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### Authors

Riesenberg, Amy N  
Conley, Kevin W  
Le, Tien T  
[et al.](#)

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## Separate and coincident expression of *Hes1* and *Hes5* in the developing mouse eye

Amy N. Riesenber<sup>1</sup>, Kevin W. Conley<sup>1,#</sup>, Tien T. Le<sup>1</sup>, and Nadean L. Brown<sup>1,2</sup>

<sup>1</sup>Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229

<sup>2</sup>Department of Cell Biology & Human Anatomy, University of California Davis, One Shields Avenue, Davis, CA 95616

### Abstract

**Background**—Notch signaling is broadly required during embryogenesis, frequently activating the transcription of two bHLH transcription factors, *Hes1* and *Hes5*. But, it remains unresolved when and where *Hes1* and *Hes5* act alone or together during development. Here, we analyzed a *Hes5*-GFP BAC transgenic mouse, as a proxy for endogenous *Hes5*. We directly compared transgenic GFP expression with *Hes1*, and particular markers of embryonic lens and retina development.

**Results**—*Hes5*-GFP is dynamic within subsets of retinal and lens progenitor cells, and differentiating retinal ganglion neurons, in contrast to *Hes1* found in all progenitor cells. In the adult retina, only Müller glia express *Hes5*-GFP. Finally, *Hes5*-GFP is upregulated in *Hes1* germline mutants, consistent with previous demonstration that *Hes1* suppresses *Hes5* transcription.

**Conclusions**—*Hes5*-GFP BAC transgenic mice are useful for identifying *Hes5*-expressing cells. Although *Hes5*-GFP and *Hes1* are coexpressed in particular developmental contexts, we also noted cohorts of lens or retinal cells expressing just one factor. The dynamic *Hes5*-GFP expression pattern, coupled with its derepressed expression in *Hes1* mutants, suggests that this transgene contains the relevant cis-regulatory elements that regulate endogenous *Hes5* in the mouse lens and retina.

### Keywords

*Hes1*; *Hes5*; lens development; retina; neurogenesis; Notch signaling

### Introduction

The Notch signaling pathway maintains a balance between dividing and differentiating cells, and releases waves of progenitors to initiate differentiation at the right time, and in the correct proportions. A *Notch* signal initiated by ligand-receptor binding, invokes a series of proteolytic events, resulting in Notch intracellular protein domain (ICD) translocation into

Author for correspondence: Nadean L. Brown, Ph.D., Department of Cell Biology & Human Anatomy, University of California, Davis, Room 4407 Tupper Hall, Davis, CA 95616, nlbrown@ucdavis.edu, Phone: 530-752-7806.

<sup>#</sup>Present address: Institute for Quality Science Teaching and Learning, Museum of Science and Industry, Chicago, IL 60637

the nucleus. The Notch-ICD forms a complex with nuclear proteins RBPJ/Su(H) and MAML to activate target gene transcription, including the *Hes1* and *Hes5* transcriptional repressors (reviewed in Davis and Turner, 2001; Kageyama et al., 2007; Kopan and Ilagan, 2009). To fully understand Notch signaling mechanisms during development, it is necessary to define the contexts that require different combinations of downstream effector genes.

The rodent eye is an established model for investigating mechanisms of progenitor cell proliferation and cell fate specification, and the ocular expression patterns of Notch pathway genes have been described over the past decade (Lindsell et al., 1996; Bao and Cepko, 1997). Lens development initiates when an ectodermally derived lens placode evaginates to form a lens vesicle, from which differentiated fiber cells arise in two separate waves. *Jag1*, *Notch1*, *Notch2*, *Rbpj*, and *Hes1* are more or less ubiquitous in early lens progenitor cells. However, once fiberogenesis initiates, the expression of both Notch receptors, *Rbpj*, *Hes1* and *Hes5* are all restricted to the anterior epithelial layer (AEL), whereas *Jag1* is localized to the equatorial transition zone, where overt differentiation occurs (Jia et al., 2007; Rowan et al., 2008; Le et al., 2009; Le et al., 2012). Multiple loss-or-gain of function studies support the idea that a *Jag1-Notch2-Rbpj* mediated signal stimulates AEL cells to either proliferate or differentiate (Jia et al., 2007; Rowan et al., 2008; Le et al., 2009; Saravanamuthu et al., 2012).

Simultaneously, the optic vesicle, which is comprised of neuroepithelial cells, invaginates into the optic cup/retina. Four retinal cell types (RGCs, cones, amacrine and horizontal neurons) appear largely before birth, whereas rods, bipolar neurons and Müller glia appear after birth. In the mouse retina *Dll1*, *Dll3*, *Dll4*, *Notch1*, *Notch3*, *Rbpj*, *Hes1* and *Hes5* are all expressed during retinal development (Ishibashi et al., 1995; Lindsell et al., 1996; Bao and Cepko, 1997; Lee et al., 2005; Nelson et al., 2006; Nelson and Reh, 2008; Rocha et al., 2009). Numerous loss- and gain-of-function studies demonstrated that a *Dll1-Notch1-Rbpj-Hes1* signal promotes retinal progenitor cell (RPC) proliferation and suppresses RGC neurogenesis, whereas a *Dll4-Notch1-Rbpj* signal stimulates progenitor cells but suppresses cone genesis (Takatsuka et al., 2004; Riesenberg et al., 2009; Rocha et al., 2009; Zheng et al., 2009; Maurer et al., 2014). The Notch pathway generally blocks neurogenesis by suppressing proneural bHLH factor expression. For example, *Hes1* transcriptionally represses multiple proneural bHLH genes (reviewed in Ohtsuka et al., 1999; Kageyama et al., 2008; Ohsawa and Kageyama, 2008), including *Atoh7*, which is essential for RGC development (Takatsuka et al., 2004; Lee et al., 2005). *Hes5* has been described to similarly suppress neurogenesis in the CNS, but its molecular mode of action during early retinal development and its roles within Müller glia of the postnatal retina remain poorly understood. Moreover, it is unclear when and where developing lens and retinal cells utilize *Hes1* or *Hes5* alone or together.

