Bhlhb5 is Required for the Subtype Development of Retinal Amacrine and Bipolar Cells in Mice

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Background: BHLHB5, an OLG-related basic helix-loop-helix transcription factor, is required for the development of a subset of gamma-aminobutyric acid–releasing (GABAergic) amacrine cells and OFF-cone bipolar (CB) cells in mouse retinas. In order to determine BHLHB5’s functional mechanism in retinogenesis, we used the Cre-loxP recombination system to genetically trace the lineage of BHLHB5+ cells in normal and Bhlhb5-null retinas. The Bhlhb5-Cre knock-in allele was used to activate the constitutive expression of a GFP reporter in the Bhlhb5-expressing cells, and the cell fates of Bhlhb5-lineage cells were identified by using specific cell markers and were compared between normal and Bhlhb5-null retinas.

Results: In addition to GABAergic amacrine and OFF-CB cells, Bhlhb5 lineage cells give rise to ganglion, glycinergic amacrine, rod bipolar, ON-bipolar, and rod photoreceptor cells during normal retinal development. Targeted deletion of Bhlhb5 resulted in the loss of GABAergic amacrine, glycinergic amacrine, dopaminergic amacrine, and Type 2 OFF-CB cells. Furthermore, in the absence of BHLHB5, a portion of Bhlhb5 lineage cells switch their fate and differentiate into cholinergic amacrine cells.

Conclusions: Our data reveal a broad expression pattern of Bhlhb5 throughout retinogenesis and demonstrate the cell-autonomous as well as non-cell-autonomous role of Bhlhb5 in the specification of amacrine and bipolar subtypes. Developmental Dynamics 000:000–000, 2013. © 2013 Wiley Periodicals, Inc.

Key words: Bhlhb5; amacrine cell; bipolar cell; neurogenesis; transcription factor; retina

Key Findings:

- Bhlhb5 expression during retinogenesis is not confined to GABAergic amacrine cell and Type 2 OFF cone bipolar cell lineage but also is in other retinal cell lineages.
- In addition to the changes in GABAergic amacrine and Type 2 OFF cone bipolar cells, loss of Bhlhb5 results in a significant loss of glycinergic amacrine cells.
- Deletion of Bhlhb5 resulted in the cell fate change of Bhlhb5 lineage cells into cholinergic amacrine cells.

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ABBREVIATIONS USED: RPC, retinal progenitor cell; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; RGC, retinal ganglion cells; hHLH, basic helix-loop-helix; TF, transcription factor; CNS, central nervous system

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INTRODUCTION

In the retina, the six major neuronal types and one glial cell type are generated from a common pool of multipotent retinal progenitor cells (RPCs) (Turner and Cepko, 1987; Turner et al., 1990), and are located in three distinguishable nuclear layers (Young, 1985; Cepko et al., 1996; Livesey and Cepko, 2001; Hatakeyama and Kageyama, 2004; Obsawa and Kageyama, 2008; Wallace, 2008). Cone and rod photoreceptors in the outer nuclear layer (ONL) receive visual signals. Interneurons including horizontal cells, amacrine cells, and bipolar cells, are primarily positioned in the inner nuclear layer (INL) and convey visual information from photoreceptor cells to retinal ganglion cells (RGCs) in the ganglion cell layer (GCL). RGCs, the only efferent neurons in retina, receive visual information from amacrine and bipolar cells and transfer it to the brain. Based on their morphology, sublaminar localization, electrophysiology, and neurotransmitter types, the six major neuronal types can be further classified into subtypes, and currently about 80 different neuronal subtypes have been identified in mouse retina (Masland, 2001a,b). However, how these subtypes arise at the molecular level remains poorly understood.

Recent studies have shown that cell type–specific transcription factors (TFs) are expressed during retinogenesis and regulate unique aspects of retinal development and function. Importantly, such TFs are required for the specification and differentiation of bipolar and amacrine cell types (Chow et al., 2001; Bramblett et al., 2002; Li et al., 2004; Feng et al., 2006; Elshatory et al., 2007; Ding et al., 2009b). Among these TFs, the basic helix-loop-helix (bHLH) TFs play a central role in regulating cell fate throughout development, including in the retina. Based on their morphology, sublaminar localization, electrophysiology, and neurotransmitter types, the six major neuronal types can be further classified into subtypes, and currently about 80 different neuronal subtypes have been identified in mouse retina (Masland, 2001a,b). However, how these subtypes arise at the molecular level remains poorly understood.

