and harvested for analysis at P1, P2, or P14 subject to the requirements of the study. DNA constructs used for gene misexpression in this study are as follows: pCAGIG (Addgene plasmid 11159, deposited by C. Cepko and modified into a Gateway destination vector in lab), pCAGIG-Hes5 (Gateway cloned from Ultimate Human ORF Collection (Life Technologies)), pCAGIG-Ldb1 (Gateway cloned from Ultimate Human ORF Collection (Life Technologies)), pCAGIG-Lhx2 (Gateway cloned from Ultimate Human ORF Collection (Life Technologies)), pCAGIG-Ngn2 (NeuroG2) (Gateway cloned from Ultimate
Human ORF Collection (Life Technologies)), pCAGIG-Rnf12 (Gateway cloned from Ultimate Human ORF Collection (Life Technologies)), pCAGIG-CD4 (Gateway cloned from Ultimate Human ORF Collection (Life Technologies)).

DNA constructs used for Notch reporter analysis in this study are as follows: pCAG (modified from pCAGIG), pCAG-DsRed (Addgene plasmid 11151, deposited by C. Cepko), pCAG-Lhx2 (Gateway cloning from Ultimate Human ORF Collection (Life Technologies)), pCBFRE-GFP (Addgene plasmid 17705, deposited by N. Gaiano). DNA constructs used for shRNA knockdown in this study are as follows: Ascl1 shRNA (clone TRCN0000075398, TRC-Open Biosystems), Rnf12 shRNA (clone TRCN0000095740, TRC-Open Biosystems), Ngn2 (Neurog2) shRNA (clone FP-301 obtained from Franck Polleux, Columbia University) (Root et al., 2006; Hand and Polleux, 2011), (Control (pLKO.1 vector control, TRC-Open Biosystems). All shRNA constructs have been previously shown to give substantial (>70%) knockdown of their target gene. DNA constructs used for Lhx2 loss of function in this study are as follows: pCAG-Cre (Addgene plasmid 13775, deposited by C. Cepko), and pCALNL-GFP (Addgene plasmid 13770, deposited by C. Cepko).

For ex vivo electroporation studies, eyes were enucleated from P0 animals and placed in cold PBS. The cornea and scleral/RPE tissue was dissected off of the retinas, leaving the retina, iris, and lens. Retinas were placed in a custom chamber containing 1µg/µl in PBS of all DNA constructs to be electroporated in a given experiment, positioning the apical retina towards the negative pole.
Electroporation occurred after 5 – 50mV; 50ms square-wave pulses from a BTX ECM830 square-wave electroporator (Harvard Apparatus). Upon electroporation, the iris and lens was dissected from the retina, and retinas were flat mounted on .2μm Nuclepore Track-Etch Membranes (Whatman) such that the apical retina was placed onto the filter. Explants were then placed in suspension culture in DMEM supplemented with 10% FBS and 100U/ml Pen/Strep for 36 hours.

Quantitative real-time PCR

Retinas were electroporated at P0 with multiple constructs together with pCAGIG-CD4. 36 hours later, explants were harvested, dissociated, and electroporated cells isolated using Dynabeads conjugated with anti-human CD4 (Thermo-Fisher). qRT-PCR was performed as previously described (de Melo et al., 2016a), with signals normalized to Gapdh. Primers used are listed in Supplemental Table 2.