Here we evaluated *Hes5*-GFP BAC Tg mice for their recapitulation of endogenous *Hes5* expression in the developing mouse eye. We found *Hes5*-GFP expression initiates at E10.5 in discrete subsets of lens pit and optic vesicle cells, but in the lens subsequently becomes expressed by all lens AEL and transition zone cells. Once retinal neurogenesis begins in the optic cup, we saw distinct cohorts of RPCs expressing *Hes5*-GFP, *Hes1* or both. At E11.5, retinal cells expressing *Hes5*-GFP only coexpressed *Pou4f+*, a definitive RGC marker.

Moreover, we found that nascent cone photoreceptors do not express Hes5-GFP. In the adult retina, Hes5-GFP is only present in Müller glia. The diversity of Hes5-GFP BAC expression by embryonic RGCs and adult Müllers is consistent with the dynamics of endogenous *Hes5* mRNA expression (Hojo et al., 2000; Nelson et al., 2011). Finally, we observed Hes5-GFP is derepressed when *Hes1* gene dosage was reduced.

## Results

Although by no means the only readout of Notch signaling, cells expressing *Hes1* and/or *Hes5* are categorized as recipients of a canonical Notch signal (Kageyama et al., 2008; Kopan and Ilagan, 2009). In the mouse there are specific antibodies that label Hes1-expressing cells (Lee et al., 2005), but no Hes5 antibody of comparable quality and specificity. Therefore it has not been possible to visualize which ocular cells express Hes5 protein, alone or in combination with Hes1. In an effort to circumvent this issue, we evaluated a Hes5-GFP BAC transgenic (Tg) mouse, obtained from the Mutant Mouse Regional Resource. We directly compared GFP expression to a battery of early eye development markers, and several Notch pathway proteins, including Hes1.

### Overlapping and distinct Hes1 and Hes5-GFP expression domains in the prenatal eye

This Hes5-GFP BAC transgene inserted onto the mouse X chromosome. Hemizygotes were propagated by mating transgenic females to wild type males, so that on average 50% of the progeny would be transgenic. Over the course of this study, we identified 92 transgenics of 198 total weanling mice (48%, n= 18 litters) using the MMRRC recommended genotyping protocol. Recently, we switched to assessing transgenic inheritance directly in our mouse colony, using a GFP flashlight and scoring expression in adult lenses (see Methods). To date, we have identified 27 GFP+ of 55 total weanling mice (49%, n=6 litters). In our embryonic analyses, we initially compared PCR genotyping to live GFP screening using a fluorescent microscope, UV light source and GFP filter. We saw 100% correlation between these methods, among 31 of 31 transgenic embryos, from 4 litters. As a consequence we switched exclusively to live embryo screening for the Hes5-GFP transgene. To date we identified 232 GFP+ out of 451 embryos (52%, n= 34 litters), including those with the transgene intercrossed with *Hes1 KO/+* or *Hes1KO/+;Atoh7<sup>LacZ</sup>/+* mice. We also observed 100% expressivity of the GFP reporter within cryosections (232 out of 232), all of which displayed the expected tissue-specific patterns. Overall we conclude that this Hes5-GFP BAC transgenic line is both stably inherited and a reliable reporter.

We first assessed the live fluorescence of Hes5-GFP BAC transgenic embryos from E9.5 through E16.5. No GFP expression was obvious at E9.5, but starting at E10.0 (Fig 5A) GFP expression was seen in restricted forebrain and midbrain domains, and more uniformly throughout the hindbrain and spinal cord (Fig 1A, 5A). Between E10.5 to E11, additional somite, olfactory epithelium and eye domains became apparent. At the gross level, Hes5-GFP mimics *Hes5* mRNA expression (compare Fig 1A to 1B). By E13.5 Hes5-GFP was very obvious in the developing eye (arrow in Fig 1C). We next analyzed fixed cryosections, labeled with anti-GFP, and noted patchy Hes5-GFP expression in peripheral RPCs at E10.5 (Fig 1D), which was not maintained at E11, where Hes5-GFP-expressing cells were now

restricted to the central cup (Fig 1E), a domain that had expanded peripherally at E12.5 (Fig 1F). These relatively rapid changes were unexpected, given that GFP transgenic lines are often prone to persistent GFP reporter expression. We also compared anti-GFP labeling of Hes5-GFP to *Hes5* mRNA in situ expression (compare Figs 1F,1G to 1H, Fig 1J to 1K). We observed that *Hes5* mRNA and Hes5-GFP are both present in lens AEL cells, but Hes5-GFP was retained in a subset of in central lens fiber cells (Fig 1G), whose nuclei are Prox1+ (Fig 1I). In the E12.5 central retina both *Hes5* mRNA and Hes5-GFP are robustly expressed, but not at the periphery, where the ciliary body forms. At E14.5, *Hes5* mRNA is restricted to subsets of RPCs, whereas both progenitor cells and nascent RGC neurons express Hes5-GFP, including their axons extending through the optic nerve head (ONH in Fig 1J,1L) and into the optic nerve. Intriguingly, optic nerve head cells, uniquely marked by Pax2 (red nuclei in Fig 1L), do not express Hes5-GFP (arrows in Fig 1F, 1J and 1L).

By antibody multiple labeling, we next compared Hes5-GFP expression with endogenous Jag1, Rbpj and Hes1 proteins, in the E10.5–E13.5 developing eye. In both the E10.5 lens pit and optic cup, Hes5-GFP expression is first seen within subsets of progenitor cells. Those lens progenitor cells expressing Hes5-GFP, co-expressed Jag1 at the cell membrane (Figs 2A, arrows in 2B), whereas other Jag1+ cells (negative for Hes5-GFP), express Hes1 (Fig 2C). All Hes5-GFP cells co-express the ubiquitous Rbpj protein throughout the developing eye (Fig 2C). We conclude that during the early phase of lens development, progenitor cells are actively signaling since they all express Jag1 and Rbpj. However, subpopulations could be identified by virtue of expression of Hes1 alone, or Hes1+Hes5GFP (Fig 2D).