Recent studies have shown that cell type-specific transcription factors (TFs) are expressed during retinogenesis and regulate unique aspects of retinal development and function. Importantly, such TFs are required for the specification and differentiation of bipolar and amacrine cell types (Chow et al., 2001; Bramblett et al., 2002; Li et al., 2004; Feng et al., 2006; Elshatory et al., 2007; Ding et al., 2009b). Among these TFs, the basic helix-loop-helix (bHLH) TFs play an important role in retinal cell specification and differentiation along with the homeodomain TFs. Some bHLH repressor genes such as *Hes1* and *Hes5* promote the proliferation of RPCs to ensure a sufficient number of RPCs for the differentiation of a full range of cell types (Williams et al., 2010; Kucukdereli et al., 2011). In contrast, the bHLH activator genes are known to promote neurogenesis. For example, in mice, *Atoh7* is required for RGC development by establishing RGC competence but not cell fate specification (Yang et al., 2003; Brzezinski et al., 2012). Targeted deletion of *Atoh7* blocks the initial differentiation of RGCs and results in an increase amacrine cell differentiation (Wang et al., 2001). The bHLH factors also specify retinal cell types in combination with homeodomain (HD) TFs (Hatakeyama et al., 2001; Reese, 2011). Studies have shown that co-expression of MASH1 and MATH3 promotes neurogenesis over gliogenesis while CHX10 specifies cells of the inner nuclear layer (Hatakeyama et al., 2001). In addition, co-expression of CHX10 and MASH1 or MATH3 promotes the generation of bipolar cells (Hatakeyama et al., 2001). Similarly, during amacrine cell development, expression of NEUROD or MATH3 together with PAX6 or SIX3 promotes the generation of amacrine cells, confirming bHLH TFs’ key roles in the neuronal fate specification (Inoue et al., 2002).

BHLHB5, a member of OLG-subclass of bHLH TFs, is expressed in the CNS, sensory organs, kidney, and hair follicles and is thought to function as a negative regulator of other bHLH proteins (Kim et al., 2002; Xu et al., 2002; Brunelli et al., 2003). In mice lacking *Bhlhb5* develop self-inflicted skin lesions and display selective loss of a subset of inhibitory interneurons in the dorsal horn that regulate itching response (Ross et al., 2002; Brunelli et al., 2003). In *Bhlhb5*-null mice, corticospinal motor neurons terminate prematurely along the pyramidal tract in the ventral hindebrain and fail to enter the spinal cord (Joshi et al., 2008). In addition, mice lacking Bhlhb5 develop self-inflicted skin lesions and display selective loss of a subset of inhibitory interneurons in the dorsal horn that regulate itching response (Ross et al., 2010). We have previously shown that BHLHB5 is expressed in GABAergic amacrine and Type 2 OFF-cone bipolar (CB) cells, and targeted deletion of Bhlhb5 results in a reduction of these neuron subtypes (Feng et al., 2006). However, whether BHLHB5 expression is restricted to GABAergic amacrine and Type 2 OFF-CB cells and has a purely instructive role in specifying their cell fate or whether BHLHB5 has a broader expression and function in cell fate specification during retinogenesis remains unknown. Here, we use a lineage-tracing strategy (Novak et al., 2000; Yang et al., 2003; Feng et al., 2010) to trace Bhlhb5 cell lineage during retinogenesis. We demonstrate that *Bhlhb5* expression is turned on in RGCs, GABAergic amacrine cells, glycinergic amacrine cells, OFF-CB cells, rod bipolar cells, ON-bipolar cells, and rod photoreceptors. In *Bhlhb5*-null retinas, GABAergic and glycinergic amacrine cells and Type 2 OFF-CB cells are decreased, which consequently leads to the reduction of the dendritic strata formed by *Bhlhb5* lineage cells in the IPL. In addition, *Bhlhb5* lineage cells take on the identity of cholinergic amacrine cells in the absence of *Bhlhb5*, suggesting a cell fate conversion among amacrine subtypes. Thus, BHLHB5 plays a broader role in regulating cell differentiation in the retina.