Immunohistochemistry

Antibodies utilized for fluorescent immunohistochemistry are as follows: goat anti-Brn3 (1:200; Santa Cruz Biotechnology), mouse anti-calbindin (Calb1) (1:200; Sigma-Aldrich), rabbit anti-Calretinin (Calb2) (1:200; Chemicon), goat anti-Chat (1:100; Chemicon), sheep anti-Chx10 (Vsx2) (1:200; Exalpha Biologicals), rabbit anti-Dab1 (1:200; EMD Millipore), rabbit anti-DsRed (1:500; Clontech Laboratories), rabbit anti-GABA (1:200; Sigma), mouse anti-Gad6 (Gad2) (1:200; Developmental Studies Hybridoma Bank, University of Iowa),
goat anti-GFP (1:500; Rockland Immunochemicals), rabbit anti-GFP (1:1000; Invitrogen), mouse anti-Glutamine synthase (Glul) (1:200; BD Biosciences), rat anti-Glycine (1:200; ImmunoSolution), mouse anti-Islet1 (1:200; Developmental Studies Hybridoma Bank), mouse anti-Ki67 (1:200; BD Biosciences), rabbit anti-Lhx2 (1:1500; generated in house with Covance), mouse anti-P27 (1:200; Invitrogen), mouse anti-Pax6 (1:200; Developmental Studies Hybridoma Bank), rabbit anti-TH (1:500; Pel Freez), mouse anti-VGlut3 (1:200; Antibodies Incorporated). Secondary antibodies used were FITC conjugated donkey anti-goat IgG (1:500; Jackson Immunoresearch), FITC conjugated donkey anti-mouse IgG (1:500; Jackson Immunoresearch), FITC conjugated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch), Texas Red conjugated donkey anti-goat IgG (1:500; Jackson Immunoresearch), Texas Red conjugated donkey anti-mouse IgG (1:500; Jackson Immunoresearch), Texas Red conjugated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch), Texas Red conjugated donkey anti-sheep IgG (1:500; Jackson Immunoresearch). All section immunohistochemical data shown was imaged and photographed on a Zeiss Meta 510 LSM confocal microscope.

**In Situ Hybridization**

Single-color *in situ* hybridization was performed as previously described (Blackshaw, 2013). RNA probes were generated using the following EST sequences as templates: *Ascl1*, GenBank accession number BE953927; *Hes6*, GenBank accession number AW048812; *Neurod1*, GenBank accession number
AI835157; Neurod4, GenBank accession number AI846749; Neurog2, GenBank accession number BC055743; Olig2, GenBank accession number AI844033.

**Immunoblotting and Immunoprecipitation**

Wildtype retinal tissues were harvested from E16 (8 litters), P2 (5 litters) and P5 (5 litters), and snap-frozen for storage. After pooling tissues from all litters, tissue homogenization was carried out by aspirating the tissue 20 times using a 23-gauge needle in lysis buffer (100mM Tris-HCl, 150mM NaCl, 25mM NaF, 50 µM ZnCl₂, 15% glycerol, 1% Triton X-100) supplemented with protease inhibitors (Roche #11697498001) and BitNuclease (Biotools #B16003) for clarification. Following a 1hr of incubation at 4°C, supernatant were collected after centrifuging at 10,000 rcf for 10mins at 4°C. Following normalization using standard BCA assay, immunoprecipitation (IP) was carried out by first incubating lysates overnight at 4°C with 5µg of anti-LHX2 (clone ID: R911.1.2E3, CDI labs Inc. catalog #15-389) and mouse pan-IgG (#sc-2025, Santa Cruz) respectively. Next, the antibody-protein complexes were pulled down by incubating 2 hours with ProteinG Dynabeads (ThermoFisher #10004D) at 4°C, washed thrice with lysis buffer and eluted in LDS-sample loading buffer (ThermoFisher #NP0008). Input lysate along with the IP samples were resolved in a SDS-PAGE gel and immunoblotted sequentially using anti-LHX2 (1:750, clone ID: R911.1.2E3, CDI labs Inc. catalog #15-389), LDB1 (1:1000 Sigma #HPA034488), and GAPDH (Sigma #G8795) antibodies. Anti-rabbit IRDye680RD (LiCor # 925-68071) and light-chain specific anti-mouse AlexaFluor 790 (Jackson Immunolabs #115-655-
174) secondary antibodies were used to visualize bands, and blots were imaged using an infra-red fluorescence imager (LiCor Clx).