Meanwhile in the adjacent optic cup, distal optic cup cells coexpress Hes5-GFP at the boundaries of a Jag1 distal expression domain (Fig 2A). Although no function has been reported for this specific Jag1 domain, it does precede the advancing wave of neurogenesis from E9.5 to E12.5 (Le et al., 2009; Maurer et al., 2014), further suggesting the potential for a Jag1-mediated signal in the distal optic cup. It is also interesting that Hes5-GFP is more easily visualized within nasal RPCs (Figs 2C, 2D). When retinal neurogenesis initiates at E11.5, the majority of Hes5-GFP+ cells coexpress Hes1 (white arrows), but smaller cohorts of Hes1+ (white arrowheads in Fig 2E) or Hes5-GFP+ cells (pink arrowheads) became distinguishable. At E12.5 a boundary appears between the forming ciliary body and retina (Marcucci et al., 2016). Here too we noted differences between Hes5-GFP and Hes1 protein expression. Although Hes5-GFP is confined to retinal cells (white arrow in Fig 2F, Fig 1F, 1H), Hes1 is expressed by both RPCs and presumptive ciliary body cells. From E12.5 to E16.5, we found central RPCs were double positive for Hes1+Hes5-GFP (arrows in Fig 2G), although some Hes1+ RPCs (arrowheads) was also present. By contrast, Hes5-GFP was uniquely expressed by inner GCL neurons (Fig 2G), corresponding to nascent RGCs (Fig 3).

### Differential Hes expression during prenatal retinal neurogenesis

A long-term goal of our work is to uncover the distinct molecular mechanisms by which Notch signaling regulates RGC versus cone photoreceptor neurogenesis. So we compared Hes5-GFP expression to that of the cell cycle marker *Ccnd1/CyclinD1*, *Atoh7<sup>LacZ</sup>* ( $\beta$ -gal) or *Pou4f*. The *Atoh7* lineage contains RPCs with competence to adopt the RGC fate (Yang et al., 2003; Brzezinski et al., 2012), whereas *Pou4f* expression indicates cells that

differentiated as RGC neurons (Xiang et al., 1993). We noted that Hes5-GFP expression localizes to the central optic cup at E11.5, coincident with the initiation of neurogenesis (Figs 1E, 2E, 3A). While most central RPCs coexpressed Hes5-GFP and *Ccnd1* (Fig 3A), we also saw sparse Hes5-GFP<sup>+</sup> cells in the innermost optic cup (arrows 3B). With the expansion of neurogenesis between E11.5 and E13.5, many more Hes5-GFP cells coexpressed Pou4f<sup>+</sup>, although occasional Pou4f<sup>+</sup> only RGCs were also observed (arrowheads in Fig 3B, Figs 3E, 3G). This is consistent with a recent study reporting that *Hes5* RPCs give rise to all contralateral-projecting RGCs (Kuwajima et al., 2017). The subset of Pou4f<sup>+</sup> only RGCs might correlate with intrinsic differences among RGC subtypes, or with some aspect of Hes5-GFP reporter kinetics. Overall, we conclude that this Hes5-GFP BAC transgene is a short-term lineage tracer for the majority of RGCs.

Next we analyzed Hes5-GFP expression in retinal sections from *Atoh7<sup>LacZ/+</sup>;BAC Tg Hes5GFP* hemizygous embryos. At E12.5 we noted mostly double-positive (GFP<sup>+</sup> β-gal<sup>+</sup>) cells (Figs 3C–3E), but also observed occasional single-labeled cells, either GFP<sup>+</sup> (pink arrowhead in 3C) or β-gal<sup>+</sup> (white arrowhead in 3C, white arrow in 3D insets). We previously showed mutually exclusive expression of Hes1 and β-gal proteins in E11.5 *Atoh7<sup>LacZ/+</sup>* retinas, consistent with Hes1 repression of *Atoh7* expression (Takatsuka et al., 2004; Lee et al., 2005). Therefore, to further highlight the differences between Hes5-GFP and Hes1 expression at the cellular level, *Atoh7<sup>LacZ/+</sup>;Hes5GFP* sections were triple labeled with β-gal, GFP and Hes1 antibodies (Fig 3D). In addition to Hes1-only, Hes5-GFP only and Hes1+Hes5GFP<sup>+</sup> cohorts, we also saw a minor population of β-gal (*Atoh7<sup>LacZ/+</sup>*) cells (arrows pointing to blue-only cells in 3D insets). Similarly, triple labeling of *Atoh7<sup>LacZ/+</sup>;Hes5GFP* sections with β-gal, GFP and Pou4f antibodies, showed many Hes5-GFP/Pou4f double positive cells, particularly in the inner retina, but also sparse GFP<sup>−</sup> or Pou4f<sup>−</sup> only cells (Fig 5E). With the onset of retinal lamination at E13.5, Hes5-GFP<sup>+</sup> cells were present in the neuroblast layer, marked by *Ccnd1* expression (bracket in Fig 3F), and in inner RGCs coexpressing Pou4f (Fig 3G). Importantly, Hes5-GFP expression was absent in Rrg<sup>+</sup> cone photoreceptors (Fig 3H). We conclude that when Hes1 expression is shut off by RPCs adopting the RGC fate, Hes5-GFP becomes activated within these differentiating neurons. Moreover, cone photoreceptors do not express Hes5-GFP.

### Hes5-GFP and adult Müller glia

Throughout the developing CNS, glial cells are generated at the end of histogenesis. This also occurs in the retina where Müller glia are among the last cell types to develop (reviewed in Jadhav et al., 2009). Here too, Notch signaling and its downstream effectors, Hes1 and Hes5 are required for Müller glial differentiation (Hojo et al., 2000; Bernardos et al., 2005; Muto et al., 2009; Nelson et al., 2011). *Hes5* mRNA was been previously shown to be restricted to Müller glia beginning at postnatal day 7 (P7) through adulthood (Nelson et al., 2011). Anti-GFP labeling of P21 BAC Tg *Hes5GFP* hemizygous cryosections showed localization to cells whose somas reside in the INL and processes span the entire retinal width (Figs 4A,B). This is consistent with Müller cell morphology. To validate the specificity of Hes5-GFP expression, we performed double immunofluorescent labeling with Müller markers Rlbp1/CRALBP and Sox9, as well as the bipolar neuronal marker Vsx2/Chx10. Essentially complete coexpression between Rlbp1 and GFP was observed (Figs 4B,

arrows in 4C) and between GFP and nuclear Sox9 in the INL (Fig 4D arrows). Hes5-GFP expression was mutually exclusive to that of Vsx2 in bipolar neuron nuclei (arrowheads in Fig 4E). Overall we conclude that Hes5-GFP accurately reports Müller-specific *Hes5* expression in the adult mouse retina.