RESULTS

Spatiotemporal Pattern of *Bhlhb5* Lineage Cells During Mouse Retina Development

Previously, we have shown that BHLHB5 is expressed in GABAergic amacrine and Type 2 OFF-CB neuronal subtypes and is required for their development (Feng et al., 2006). To determine if *Bhlhb5* expression is sufficient to specify the fate of GABAergic amacrine and Type 2 OFF-CB cells, we first investigated whether *Bhlhb5* expression is limited to these cells during mouse retinal development. We crossed *Bhlhb5*<sup>Cry</sup> (Joshi et al., 2008) and *Bhlhb5*<sup>Cry</sup>;*Z/<sup>EG</sup>* knock-in mice with *Z/EG* conditional GFP reporter mice (Novak et al., 2000) to trace the lineage of Bhlhb5<sup>Cry</sup> cells. The Bhlhb5<sup>Cry</sup> knock-in mice express Cre recombinase under *Bhlhb5* regulatory sequences. Crossing Bhlhb5<sup>Cry</sup> mice with the *Z/EG* mice leads to the constitutive expression of GFP in the cells once expressing Bhlhb5, allowing the lineage tracing and characterization of these cells (Fig. 1A). We crossed Bhlhb5<sup>Cry</sup>;*Z/+*, Bhlhb5<sup>Cry</sup>;*Z/EG*, and *Z/EG* to obtain the Bhlhb5 heterozygotes (Bhlhb5<sup>Cry</sup>;*Z/EG*) and Bhlhb5 homozygous null (Bhlhb5<sup>Cry</sup>;*Z/EG*) mice. Consistent with our previous results (Feng et al., 2006), the Bhlhb5<sup>Cry</sup> heterozygotes were indistinguishable from their wild type littermates and were used as controls. The Bhlhb5-null mice were born at...
Mendelian frequencies and survived into adulthood with apparent reduction in size and body weight. Consistent with BHLHB5 expression, the onset of GFP expression in Bhlhb5\(^{Cre/\text{loxp}}\);Z/EG retinas was first detected at E13.5 in the newly formed GCL (Fig. 1B). Later, GFP\(^{+}\) Bhlhb5 lineage cells were seen in both the neural blast layer (NBL) and the GCL at E15.5 (Fig. 1C). At P0, Bhlhb5 lineage cells were found in the GCL and the NBL (Fig. 1D). When the retinal layer structure is formed at P14, Bhlhb5 lineage cells were observed in all three cellular layers with a majority of Bhlhb5 lineage cells distributed in the INL and their GFP\(^{+}\) strata in different sublaminar layers (Fig. 1E), suggesting that Bhlhb5 expression alone is not limited to GABAergic amacrine and Type 2 OFF-CB cells and that Bhlhb5 expression is insufficient to specify the fate of these cells.

To assess the mosaicism of GFP expression, we co-labeled mature Bhlhb5\(^{Cre/\text{loxp}}\);Z/EG mouse retinas...
with anti-GFP and anti-BHLHB5 in whole mounts. As shown in Figure 1F–K, the total number of GFP\(^{+}\) cells is greater than that of BHLHB5\(^{+}\) cells because the lineage tracing method labels all cells presently and previously (transiently) expressing Bhlhb5 while anti-BHLHB5 labeled the cells with present BHLHB5 expression only. The BHLHB5\(^{+}\) cells in GCL and INL are mostly GFP\(^{+}\) in Bhlhb5\(^{Cre/+}\);Z/EG mice (Fig. 1F–K). Quantification of the immunolabeled retinal flat mounts revealed that about 85.7% of the BHLHB5\(^{+}\) cells in the GCL are GFP\(^{+}\) (48 ± 6 out of 56 ± 6, n=3) and that 90% of the BHLHB5\(^{+}\) cells in the INL are GFP\(^{+}\) (206 ± 21 out of 229 ± 16, n=3). This slight mosaicism is likely due to the incomplete Cre-mediated recombination event or to mosaic expression of the GFP transgene or both. Nevertheless, this genetic tracing approach appears suitable for the spatiotemporal analysis of the Bhlhb5 lineage cells throughout retinogenesis.