**Densitometry and Statistical Analysis**

Three technical repeats of the co-IP experiments were performed, followed by three independent immunoblots. Densitometry signal from lanes corresponding to LHX2 input, LHX2 IP, LDB1 IP and GADPH loading controls of all three SDS-PAGE gels was measured using LiCor image studio software. Following normalization of signal from LHX2 IP to its respective input, the ratio of LDB1 co-IP with LHX2 was calculated. Statistical analysis was performed using R software. We performed linear regression (r, lm) to adjust batch effects in the ratio of LDB1 co-IP with LHX2 between the three blots. Next, we performed one-way ANOVA (r, aov) using the adjusted values to test if there were any statistically significant differences between the means of LDB1 signal that co-IPed with LHX2 in the E16, P2 and P5 samples. For post-hoc pairwise comparisons, we performed a t-test (r, t-test).

**Acknowledgements:**

We thank K. Yang for help with statistical analysis and W. Yap for comments on the manuscript. This study was supported by grants from the NIH to B.S.C. (F32EY024201, K99EY027844) and S.B. (R01EY020560).
References:


Figure 1. Electroporation of \textit{Lhx2} blocks Müller gliogenesis, bipolar cell formation and changes amacrine cell morphology. (a, b, d-f, h, i) Electroporation of \textit{Lhx2} at resulted in a significant (P<0.05) decrease at P14 of MG (P27^{kip1} and GLUL +ve) [4.68\% (SE= 0.60\%, N=6, P27^{kip1}), 4.65\% (SE=0.21\%, N=6 GLUL) vs. 0.8\% (SE=0.29\%, N=6, P27^{kip1}); 0.85\% (SE=0.25\%, N=6, GLUL)]. (a-c, g, j) \textit{Lhx2} electroporation resulted in decreased (P<0.05) bipolar interneurons (VSX2 +ve) [7.81\% (SE=0.38\%, N=6) vs. 3.17\% (SE=0.26\%, N=6)] and increased photoreceptors [77.3\% (SE=2.4\%, N=5) vs. 82.44\% (SE=2.1\%, N=5)]. (b) Amacrine cell morphology changed from narrow field cells with diffuse dendrites to wide-field amacrine cells, which stratified into the S1, S2, and S3 sublamina of the inner plexiform layer (b, white arrows). (k-m) Cells electroporated with pCAGIG-Lhx2 at P0 showed significant (P<0.05) down-regulation of both VSX2 and KI67 by P2 [pCAGIG, 45.75\% (SE= 2.6\%, N=5, VSX2); 44.8\% (SE=1.79\%, N=5 KI67); pCAGIG-Lhx2, 15.3\% (SE=0.42\%, N=5, VSX2); 22.8\% (SE=1.97\%, N=5, KI67)]. (n-p) Electroporation of \textit{Lhx2} at P0 results in a significant decrease (P<0.05) of pCBFRE-GFP Notch reporter expression at P1 and P2 [pCAG, 95.49\% (SE=0.4\%, N=5, P1); 87.34\% (SE=1.57\%, N=5, P2); pCAG-Lhx2, 63.43\% (SE=2.86\%, N=5, P1); 75.39\% (SE=1.5\%, N=5, P2)]. * Indicates statistically significant decrease. ^ Indicates statistically significant increase.