### Hes5-GFP transgenic expression is modulated by Hes1 activity

*Hes1* and *Hes5* mRNA are each widely expressed in the developing nervous system, but the patterns are fairly complementary to one another (Hatakeyama et al., 2004). For example *Hes1* is expressed in the anterior neural folds and optic vesicles, whereas *Hes5* normally does not appear in the eye until after E10.5 (Hatakeyama et al., 2004; Lee et al., 2005). These differences in spatiotemporal expression are suggestive of compensatory gene activates, which was demonstrated in double and triple *Hes* mutant embryos (Hatakeyama et al., 2004; Baek et al., 2006). However, *Hes* genes also suppress one another in particular developmental contexts, including in the optic vesicle, where *Hes5* mRNA is upregulated in *Hes1*<sup>-/-</sup> embryos (Hatakeyama et al., 2004). Whether this suppression occurs via direct transcriptional repression or indirectly through canonical Notch signaling has not been addressed in vivo. Therefore, we tested the possibility that Hes1 regulates Hes5-GFP BAC Tg expression by crossing this transgene into a *Hes1* germline mutant background.

With the caveat that our data are not quantitative, we saw that GFP live fluorescence and anti-GFP staining of fixed sections strongly suggest derepression of transgenic expression in *Hes1* mutants (Figs 5A–5C). Antibody labeling of tissue sections verified that there is a complete loss of Hes1 nuclear protein (Figs 5D–5F). Derepression of Hes5-GFP in ocular progenitor cells (lens and retina) persisted through E11.5 (Figs 5G–I) and E12.5 (Figs 5J–5L), when additional eye phenotypes of *Hes1* mutants became manifested. These included premature neuronal differentiation, as marked by *Tubb3* expression (Fig 5I), a morphologically abnormal optic cup (Fig 5I), ectopic neurons in the RPE (Fig 5I) and lens microphthalmia (Fig 5L) (Tomita et al., 1996; Takatsuka et al., 2004; Lee et al., 2005). *Hes1*<sup>+/-</sup> eyes were indistinguishable from wild type littermate controls. These findings are consistent with previous demonstration of *Hes5* mRNA upregulation in *Hes1* mutant ocular progenitor cells (Hatakeyama et al., 2004). Our data favor the idea that *Hes1* normally suppresses *Hes5*, via a Hes1-responsive element contained in *Hes5* noncoding DNA contained within the BAC transgene.

## Discussion

The goal of this study was to evaluate a Hes5-GFP BAC transgenic mouse line as a reporter of Hes5 expression in the developing mouse eye. Hes5-GFP expression is first observable in E10.5 ocular sections, in both lens pit and optic vesicle cells. Once lens fiber cells appear, Hes5-GFP expression became largely localized to progenitor cells in the AEL and transition zone, although transient GFP expression in nascent fiber cells was noted. At the initiation of retinal neurogenesis particular subsets of RPCs expressed Hes5-GFP, Hes1, or both. The Hes5-GFP only retinal cells were most often located in the inner retina and coexpressed the RGC marker *Pou4f*. Yet in the adult retina, Hes5-GFP is specifically expressed by Müller glia (Fig 4 and Nelson et al., 2011). These retinal expression domains are consistent, but not

identical, to those of another *Hes5*-GFP transgene, containing 3KB of genomic DNA from the *Hes5* locus (Basak and Taylor, 2007; Nelson et al., 2011). This conventional transgenic line contains 1.6Kb of 5' and 1.4Kb of intronic and 3' noncoding DNA driving GFP expression from the endogenous promoter. These DNA segments contain 5 putative Rbpj and 1 Smad binding site, with a single Rbpj consensus site sufficient for all Notch1-inducible activity (Basak and Taylor, 2007). In addition, *Hes5* 3' sequences have a negative effect on transgenic expression (Basak and Taylor, 2007). However, it remains unknown if this transgene contains all *Hes5* cis-regulatory elements. Here we not only analyzed the ocular expression profile of a *Hes5*-GFP BAC transgenic mouse line, but compared GFP and Hes1 protein expression on a cell-by cell-basis, during key stages of early eye development.

During eye morphogenesis, *Hes5*-GFP labeled subsets of lens pit and optic vesicle progenitor cells, which along with Jag1 ligand expression in the optic vesicle (Le et al., 2009), is suggestive of differential Notch signaling. *Notch1*, *Rbpj* and *Hes1* exhibit uniform mRNA and/or protein expression patterns at this age (Lindsell et al., 1996; Bao and Cepko, 1997; Lee et al., 2005; Riesenberg et al., 2009), and alpha-Cre;*Jag1* conditional or *Hes5* germline mutants display no optic cup phenotypes (Hojo et al., 2000; Zhou et al., 2013). Therefore, to test the biologic significance of these restricted patterns will necessitate laborious multiple ligand, receptor or effector mutant combinations. *Hes1* and *Hes5* can act redundantly to suppress neurogenesis, including within the embryonic retina. But, the compensatory mechanisms remains incompletely defined, in large part because the types of cells and developmental ages when one or both are expressed are still unknown. The most stringent method for addressing this issue would be to assess protein colocalization, but a specific *Hes5* antibody is not available. Most transgenes are imperfect reporters, largely because their dynamics rarely match the endogenous protein. With this limitation in mind, the *Hes5*-GFP BAC transgenic mouse displayed relatively rapid changes in gene expression, particularly during early stages of eye development. This reporter was excluded from the developing ciliary body, RPE and optic nerve head, which all express Hes1. We also found that unlike *Hes5* mRNA, *Hes5*-GFP expression persists in differentiating RGCs. This last observation confirms a recent study of contralateral-projecting RGCs, which suggested that the major class of RGCs derive from *Hes5* mRNA-expressing progenitor cells (Kuwajima et al., 2017). Finally, we also observed that *Hes5*-GFP is expressed by a fraction of *Atoh7*<sup>LacZ/+</sup> cells. The *Atoh7* retinal lineage gives rise to all 7 cell major cell classes, with ~10% differentiating as RGCs (Brzezinski et al., 2012). Thus it is provocative to think that the GFP/ $\beta$ -gal+ population identifies those progenitors cells differentiating into RGCs.