### Analysis of Bhlhb5 Cell Lineage in Amacrine Subtypes

To determine the amacrine subtype identities of Bhlhb5 lineage cells, we co-labeled Bhlhb5\(^{Cre/+}\);Z/EG retinas with anti-GFP and various retinal amacrine cell markers at P21 (Fig. 2). In the inner part of the INL, co-labeling of GFP and Pax6, a panamacrine marker, revealed that 32.3 ± 7% of the amacrine cells originated from the Bhlhb5 lineage cells (Fig. 2A–C, arrows). A majority of these GFP\(^{+}\) amacrine cells in the INL and nearly all of the GFP\(^{+}\) cells in the GCL are GAD65\(^{+}\), accounting for 45.8 ± 4% of all GAD65\(^{+}\) GABAergic amacrine cells (Fig. 2D–F, arrows). Immunolabeling of GFP also revealed four distinguishable GFP\(^{+}\) strata within the IPL. These sublaminae were formed by the dendrites of Bhlhb5 lineage cells in the INL and GCL, and precisely overlapped the strata of GAD65\(^{+}\) GABAergic amacrine cells (Fig. 2D–F). The remaining, small portion of GFP\(^{+}\) cells in the INL were co-localized with 12.5 ± 4% of GLYT1\(^{+}\) cells (Fig. 2G–I, arrows), indicating their glycinergic amacrine cell identity. However, none of the GFP\(^{+}\) cells was ChAT\(^{+}\) cholinergic amacrine cell or calretinin\(^{+}\) amacrine cell (Fig. 2J–O). Consistently, the GFP\(^{+}\) strata did not overlap those formed by ChAT\(^{+}\) or calretinin\(^{+}\) amacrine cells (Fig. 2J–O). Furthermore, GFP\(^{+}\) Bhlhb5 lineage cells did not express tyrosine hydroxylase (TH), a dopaminergic amacrine cell marker (Fig. 2P–R). Thus, by immunostaining of GFP with different amacrine cell markers, we demonstrate that Bhlhb5 is selectively expressed in both GABAergic and glycinergic amacrine cells in the mouse retina.

### Subtype-Specific Loss and Cell Fate Conversion of the Bhlhb5 Lineage Cells in the Absence of Bhlhb5

The above Bhlhb5 cell lineage tracing experiments in Bhlhb5\(^{Cre/+}\);Z/EG mice reveals that Bhlhb5 is expressed in a variety of retinal cell types during retinogenesis. To further elucidate the role of Bhlhb5 and to determine whether BHLHB5 is cell-autonomously required for the differentiation of these cells, we generated Bhlhb5-null (Bhlhb5\(^{Cre/lox}\);Z/EG) mice and analyzed the Bhlhb5 cell lineage in the absence of Bhlhb5. First, we performed double-immunostaining for GFP and various amacrine cell markers to test the effect of Bhlhb5 knockout on amacrine subtypes. Without Bhlhb5, the GAD65-positive GABAergic amacrine cells were reduced by about 51% and the GFP/GAD65 cohorts by approximately 49% (Fig. 5A,B, and K; arrows). Similarly, GLYT1\(^{+}\) glycinergic amacrine cells were reduced by about 70% compared to those in the Bhlhb5 heterozygous retinas, and interestingly, most of the remaining GLYT1\(^{+}\) glycinergic cells were GFP-negative, thus not originating from Bhlhb5 cell lineage (Fig. 5C,D, and K). Although Bhlhb5 is not expressed in ChAT\(^{+}\) or calretinin\(^{+}\) amacrine cells during normal development, approximately 9% ChAT\(^{+}\) cells and 11% calretinin\(^{+}\) cells were formed by the dendrites of Bhlhb5 lineage cells in the ONL and precisely overlapped the strata of GAD65\(^{+}\) GABAergic amacrine cells (Fig. 2D–F). The remaining, small portion of GFP\(^{+}\) cells in the INL were co-localized with 12.5 ± 4% of GLYT1\(^{+}\) cells (Fig. 2G–I, arrows), indicating their glycinergic amacrine cell identity. However, none of the GFP\(^{+}\) cells was ChAT\(^{+}\) cholinergic amacrine cell or calretinin\(^{+}\) amacrine cell (Fig. 2J–O). Consistently, the GFP\(^{+}\) strata did not overlap those formed by ChAT\(^{+}\) or calretinin\(^{+}\) amacrine cells (Fig. 2J–O). Furthermore, GFP\(^{+}\) Bhlhb5 lineage cells did not express tyrosine hydroxylase (TH), a dopaminergic amacrine cell marker (Fig. 2P–R). Thus, by immunostaining of GFP with different amacrine cell markers, we demonstrate that Bhlhb5 is selectively expressed in both GABAergic and glycinergic amacrine cells in the mouse retina.