GCL, ganglion cell layer; INL, inner nuclear layer; NBL, neuroblastic layer; ONL, outer nuclear layer; P, postnatal day; s inner plexiform layer sublamina. Scale bars, 50 µm (all panels).
Figure 2. *Lhx2* regulates expression of bHLH factors in the retina. (a-f) In situ hybridization analysis of *Lhx2ΔcKO (Pdgfra-Cre; Lhx2^{loxp/loxp})* retinas at P0 reveals the requirement of *Lhx2* for bHLH expression. Lower power images are 5X magnification, while high power images are 20X. (g) ChIP performed on retinal tissue collected at postnatal days 2 and 8. Graphs show the mean percentages of input recovery for the IP fractions and the isotype controls. * Indicates statistical significance (P<0.05). Indicated bars represent the standard error. (h) The normalized ratio of LHX2 binding to target loci reveals decreasing occupancy from P2 to P8. (i-k) Co-electroporation of *Lhx2* with *Hes5* blocked the gliogenic effect of *Hes5* electroporation [pCAGIG-Hes5, 23.75% (SE=2.63%, N=6, P27^{Kip1}); 20.83% (SE=2.46%, N=6, GLUL); pCAGIG-Hes5/pCAGIG-Lhx2, 5.33% (SE= 1.4%, N=6, P27^{Kip1}); 0.43% (SE=0.1%, N=6, GLUL)]. (l-n) shRNA knockdown of *Ascl1* rescues (P<0.05) P27^{Kip1} expression [pCAGIG-Lhx2, 0.8% (SE=0.29%, N=6, P27^{Kip1}); 0.85% (SE=0.25%, N=6, GLUL); pCAGIG-Lhx2/Ascl1 shRNA, 2.95 % (SE=0.48%, N=6, P27^{Kip1}); 1.62% (SE=0.36%, N=6, GLUL)]. * Indicates statistical significance for panels (g, h), but represents a significant decrease (k, n). ^ indicates significant increase (k, n). Scale Bars, 1000 µm (5 X mag, a-f), 250 µm (20 X mag, a-f), 50 µm (j, l, m).
**Figure 3.** *Lhx2* synergistically promotes the formation of wide field amacrine cells with *Neurog2*. (a, f) Electroporation of *Neurog2* results in an increase in the formation of narrow field diffusely arborizing amacrine cells. (b, f) Co-electroporation of *Lhx2* with *Neurog2* transforms the morphology of ACs from narrow field/diffusely arborizing to wide field/selectively stratified. The overall fraction of ACs is unchanged relative to pCAGIG-Lhx2 or pCAGIG electroporated retinas. (c-e) Electroporation of *Lhx2* with *Neurog2* results in a synergistic expansion of the width of the dendritic field. (g, h) shRNA mediated knockdown of *Neurog2* blocks the formation of wide field amacrine cells generated by electroporation of *Lhx2*. (h arrows) the lateral S1, S3, and S5 stratified dendritic arbors are lost. (i) Co-electroporation of *Lhx2* with *Neurog2* results in significant increases in CALB2+ amacrine cells (primarily AII), bipolar cells, and photoreceptors compared to electroporation of *Neurog2* alone (P<0.05). S, inner plexiform layer sublamina. Scale bars, 50 μm (a, b, d, e, g, h), 200 μm (c).
Figure 4. Co-electroporation of Lhx2 with Ldb1 or Rnf12 differentially affects Müller gliogenesis. (a-d, i) Electroporation of Ldb1 inhibits the formation of MG, and co-electroporation of Lhx2 with Ldb1 generates an identical phenotype as electroporation of Lhx2 alone. (e-h arrows, j) Electroporation of Rnf12 significantly increases the proportion of MG generated [7.73% (SE=0.53%, N=6, P27Kip1) and 8.3% (SE=0.85%, N=6 GLUL)], while co-electroporation of Lhx2 with Rnf12 rescues MG [3.53% (SE=0.19%, N=6, P27Kip1) and 4.15% (SE=0.45%, N=6 GLUL)]. (k,l arrows; m-o) shRNA knockdown of Rnf12 significantly blocks the formation of MG compared to shRNA controls [5.45% (SE=0.75%, N=6, P27Kip1); 5.72% (SE=0.74%, N=6 GLUL) vs. 0.63% (SE=0.18%, N=6, P27Kip1); 0.64% (SE=0.31%, N=6, GLUL)]. (p-r) Rnf12 requires functional Lhx2 to promote MG development (P<0.05; N=3) P27Kip1, (P<0.05; N=3) GLUL. * indicates significant decrease. ^ indicates significant increase. Scale bars, 50 µm (all panels).