Basak and Taylor previously demonstrated that *Hes5* responsiveness to activated-Notch1 is mediated through a single Rbpj binding site situated near the 5' promoter (Basak and Taylor, 2007), but the factors required to shut *Hes5* expression off are largely unknown. This same study showed that 3' noncoding sequences reduce *Hes5* transcriptional levels, but the underlying basis, for this was not explored further. We found that a loss of *Hes1* activity derepresses *Hes5*-GFP BAC transgenic expression, thereby extending the established idea of Hes1 repression of *Hes5*, to further support the possibility that it is direct at the *Hes5* locus. In the future, it will be interesting to ask whether this regulatory relationship might be mediated by a particular Notch pathway signal, which stimulates Hes1 to block *Hes5* transcription and how this correlates with the timing of neuronal and glial development.



## Experimental Procedures

### Animals

BAC Tg(Hes5-EGFP)CV50Gsat/Mmmh mice (stock strain 000316-MU) were generated in the GENSAT/NINDS Project by recombineering mouse BAC clone BX480 (RP24-341110) to contain a GFP expression cassette (Gong et al., 2003). The resulting mouse transgenic line was maintained as a hemizygous stock on a CD-1 background, then cryoarchived at the MMRRC. After resuscitation this transgenic line, inserted on the X chromosome, was maintained by mating Hes5-GFP hemizygous females to CD-1 males, and PCR genotyping with recommended primers and protocol ([https://www.mmrrc.org/catalog/sds.php?mmrrc\\_id=316](https://www.mmrrc.org/catalog/sds.php?mmrrc_id=316)), or by visualizing live GFP fluorescence in adult mouse lenses with a GFP flashlight and Royal Blue filter (NightSea). Transgenic embryos were similarly PCR genotyped and/or scored for live GFP expression using an MZ12 Leica dissection scope, equipped with an LED UV light source and GFP filter set.

*Hes1* germline mutant mice (*Hes1<sup>tm1Fgu</sup>*) were maintained on an ICR background and genotyped using published protocols (Ishibashi et al., 1995). *Atoh7<sup>LacZ/+</sup>* mice (*Math5<sup>LacZ</sup> = Atoh7<sup>tm1Gla</sup>*) were maintained on a CD-1 background and genotyped using published protocols (Brown et al., 2001). In some experiments, BAC Tg Hes5-GFP hemizygous females were mated to *Hes1<sup>+/-</sup>* males, creating compound heterozygous females, which were subsequently timed mated with *Hes1<sup>+/-</sup>* male mice for embryonic litters. BAC-Tg Hes5-GFP; *Atoh7<sup>LacZ/+</sup>* embryos were similarly generated from timed matings between BAC Tg Hes5-GFP hemizygous females and *Atoh7<sup>LacZ/LacZ</sup>* males.

The date a vaginal plug was noted was assigned as embryonic age E0.5. A minimum of three embryos per age and genotype, from 2 litters were analyzed in each experiment. All mice were housed and cared for in accordance with guidelines provided by the National Institutes of Health, Bethesda, MD; Association for Research in Vision and Ophthalmology; and conducted with approval and oversight from the Cincinnati Children's Hospital Research Foundation and UC Davis Institutional Animal Care and Use Committees.

### Immunohistochemical analyses

Embryonic and adult eye tissues were fixed in 4% paraformaldehyde/PBS for 40–60 minutes at 4°C, processed through a sucrose/PBS series and cryoembedded. Antibody labeling of 10 micron cryosections used rat anti-βgal (gift from Tom Glaser, 1:1000)(Saul et al., 2008), rabbit anti-Ccnd1/CyclinD1 (Neomarkers clone SP4 1:100); chick anti-GFP (Invitrogen or Abcam 1:1000), rabbit anti-Hes1 (1:1000)(Lee et al., 2005), goat anti-Jag1 (Santa Cruz Biotechnology C-20 1:1000), rabbit anti-Ki67 (Vector Labs 1:1000), goat anti-pan Pou4f (Santa Cruz sc6026 1:50); rabbit anti-Prox1 (Biolegend/Covance 1:6000), rabbit anti-PAX2 (Biolegend/Covance 1:1000); rat anti-Rbpj (CosmoBio USA Clone T6709 1:100), mouse anti-Rlbp1/CRALBP (Affinity Bioreagents 1:100); rabbit anti-Rxrg (Santa Cruz Biotech 1:200); rabbit anti-Sox9 (Chemicon/Millipore 1:200); rabbit anti-Tubb3 (Biolegend/Covance 1:1000); sheep anti-Vsx2/Chx10 (Exalpha Biologicals N-terminus 1:1000); DAPI staining (Sigma Chemical 1:1000 dilution of 1mg/ml stock). Secondary antibodies were directly conjugated to Alexa Fluor350, Alexa Fluor488, or Alexa Fluor594 (Invitrogen), or were

biotinylated (Jackson ImmunoResearch) and then sequentially labeled with Alexa488- or Alexa594-Streptavidin (Jackson ImmunoResearch).

Whole mount or cryosection in situ hybridization were performed as described in (Brown et al., 1998), using a *Hes5* cDNA plasmid as a template for a digoxigenin-labeled antisense riboprobe.

## Imaging

Digital Tiff images of live whole embryo GFP expression or embryos post whole mount in situ hybridization were captured using a Leica MZ12 dissecting microscope equipped with a UV light source, Optronics Magnafire digital B&W and color cameras and software. Digital Tiff images of embryonic eye cryosections, either post in situ or post- antibody labeling were collected using a Zeiss microscope equipped with color and black and white cameras and an Apotome deconvolution device, Axiovision (v7.0). The Adobe Photoshop (CS4) software program was used to convert Tiff images to Photoshop format and equivalently adjust their contrast.

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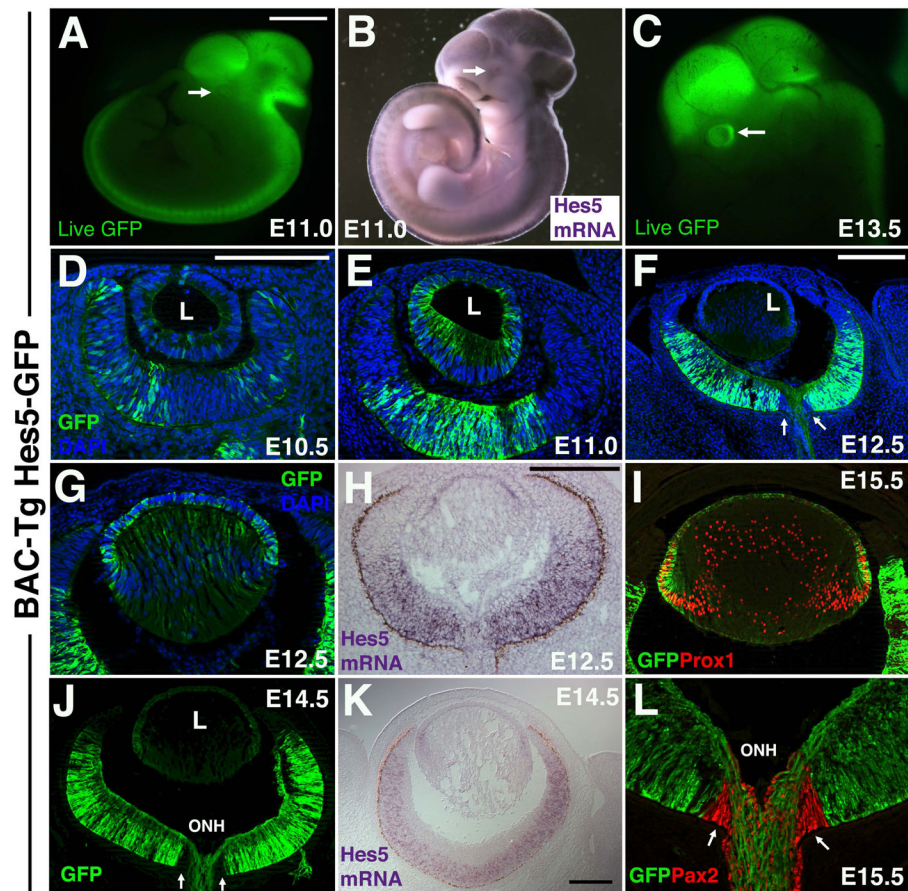
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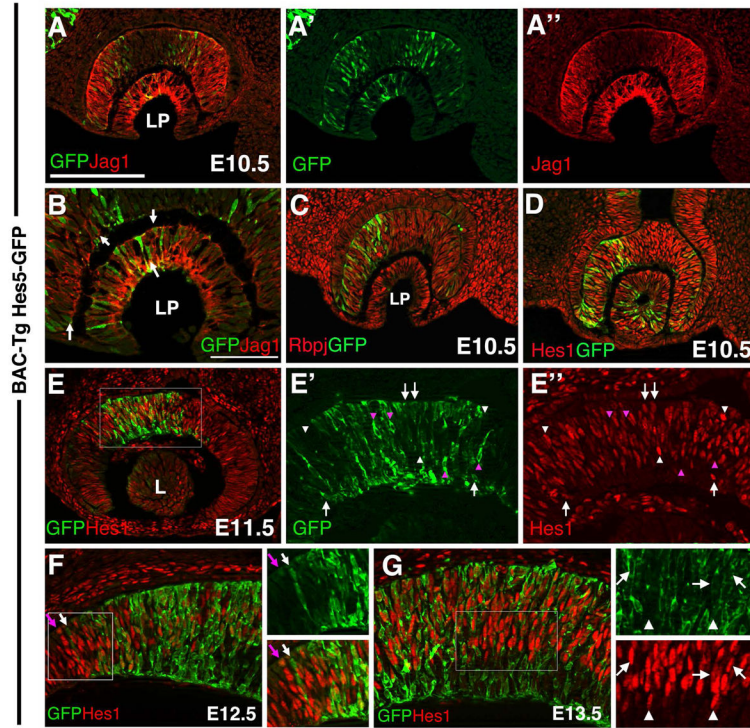
**Key Findings**

- Dynamic Hes5-GFP expression during mouse eye development
- Hes1 and Hes5-GFP delineate subpopulations of lens and retinal progenitor cells
- Hes5-GFP labels embryonic RGCs and their axons
- Hes5-GFP is specifically expressed by adult Müller glia



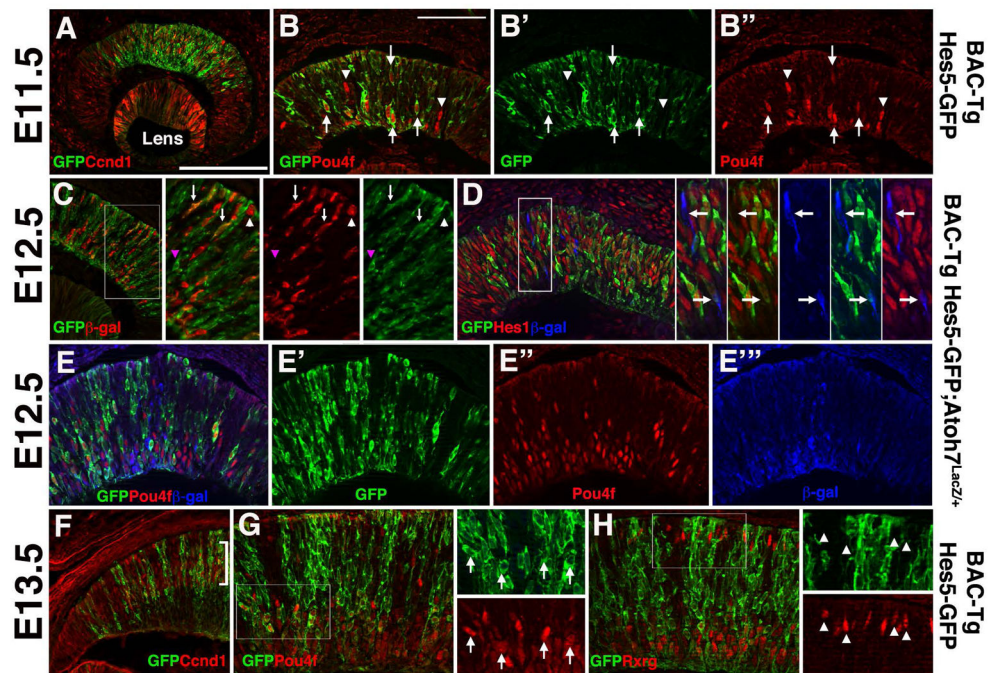
**Figure 1. Hes5-GFP BAC-Tg reporter expression relative to *Hes5* mRNA**