### Expression of Bhlhb5 in photoreceptor cells and RGCs

Although a majority of GFP\(^{+}\) cells were observed in the INL, some GFP\(^{+}\) cells were also seen in the GCL and the ONL. In order to identify these cells, we triple-immunolabeled Bhlhb5\(^{Cre/+}\);Z/EG retinal sections with anti-GFP, anti-RXRx (a cone photoreceptor marker), and anti-rhodopsin (a rod photoreceptor marker), and revealed that all of the Bhlhb5 lineage cells in the ONL expressed rhodopsin, but not RXRy, and were thus rod photoreceptors (Fig. 4A–D). Moreover, we did not detect the expression of calbindin and glutamine synthetase (GS) in the GFP\(^{+}\) Bhlhb5 lineage cells, thus excluding their identities as horizontal cells or Müller glial cells, respectively (Fig. 4E–J). Double-labeling of GFP with RGC marker anti-POU4F1 and anti-POU4F2 revealed a small number of Bhlhb5 lineage cells as RGCs (5.8 ± 2% RGCs express GFP) (Fig. 4K–N, arrows). Therefore, in addition to the bipolar and amacrine subtypes, a small subset of RGCs and rod photoreceptors are generated from the Bhlhb5 cell lineage.
were GFP$^+$ in Bhlhb5-null retinas, indicating a cell fate conversion (Fig. 5F–H, arrows). Furthermore, about 90% TH$^+$ dopaminergic amacrine cells were lost in Bhlhb5-null retinas even though Bhlhb5 was not expressed in dopaminergic amacrine cells (Fig. 5I,J). Therefore, our cell lineage tracing results demonstrate that BHLHB5 regulates amacrine cell specification in a subtype-specific manner.
Subtype-Specific Loss of Bhlhb5 Lineage Bipolar Cells in Bhlhb5-Null Retinas

To assess the role of Bhlhb5 in bipolar cells, we similarly performed the double immunolabeling of anti-GFP and different bipolar cell markers. We found that the total number of VSX1⁺ OFF-CB cells was reduced by approximately 51% while the GFP/VSX1 cohorts were decreased by about 75% (Fig. 6C,D, arrows). Strikingly, targeted deletion of Bhlhb5 resulted in the complete loss of Type 2 OFF-CB cells coming from Bhlhb5 cell lineage (GFP⁻ and recoverin⁻) (Fig. 6C,D, arrows). In comparison, the number of PKCa⁺ rod bipolar cells and GOα⁺ ON-bipolar cells were not affected by Bhlhb5-null mutation (Fig. 6E–H). Thus, Bhlhb5-null mutation also affects the development of bipolar cells in a subtype-specific manner.

DISCUSSION

Previously we have shown that BHLHB5 expression is detected by anti-BHLHB5 in GABAergic amacrine and Type 2 OFF-CB cells in adult mouse retinas (Feng et al., 2006). Since most retinal subtype-specific markers are not expressed or their expression has yet to be confined to individual retinal subtypes during early retinogenesis, we have not been able to conclude that Bhlhb5 expression is limited to and is specifically required for the development of these two retinal subtypes during retinogenesis. This Bhlhb5 cell lineage study demonstrates that Bhlhb5 is expressed in more retinal neuronal
subtypes than those detected by anti-BHLHB5. In addition to GABAergic amacrine and Type 2 OFF-CB cells, we found that Bhlhb5 is expressed in ganglion, glycinergic amacrine, rod bipolar, ON-bipolar, and rod photoreceptor cells. The multiple cell lineage expression of Bhlhb5 suggests its broader role in retinogenesis. However, Bhlhb5 lineage cells do not give rise to cone, horizontal, and Müller glial cells, suggesting that Bhlhb5 may be expressed in a selective pool of progenitors or cell lineage. Moreover, this expression of Bhlhb5 is transient during retinal development and when retinogenesis is completed, its expression is turned off in all cells except the GABAergic amacrine and Type 2 OFF-CB cells. Previously, we have shown that the expression of Bhlhb5 starts at E11.5, a time when retinal neurogenesis begins, and persists through adulthood (Feng et al., 2006).

By tracing the lineage of Bhlhb5, we detect the expression of BHLHB5 in both early born (ganglion and amacrine cells) and late born neurons (bipolar and rod photoreceptor cells), indicating that Bhlhb5 is expressed in and regulates the differentiation of two developmentally different pools of RPCs.

We have previously reported that targeted deletion of Bhlhb5 results in the loss of one-half of the GABAergic amacrine and one-third of the VSX1+ CB cells in adult retinas (Feng et al., 2006). It remains unknown whether BHLHB5 expression is limited to these and cell-autonomously specifies their cell fate. Using the lineage tracing approach, we have analyzed the full impact of Bhlhb5-null mutation on retinal neurons. We have been able to confirm the cell type-specific loss of GABAergic amacrine cells. Our cell lineage tracing experiments similarly reveal that in Bhlhb5-null retinas, there is a loss of about 50% GAD65+ amacrine cells that originated from the GFP+ Bhlhb5 cell lineages (Fig. 5A,B). However, compared with the loss of one-third of the VSX1+ CB cells, the loss of bipolar cells positive for VSX1 and GFP is greater at about 75% (Fig. 6A,B). Based on their expression of Bhlhb5 and the dependence on Bhlhb5 expression, the GABAergic amacrine and the VSX1+ CB cells could be further divided into additional subtypes. Moreover, not all GABAergic amacrine and VSX1+ CB cells originating from Bhlhb5 lineages are lost in Bhlhb5-null retinas, suggesting a partial cell-non-autonomous function of BHLHB5 in the specification of GABAergic amacrine cells and VSX1+ CB cells. BHLHB5 likely executes its unique functions in different cells, likely in combination with other regulatory proteins.
Our analysis of Bhlhb5 lineage cells also reveals that Bhlhb5-null mutation ablates the glycinergic amacrine cells and recoverin+ Type 2 OFF-CB subtypes originated from Bhlhb5 cell lineage (Figs. 5C,D and 6C,D). Thus, BHLHB5 likely plays a cell-autonomous role in these amacrine and bipolar subtypes. Conversely, about 90% of TH+ dopaminergic amacrine cells are missing in Bhlhb5-null retina even though BHLHB5 is not expressed in dopaminergic amacrine cells during normal development, suggesting its non-cell-autonomous role. Moreover, during normal retinal development, Bhlhb5 lineage cells do not produce ChAT+ cholinergic amacrine nor calretinin+ amacrine cells (Fig. 2). However, in the absence of Bhlhb5, GFP+ Bhlhb5 lineage cells develop into ChAT+ or calretinin+ amacrine cells, indicating that BHLHB5 likely acts as a repressor to negatively regulate the differentiation of ChAT+ and calretinin+ amacrine cells and that in its absence, Bhlhb5 lineage cells switch the fate to become ChAT+ or calretinin+ amacrine cells (Fig. 5). Furthermore, though some RGCs are generated from Bhlhb5 lineage, there is no significant difference in the number of RGCs positive for POU4F1 or POU4F2 (data not shown), suggesting that Bhlhb5 should be transiently expressed in RGC lineage but might be required for RGC development.

In the developing CNS, BHLHB5 is expressed in layers II–V of the developing neocortex and acts as an area-specifying transcription factor. Ablation of Bhlhb5 resulted in the abnormal area-specific gene expression and disorganized somatosensory and caudal motor cortices structure (Joshi et al., 2008). Bhlhb5 also plays an essential role in itch neural circuit and is required for specific inhibitory interneurons that regulate pruritus. Deletion of Bhlhb5 caused a significant loss of inhibitory synaptic inputs and resulted in abnormal itch (Ross et al., 2010). During spinal cord development, BHLHB5 expression is found in the dI6 dorsal interneurons, V1 and V2 ventral interneurons at early stage, and a specific subset of late-born dorsal association interneurons at late stage (Liu et al., 2007). BHLHB5 regulates the spatially...
restricted expression of the Notch ligands and Fringe proteins by transforming the spatial information into local cell interactions to control the neurogenesis and cell specification in spinal cord (Skaggs et al., 2011). Recent study shows that BHLHB5 and PRDM8, a PR/SET domain protein, form a repressor complex to regulate the normal neural circuit formation by repressing the cadherin-11 function (Ross et al., 2012). In the retina, Prdm8 expression is detected in a subset of bipolar cells and in the non-cholinergic amacrine cells in the INL (Komai et al., 2009), resembling that of Bhlhb5. Interestingly, in the absence of Prdm8, there is a 33% reduction in INL cell number and, specifically, Type 2 OFF-bipolar cells are virtually absent (Jung, 2008). Thus, it is likely that a similar, repressive regulatory pathway mediated by BHLHB5/PRDM8 exists in the retina and is required for the development of Type 2 OFF-bipolar cells. Further studies are warranted to compare the roles of these two genes in mice null for Bhlhb5 or Prdm8 or both.

Conclusions
In our study, we used Cre-loxP recombination strategy to trace the fate of the Bhlhb5 lineage cells in the retinas of Bhlhb5 heterozygous and homozygous null mice. We demonstrate that during retinal development, Bhlhb5 expression occurs in a variety of retinal cell types including RGCs, GABAergic amacrine cells, glycinergic amacrine cells, Type 2 OFF-CB cells, rod bipolar cells, ON-bipolar cells, and rod photoreceptor cells. Targeted deletion of Bhlhb5 results in a significant loss of GABAergic, glycinergic, and dopaminergic amacrine subtypes, and VSX1+ CB and recoverin+ Type 2 OFF-CB subtypes. The Bhlhb5 cell lineage analysis also demonstrates that Bhlhb5 is cell-autonomously and non-cell-autonomously required for the development of glycinergic amacrine cells and recoverin+ Type 2 OFF-CB subtypes as well as for a majority of GABAergic amacrine and VSX1+ CB cells. In addition, some Bhlhb5 lineage cells switch their fate into cholinergic amacrine cells in the absence of Bhlhb5. Thus Bhlhb5 is required for retinal interneuron subtype specification and plays a role in amacrine cell subtype fate choice.

EXPERIMENTAL PROCEDURES

Animals
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the University Committee of Animal Resources (UCAR Protocol No. 101414) at the University of Rochester. Bhlhb5Cre/+ and Bhlhb5lacZ/+ mice were generated previously (Feng...
et al., 2006; Joshi et al., 2008). The Z/EG conditional enhanced GFP reporter mice (Novak et al., 2000) were purchased from the Jackson Laboratory (Bar Harbor, ME; stock number 003920). Embryos were designated as E0.5 at noon on the day at which vaginal plugs were observed. The day of birth was considered as P0.

Histochemistry and Immunohistochemistry

Staged mouse embryos were dissected and immediately fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C for 2–3 hr. After dehydration using graded sucrose, samples were embedded and frozen in OCT medium (Tissue-Tek), and sectioned at 14-μm thickness. For samples harvested after birth, vascular perfusion was performed to eliminate the blood in the vessels, and then retinas were dissected and fixed in PFA. Retinal flat mount immunostaining was performed as previously described (Ding et al., 2009a). Dilution and sources of antibodies used in this study were: chicken anti-GFP (1:1,000, Abcam, Cambridge, MA), goat anti-BHLHB5 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-POU4F1 (1:500, Santa Cruz), mouse anti-calbindin (1:500, Sigma, St. Louis, MO), rabbit anti-calretinin (1:2,000, Oncogene, Cambridge, MA), rabbit anti-CHAT (1:200, Chemicon, Temecula, CA), sheep anti-CHX10 (1:200, Exalpha, Shirley, MA), mouse anti-GAD65 (1:200, BD Biosciences, Franklin Lakes, NJ), mouse anti-PAX6 (1:200, DSHB, Iowa City, IA), rabbit anti-PKCa (1:5,000, Sigma), rabbit anti-recoverin (1:200) (Chow et al., 2001), rabbit anti-TH (1:200, Chemicon), rabbit anti-Vsx1 (1:100) (Chow et al., 2001). Alexa-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used at a dilution of 1:1,000. Images were captured with a Zeiss (Thornwood, NY) 510 META confocal microscope.

Statistical Analysis

Cell number quantification of different retina cell markers was performed with retina sections from at least three age-matched animals for each cell type. Data are represented as mean ± SEM. Statistical analysis was performed using paired two-sample Student’s t-test. A value of P < 0.05 was considered statistically significant.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

LH, XYZ, JLY, and LG conceived and designed the experiments; LH, FH, LF, XJL, and GQL performed the experiments; LH and LG analyzed the data; LH and LG wrote the paper.

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REFERENCES


Liu B, Liu Z, Chen T, Li H, Qiang B, Yuan J, Peng X, Qi M. 2007. Selective expression of Bhlhb5 in subsets of...