Figure 5. Reduced levels of the LHX2-LDB1 are seen following the onset of retinal gliogenesis. (a) Co-immunoprecipitation (co-IP) of LDB1 with LHX2 in retinal tissue collected at E16, P2 and P5. Decreased LDB1 interaction with LHX2 is observed at P5. IgG IP is used as negative control. * indicates non-specific bands detected by the anti-LHX2 antibody that do not appear in IP lanes. (b) One-way ANOVA analysis that compared the densitometry signals of LDB1 that co-IPs with LHX2 at each timepoint was found to be statistically significant (p=0.0016). The relative levels of LDB1 recovered following immunoprecipitation with anti-LHX2 following normalization to the total amount of LHX2 protein in the input lane. This controls for developmental changes in LHX2 levels. Post-hoc t-test indicates that the decrease in levels of LDB1 that co-IP with LHX2 decreases significantly with E16>P2>P5. # and ## indicate statistical significance at p = 0.016 and p = 0.003 in the post-hoc t-test respectively.
**Supplementary Figure 1.** Electroporation of *Lhx2* promotes the formation of wfACs. (a-c) Morphology of a wfAC generated following electroporation of *Lhx2*. (d) Generated wide field amacrine cells co-express the pan-amacrine marker PAX6. (e-m) Co-labeling with amacrine cell subtype selective markers reveals that amacrine cells generated by *Lhx2* electroporation do not fall within any well-established molecular category. ISLET1, CHAT- cholinergic starburst amacrine cells; GABA, GAD2- GABAergic amacrine cells; GLYCINE- glycinergic amacrine cells; VGLUT3- glutamatergic amacrine cells; CALB2- mixed population primarily All amacrine cells, A19 amacrine cells, and non-All glycine immunoreactive amacrine cells; TH- dopaminergic wide field amacrine cells; DAB1- All amacrine cells. Arrows indicate wfACs throughout. GCL, ganglion cell layer; INL, inner nuclear layer; outer nuclear layer; s inner plexiform layer sublamina. Scale bars, 50 µm (all panels).
de Melo, et al. Figure S2
Supplementary Figure 2. (a-d) Expression of inner retinal cell class markers at P21 and P120 in \textit{Pdgfra-Cre}; \textit{R26YFP}; \textit{Lhx2}^{\text{lox/lox}} retinas. Expression of the retinal ganglion cell marker Brn3 (a), retinal ganglion, amacrine, and horizontal cell marker Pax6 (b), and bipolar cell marker Vsx2 (c) are detectable at both P21 and P120. (d) Expression of Lhx2 is not detectable at both P21 and P120. (e-h) expression of amacrine cell subclass specific markers at P21 and P120 in \textit{Pdgfra-Cre}; \textit{R26YFP}; \textit{Lhx2}^{\text{lox/lox}} retinas. Expression of choline acetyltransferase, Chat (e), calretinin, Calb2 (f), GABA, (g), and calbindin, Calb1 (h) are detectable at both P21 and P120. Scale bars, 50 \(\mu\)m (d, h).
**Supplementary Figure 3.** Knockdown of *Ascl1* exacerbates *Lhx2*-mediated Notch inhibition. (a-d) Examples of *ex vivo* explant electroporations of P0 retinas cultured 2 days *in vitro* expressing the pCBFRE-GFP (Notch reporter), an electroporation control (pCAG-DsRed) and either: (a) pCAG control construct, (b) pCAG-Lhx2, (c) *Ascl1* shRNA or (d) pCAG-Lhx2 and *Ascl1* shRNA. (e) Quantification of the proportions of electroporated cells (red) that are expressing the pCBFRE-GFP transgene, with significant findings from a one-way ANOVA (*p* < 0.0001) analysis and Tukey multiple comparisons test indicated. *
Supplementary Figure 4. RNA expression of Rnf12 and Ldb1 contrasted with Lhx2 during mouse retinal development. (a-c) RNA expression of Lhx2 is restricted to RPCs during embryonic time points with down regulation occurring in early-born neurons in the GCL. (d-g) Down regulation of Lhx2 in newly generated neurons continues in postnatal retina, with Lhx2 becoming restricted to