**A,B)** Whole embryo Hes5-GFP fluorescence, compared to *Hes5* mRNA in situ expression. Arrows point to forming eye. **C)** At E13.5 GFP fluorescence in the developing eye is obvious (arrow). In all other panels, GFP expression was visualized by anti-GFP labeling of fixed cryosections, collected in the horizontal plane of sectioning. **D–F)** Dynamic changes in Hes5-GFP during early eye formation. **F–H)** Comparison of Hes5-GFP expression at E12.5 in the developing lens and retina. Arrows in F point to the optic nerve head, which lacks anti-GFP labeling. **I)** E15.5 Hes5-GFP expression in lens AEL and transition zone (bracket), compared to Prox1 nuclear expression (red) within transition zone and lens fiber cell nuclei. **J,K)** Comparison of Hes5-GFP and *Hes5* mRNA expression at E14.5. Hes5-GFP is present in RPCs (like the endogenous gene), but also within RGCs, including their axons extending into the optic nerve head (ONH). **J,L)** Pax2+ optic nerve head (arrows) and optic stalk cells do not express GFP. Dorsal is up in A–C; Anterior is up, and nasal left in panels D–L. L = lens in D–F,J; ONH = optic nerve head in J,L. Scale bar in A=1mm (for A–C); in D (for D,E,G,L) or H=20 microns; in F for F,I,J) or K=10 microns.



**Figure 2. Hes5-GFP expression in the early eye relative to other Notch signaling pathway components**

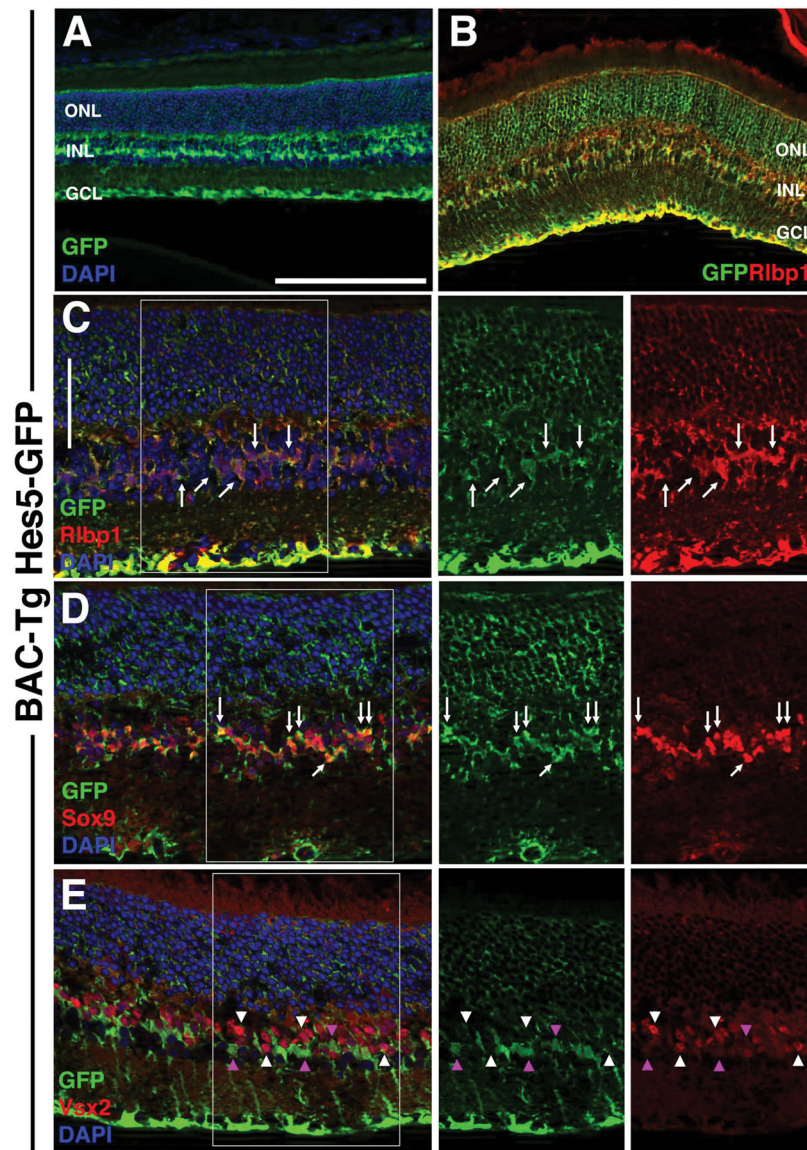
**A,A',A''**, **B**) Hes5-GFP and Jag1 protein overlap in subsets of distal optic cup and lens pit cells. Arrows in **B** point to cells with Jag1 expression (red) at the cell membrane, surrounding cytoplasmic GFP. Nasal is to the left here, and in **C,D**. **C**) All Hes5-GFP cells co-express Rbpj. **D**) At this same age, every Hes5-GFP lens, optic cup and stalk cell also coexpresses Hes1 protein. **E, E',E''**) Starting at E11.5, some GFP + only (pink arrowhead) and some Hes1+ only (white arrowheads) cells can be identified, although the majority of Hes5-GFP+ cells coexpress Hes1 (white arrows). **E'** and **E''** are 8X zoomed magnifications of boxed area in **E**. **F**) Comparison of Hes5-GFP and Hes1 expression at the E12.5 ciliary margin. 4X zoom of boxed area is shown in adjacent panels. Hes1 is present in RPCs and throughout the forming ciliary margin (left boxed area), but, Hes5-GFP+ is confined to the retina, like *Hes5* mRNA (Fig 1H). White arrow points to distal-most extent of the Hes5-GFP domain, and the pink arrow denotes adjacent distal row of Hes1+ cells. **G**) Comparison of Hes5-GFP and Hes1 protein in E13.5 central retina. Arrows denote coexpressing cells, whereas white arrowheads point to Hes1+ only progenitors. Nascent RGCs in the inner retina express Hes5-GFP+. All images are in the horizontal plane of section. Scleral/apical is up in all panels; LP = lens pit; L= lens vesicle; Mag bar in **A** (for **A,B,C,E**) = 20 microns; in **D** (for **D,E',E'',F,I**) = 50 microns.



**Figure 3. Hes5-GFP expression correlates with RGC but not cone neurogenesis**

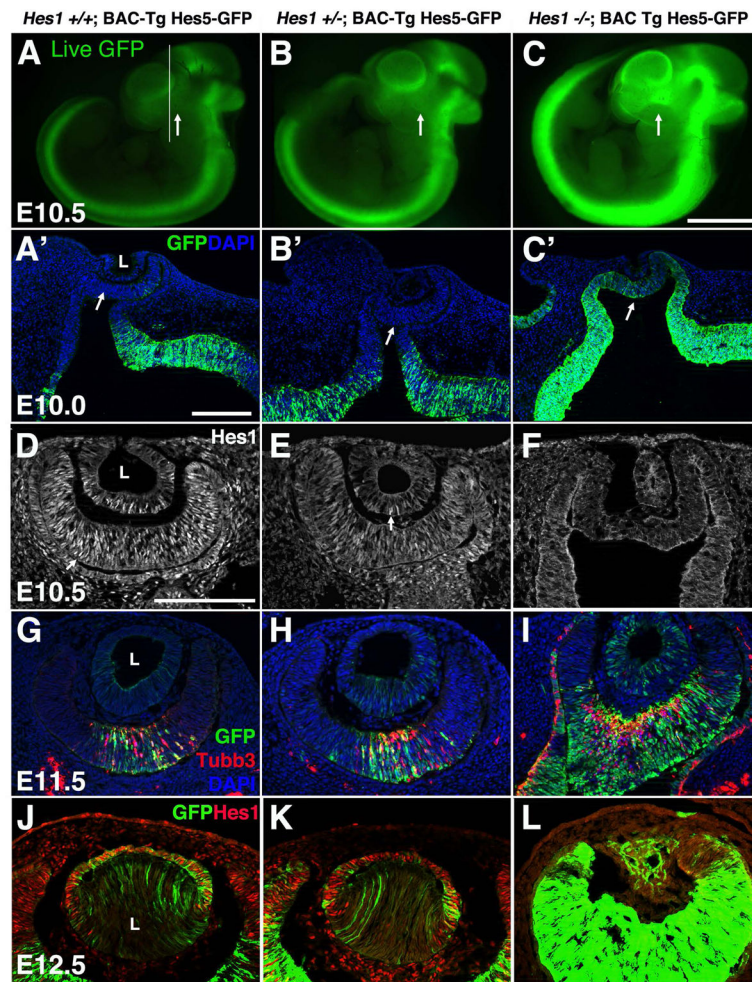
**A)** Most Hes5-GFP cells in the E11.5 central optic cup coexpress the mitotic marker Ccnd1. **B, B', B''**) But a subset of nascent Pou4f+ RGCs also express Hes5-GFP (arrows). Arrowheads denote Pou4f+ nuclei that are Hes5-GFP-negative. **C)** In Hes5-GFP/+;Atoh7<sup>LacZ/+</sup> eyes, anti-GFP and anti-βgal colabeling highlight three types of RPCs: double positive (white arrows), βgal-only (white arrowhead) GFP-only (pink arrowhead). **D)** Anti-GFP, anti-Hes1 and anti-βgal triple labeling further distinguishes a subset of Atoh7<sup>LacZ/+</sup> cells that do not express Hes1 or Hes5-GFP proteins (arrows in zoomed insets of boxed area). **E, E', E'', E'''**) Subpopulations of E12.5 RPCs express different combinations of GFP, Pou4f (red) and β-gal (blue). **F)** At E13.5, a proportion of Hes5-GFP cells reside in the outer, neuroblast layer of the retina (bracket), as marked by Ccnd1 expression. **G)** Nearly all Pou4f+ RGCs in the E13.5 central retina coexpress Hes5-GFP. Arrows in 4X zoomed inset point to double positive cells. **H)** The Rxrg transcription factor is expressed by early RGCs (inner retina) and cone photoreceptors (outer retina) (Roberts et al., 2005). Although inner Rxrg+ RGCs coexpress Hes5-GFP, Rxrg+ cones were devoid of Hes5-GFP. Arrowheads in 4X zoomed insets point to Rxrg+ cones. Scleral/apical is up in all panels; mag bars in A (for A,C,F) = 20 microns; in B (for B, D, E, G, H) = 50 microns.





#### Figure 4. Specific Hes5-GFP expression in adult Müller glia

**A)** Anti-GFP labeling of P21 transgene hemizygous cryosection showing Hes5-GFP localization within particular INL cells and their cellular processes spanning the retina (from outer limiting membrane to nerve fiber layer). **B)** GFP coexpression with Rbp1/Cralbp (arrows) in Müller cell bodies. **C)** Arrows point to coexpressing Müller cell bodies at higher magnification. **D)** Hes5-GFP was also co-expressed with Sox9 (arrows), another Müller cell nuclear marker. The circular structure within the GCL of boxed area is a blood vessel. **E)** No GFP+ INL cells (pink arrowheads) coexpress Vsx2, a bipolar marker (white arrowheads). Boxed areas in C,D,E are shown at same magnification at right, with marker channels separated. Scleral is up in all panels; bar in A,B = 20 microns; C–E = 50 microns, n = 3 adult eyes analyzed per marker. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.



**Figure 5. Hes5-GFP BAC Tg expression is regulated by *Hes1***

In each experiment (row), although not quantitative, labeling conditions and imaging time/parameters were held constant among genotypes. **A–C)** Live GFP expression among littermates. Line in A indicates sectioning plane for **A'–C'** and arrows point to forming eye. Hes5-GFP expression is upregulated in *Hes1* mutants, consistent with Hes5 mRNA expression (ref). **A'–C')** Anti-GFP labeling of fixed ocular sections also highlights changes in Hes5-GFP expression in *Hes1* mutants. Arrows point to forming optic cup. **D–F)** Loss of *Hes1* protein expression from E10.5 *Hes1* mutants. Arrows in D,E point to *Hes1* nuclear protein expression. **G–I)** In the absence of *Hes1* activity, neuronal differentiation (*Tubb3*+ cells in red) is also derepressed in the optic cup and RPE. **J–L)** Removal of *Hes1* results in lens microphthalmia, although some Hes5-GFP expression persists (L). Bar in C (for A–C) = 1 mm; in A' (for A'–C') = 10 microns and in D (for D–L) = 20 microns; n = 3 embryos per age and genotype analyzed.