MG and subsets of amacrine cells consistent with previous reports. (a’-c’) Low levels of Rnf12 RNA expression are seen in the NBL during embryonic time points, with higher expression detected in the GCL at E14 and E18. (d’-f’) Rnf12 expression is up regulated in the medial NBL at P0 and remains robustly expressed in the NBL and medial INL at P2 and P5. (g’) Adult expression of Rnf12 is located in all three retinal layers, with the INL showing the strongest labeling. (a’’-c’’) Ldb1 expression was detected throughout the embryonic retina. (d’’-e’’) Neonatal expression of Ldb1 is enriched in the NBL. (f’’) At P5 enrichment of Ldb1 in the medial INL is detected. (g’’) Adult expression of Ldb1 is localized in the INL and subsets of cells in the GCL. Weaker expression of Ldb1 was also detected in the ONL. Scale bars, 200 µm (a’’), 100 um (b’’-g’’).
De Melo, et al. Figure S5
Supplementary Figure 5. Co-electroporation of *Lhx2* with *Ldb1* or *Rnf12* results in changes in neurogenesis. (a-c, g) Electroporation of *Lhx2* or *Ldb1* does not alter the proportion of amacrine cells (PAX6 +ve) generated. (d, g) Co-electroporation of *Lhx2* with *Ldb1* generates an identical wide field amacrine cell phenotype as electroporation of *Lhx2* alone. (e-g) Electroporation of *Rnf12* inhibits the formation of amacrine cells, while co-electroporation of *Rnf12* with *Lhx2* blocks the formation of wide-field amacrine cells generated by electroporation of *Lhx2* alone (P<0.05; N=6; PAX6 +ve, pCAGIG-Rnf12 vs. pCAGIG; (P<0.05; N=6; PAX6 +ve, pCAGIG-Rnf12/Lhx2 vs. pCAGIG). (b, e-g) Electroporation of Lhx2, Rnf12, or Lhx2 and Rnf12 results in mild increases in photoreceptor numbers (P<0.05; N=6). *, indicates significant decrease. Scale bars, 50 µm (all panels).
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*de Melo, et al. Figure S6*
Supplementary Figure 6. Electroporation of a dominant-negative Ldb1 construct (pCAGIG-Ldb-DN) phenocopies Lhx2 loss of function in postnatal retina. (a, b) Electroporation of pCAGIG-Ldb1-DN at P0 by electroporation resulted in a significant decrease at P14 of MG (P27Kip1 and GLUL +ve). (c) Quantification of MG (P27Kip1 and GLUL +ve), bipolar cells, and photoreceptors in pCAGIG vs. pCAGIG-Ldb1-DN electroporated retinas. Scale bars, 50 µm (a, b).
Supplementary Figure 7. (a) qRT-PCR assessing fold enrichment of electroporated cells after hCD4 immunosorting. Fold enrichments indicated transcripts enrichment from CD4+ RNA extractions compared to CD4- fractions. (b) qRT-PCR comparing normalized expression of mLhx2, hLdb1 and hRnf12 transcript expression in CD4+ RNA fractions to control pCAGIG CD4+ fractions in Lhx2-Ldb1 or Rnf12 overexpression experiments. (c) qRT-PCR comparing normalized expression of Notch-pathway and neurogenic gene transcript expression in CD4+ RNA fractions in Lhx2-Ldb1 or Rnf12 overexpression experiments to control pCAGIG CD4+ electroporations. Analysis of significance was determined by (a + b) paired, two-tailed t-Tests on ΔcT values or (c) one-way ANOVA followed by Tukey’s multiple comparisons tests. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001; **** indicates p < 0.0001. nd – not detected.
Supplemental Table 1: Reduced expression of neurogenic bHLH factors in P0.5 Lhx2 cKO retina

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RNA-Seq data from P0.5 Pdgfra-Cre;Lhx2lox/lox retina was previously described in (1). RPKM values for each gene are listed.

Supplemental Table 2: Primers used for qRT-PCR analysis.

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* Primer sequences from Harvard Primer Bank are previously published in (Spandidos et al., 2008; 2010; Wang and Seed, 2003)
Supplementary References:

