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## Integral bHLH factor regulation of cell cycle exit and RGC differentiation

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### Abstract

**Background**—In the developing mouse embryo, the bHLH transcription factor *Neurog2* is transiently expressed by retinal progenitor cells and required for the initial wave of neurogenesis. Remarkably, another bHLH factor, *Ascl1*, normally not present in the embryonic *Neurog2* retinal lineage, can rescue the temporal phenotypes of *Neurog2* mutants.

**Results**—Here we show that *Neurog2* simultaneously promotes terminal cell cycle exit and retinal ganglion cell differentiation, using mitotic window labeling and integrating these results with retinal marker quantifications. We also analyzed the transcriptomes of E12.5 GFP-expressing cells from *Neurog2<sup>GFP/+</sup>*, *Neurog2<sup>GFP/GFP</sup>*, and *Neurog2<sup>Ascl1KI/GFP</sup>* eyes, and validated the most significantly affected genes using qPCR assays.

**Conclusions**—Our data support the hypothesis that *Neurog2* acts at the top of a retinal bHLH transcription factor hierarchy. The combined expression levels of these downstream factors are sufficiently induced by ectopic *Ascl1* to restore RGC genesis, highlighting the robustness of this gene network during retinal ganglion cell neurogenesis.

### Keywords

Neurog2; Atoh7; Ascl1; retinal ganglion cell; neurogenesis; bHLH

### Introduction

In the vertebrate retina, seven neuronal and glial cell classes arise from a common pool of retinal progenitor cells (RPCs), in a highly ordered and partially overlapping sequence (Young, 1985; Turner and Cepko, 1987; Turner et al., 1990; Rapaport et al., 2004). The RPC population expands through continuous rounds of mitotic cell division, which must be integrated with tissue morphogenesis and cell fate determination. The timing of the terminal

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S-phase (or birthdate) of an RPC strongly influences its postmitotic identity. Retinal neurogenesis initiates centrally and expands outwards towards the periphery (Prada et al., 1991; Hu and Easter, 1999; McCabe et al., 1999). In mice, the first wave of neurogenesis begins on embryonic day (E)11.0 and is complete by E13.5 (Sidman, 1961; Hufnagel et al., 2010). Since retinal ganglion cells (RGCs) are the first cell class to differentiate in all vertebrate eyes, their formation is synonymous with the initial wave of neurogenesis.

The onset of ganglion cell formation is characterized by activation of the basic helix-loop-helix (bHLH) transcription factor *Atoh7*, in newly postmitotic RPCs. Although *Atoh7*-expressing cells give rise to all seven major cell classes (Feng et al., 2010; Brzezinski et al., 2012), *Atoh7* bestows competence to a subset of these cells to develop as RGC neurons. The complete absence of RGCs and optic nerves in *Atoh7* mutant mice illustrates its importance (Brown et al., 2001; Wang et al., 2001). However, downstream of *Atoh7*, the transcription factors *Pou4f2* and *Isl1* are essential to lock-in the RGC differentiation program (Mu et al., 2008; Pan et al., 2008; Li et al., 2014; Wu et al., 2015). In the absence of either gene, RGCs are specified, but subsequently undergo significant apoptosis (65-80%) prior to birth (Gan et al., 1996; Gan et al., 1999; Mu et al., 2008; Pan et al., 2008). *Pou4f2* and *Isl1* double mutant retinas have an even greater loss of RGCs (>95%), highlighting their synergistic relationship (Pan et al., 2008; Li et al., 2014; Wu et al., 2015). *Pou4f1*, *Myt1*, *Ebf3*, *Onecut1* and *Onecut2* either act in parallel or downstream of *Pou4f2* and *Isl1* during RGC genesis (Erkman et al., 1996; Mu et al., 2008; Jin et al., 2010; Wu et al., 2012; Shi et al., 2013; Gao et al., 2014). However, a better understanding of the regulatory relationships among these genes is still lacking.

Other bHLH proneural factors are also active during retinogenesis. Indeed, *Neurog2* initiates retinal expression in mice at E11.0, within a subset of mitotic RPCs, including those at the leading edge of neurogenesis (Yan et al., 2001; Ma and Wang, 2006; Hufnagel et al., 2010; Brzezinski et al., 2011). In these RPCs, *Neurog2* directly activates *Atoh7* transcription through an evolutionarily conserved E-box in the primary *Atoh7* retinal enhancer (Riesenberg et al., 2009; Skowronska-Krawczyk et al., 2009). In the absence of *Neurog2*, *Atoh7* expression is delayed along with the advancement of *Pou4f2*<sup>+</sup> RGCs (Hufnagel et al., 2010). Another bHLH factor, *Ascl1*, is also expressed by a cohort of proliferating RPCs, beginning at E12.5 (Jasoni and Reh, 1996; Brzezinski et al., 2011). Despite partially overlapping expression domains in the prenatal retina, *Neurog2* and *Ascl1* demarcate distinct lineages (Brzezinski et al., 2011). Thus, it was unexpected that misexpression of *Ascl1* in the *Neurog2* lineage rescued *Atoh7* expression and the wave of (RGC) neurogenesis (Hufnagel et al., 2010). One explanation is that *Neurog2* and *Ascl1* are largely expressed by proliferating RPCs thus, share a common set of downstream targets in the retina. (Jasoni and Reh, 1996; Yan et al., 2001; Ma and Wang, 2006). Alternatively, the presence of multiple bHLH factors, which include *Neurod1*, *Neurod4/Math3*, and *Olig2*, endow the RGC gene network with sufficient redundancy for the establishment of functional optic nerves. To distinguish among these possibilities, we used transcriptomics and gene expression analyses to identify genes that require *Neurog2* for their expression but are also upregulated upon *Ascl1* rescue of RGC development.

## Results

### **Neurog2 regulation of cell cycle exit**

We hypothesized that *Neurog2* must normally regulate some aspect of cell cycle exit, because the percentages of both actively mitotic and apoptotic RPCs did not differ among *Neurog2<sup>GFP/+</sup>* (control), *Neurog2<sup>GFP/GFP</sup>* (mutant), and *Neurog2<sup>Ascl1KI/GFP</sup>* (rescue) embryos (Hufnagel et al., 2010). To test this idea, we performed a BrdU window labeling on embryos of all three genotypes (Repka and Adler, 1992). A single injection of BrdU was given intradermally to pregnant dams at either 1.5 or 18 hours prior to sacrifice at E12.0 (Figure 1A). RPCs in terminal S-phase at the time of injection retain BrdU label indefinitely, whereas, mitotic RPCs dilute BrdU in subsequent rounds of mitosis. The short window provided a baseline RPC mitotic index, with the long window chosen based on average RPC cell cycle length at this developmental stage (Alexiades and Cepko, 1996). Retinal sections were colabeled for BrdU, Ki67, and GFP (to mark the *Neurog2* lineage). We then quantified the percentage of GFP+ RPCs that remained mitotic (BrdU+Ki67+) versus those that exited the cell cycle (BrdU+Ki67-)(Chenn and Walsh, 2002; Kee et al., 2002; Pei et al., 2011). In the short window, there was a significant increase in mitotic RPCs in *Neurog2<sup>GFP/GFP</sup>* retinas, compared to *Neurog2<sup>GFP/+</sup>* or *Neurog2<sup>Ascl1KI/GFP</sup>* (Figure 1B). This increase was more pronounced in the longer time frame, but *Ascl1* provided full rescue during both labeling windows (Figures 2B-D). Interestingly, this outcome is not the same as ectopic expression of *Ascl1* in the *Atoh7* lineage. In that gene replacement mouse, both cell cycle exit and RGC differentiation were blocked, and the *Atoh7<sup>Ascl1KI/+</sup>* RPCs uniquely underwent extra rounds of mitosis (Hufnagel et al., 2013). Although the phenotypes of *Atoh7<sup>Ascl1KI/+</sup>* and *Neurog2<sup>Ascl1KI/GFP</sup>* mice differed, in both situations ectopic *Ascl1* expression induced cell cycle progression.

### **Neurog2 regulation of RGC genesis**

We next wished to correlate the window labeling findings with the progress of RGC differentiation. The RGC markers Pou4f and Isl1 had abnormal expression patterns in *Neurog2<sup>GFP/GFP</sup>* mutants, which were restored in *Neurog2<sup>Ascl1KI/GFP</sup>* eyes (Hufnagel et al., 2010). However, these outcomes were not quantified. Using specific pan-Pou4f or Isl1 antibodies, we labeled E12.5 retinal sections, along with anti-GFP (Figures 2A-F), to determine the percentages of marker+GFP+ per total GFP+ cells (Figures 2G,H). Overall, we noted a reduction of Pou4f+ (8%) or Isl1+ (9%) cells in *Neurog2<sup>GFP/GFP</sup>* retinas, compared to *Neurog2<sup>GFP/+</sup>*. There was also a rebound of Pou4f+ (3%) or Isl1+ (6%) cells in *Neurog2<sup>Ascl1KI/GFP</sup>* eyes, as compared to mutants. These shifts in nascent RGCs complemented the increased mitotic index found in *Neurog2<sup>GFP/GFP</sup>* eyes (4%) and its return to a nearly wild type rate in *Neurog2<sup>Ascl1KI/GFP</sup>* eyes (4%)(Figure 1B, short window). Thus, we concluded that *Neurog2* normally regulates both the terminal cell cycle exit and differentiation of early RGC neurons.

### **Genes expressed downstream of Neurog2**

To generate an unbiased view of gene expression during the initial wave of neurogenesis, we compared the retinal transcriptomes of E12.5 *Neurog2<sup>GFP/+</sup>*, *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl1KI/GFP</sup>* embryos. Because *Neurog2* heterozygotes are phenotypically

indistinguishable from wild type (Hufnagel et al., 2010), we took advantage of the live reporter in *Neurog2<sup>GFP/+</sup>*, *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl11KI/GFP</sup>* to isolate GFP+ RPCs by flow cytometry, using the 7AAD dye to gate out any dying cells (Figure 3A). An average of 22,000 GFP+7AAD- RPCs were used for total RNA isolations from *Neurog2<sup>GFP/+</sup>* (n=14), *Neurog2<sup>GFP/GFP</sup>* (n=6), and *Neurog2<sup>Ascl11KI/GFP</sup>* (n=14) embryos (see Experimental Procedures for details). The resulting cDNA libraries then underwent Next-Gen sequencing. We used the Galaxy bioinformatics platform ([www.galaxy.org](http://www.galaxy.org)) to analyze the resulting datasets (n = 3 biologic replicates/genotype). First we compared *Neurog2<sup>GFP/+</sup>* and *Neurog2<sup>GFP/GFP</sup>* transcriptomes, and then we compared the *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl11KI/GFP</sup>* transcriptomes (Figures 3,4). Genes whose transcript levels significantly differed (q < 0.05) were classified further by ontology, using the PANTHER program ([www.geneontology.org](http://www.geneontology.org)). Those biologic processes with statistically valid changes in fold enrichment (p < 0.05) were then graphed relative to one another (Figures 3B, C). Groups associated with neurogenesis, neuronal differentiation and axon guidance were most highly downregulated in *Neurog2<sup>GFP/GFP</sup>* retinal cells, whereas smaller groups of genes regulating differentiation, or acting during morphogenesis were upregulated (Figure 3B). Remarkably, just two ontologic groups, neurogenesis and generation of neurons, were upregulated in *Neurog2<sup>Ascl11KI/GFP</sup>* eyes, with none of the groups listed in Figure 3B undergoing significant downregulation (Figure 3C).

As a proof of principle, we expected to see the absence of *Neurog2* transcripts in the *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl11KI/GFP</sup>* transcriptomes. Instead, the *Neurog2* RPKM values for both genotypes were higher than for heterozygotes (Figure 4). To explain this puzzling result, we visualized the distribution of the sequence reads at the *Neurog2* locus for all three genotypes, using the IGV program (Figure 5A). The *Neurog2* gene has two exons and one intron, with the open reading frame (ORF) located in the second exon (Gradwohl et al., 1996; Sommer et al., 1996; Fode et al., 1998). In both gene replacement strategies, *Neurog2* protein coding sequences were swapped out for either a GFP or *Ascl1* cDNA, thereby creating functionally null alleles that retained *Neurog2* 5' and 3' UTR sequences (Fode et al., 2000; Seibt et al., 2003). Our aligned sequence reads highlighted a bias among all three genotypes for the *Neurog2* UTRs. However, for both mutant alleles no sequence reads mapped to the ORF (Figure 5A). To independently verify this outcome, we performed real-time PCR using total RNA templates from E12.5 littermate GFP-sorted cells not used for the RNA libraries (Figure 5B). Direct comparison of the RQ values for Exon1 versus Exon2 ORF amplicons demonstrated significantly elevated Exon1 transcript expression in *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl11KI/GFP</sup>* retinas yet, undetectable levels of the Exon2 ORF (Figure 5B). We conclude that both mutant alleles are *Neurog2* nulls in the embryonic retina. Furthermore, biased distributions of *Neurog2* sequence reads containing 5' and 3' UTR segments obscured the lack of those for the protein-coding ORF.

Another gene predicted to be highly downregulated in *Neurog2* mutants was *Atoh7*, given that it is a direct transcriptional target (Skowronska-Krawczyk et al., 2009). However, only a small, -0.4X fold reduction was found (Figure 4; p=0.01). We attributed this to the developmental age of the starting material (E12.5). *Atoh7* expression levels were presumably even lower at the initiation of retinal neurogenesis (E11-E11.5), but in mutant cells isolated for sequencing, *Atoh7* transcription had probably begun to recover (Hufnagel

et al., 2010). So, we independently validated a significant downregulation of *Atoh7* mRNA in *Neurog2<sup>GFP/GFP</sup>* eyes by qPCR (Figure 6). Other bHLH factors also participate in aspects of RGC genesis, and subsets of *Atoh7*-lineage cells coexpress *Neurod1* and/or *Neurod4/Math3* (Mao et al., 2008; Mao et al., 2013). Interestingly, the substitution of either gene into the *Atoh7* locus rescued RGC genesis (Mao et al., 2008; Mao et al., 2013). Yet another bHLH factor, *Olig2*, is expressed broadly by RPCs, although functions related to RGC neurogenesis have not been described (Nakamura et al., 2006; Shibasaki et al., 2007; Hafler et al., 2012). In other CNS tissues *Olig2* specifies oligodendrocyte fates. All three factors, *Neurod1*, *Neurod4* and *Olig2*, were significantly downregulated in *Neurog2* mutants, with the latter two more severely affected (Figure 4).

Work by multiple labs has elucidated a transcription factor hierarchy acting during vertebrate RGC development (reviewed in Centanin and Wittbrodt, 2014; Stenkamp, 2015). Here *Atoh7* has been suggested to sit at a critical node, due to its early expression and phenotype, namely a total block of optic nerve formation, and reduced expression of many genes in the early RGC network. Immediately downstream of *Atoh7* are the factors *Pou4f2* and *Isl1*, which act synergistically to cement the RGC fate (Mu et al., 2008; Pan et al., 2008; Li et al., 2014; Wu et al., 2015). Additional relevant RGC factors are *Pou4f1,3*, *Isl2*, *Onecut1,2,3*, *Myt1* and *Ebf1,2,3*, which drive particular terminal differentiation processes, axonogenesis, and/or specify functional subclasses of RGCs (Xiang et al., 1995; Erkman et al., 1996; Gan et al., 1999; Mu et al., 2008; Jin et al., 2010; Wu et al., 2012; Shi et al., 2013). Thus, it was not unexpected to find a highly significant reduction in transcript levels for *Pou4f1*, *Pou4f2*, *Onecut2*, *Onecut3* and *Ebf3* in *Neurog2* mutants (Figures 4,6). Although downregulation of this entire set of early RGC regulators might be predicted, the transient nature of the *Neurog2* mutant phenotype, and cross-regulation among subsets of downstream factors could effectively mask changes in particular genes. For example, while *Ebf1*, *Ebf2*, and *Ebf3* are all expressed by nascent RGCs, only *Ebf3* is a direct target of *Pou4f2*, and was the sole *Ebf* paralogue significantly downregulated here (Jin et al., 2010; Gao et al., 2014) (Figures 4,6). We also noted a significant loss of the RGC axon guidance molecule, *Dcc* (Figures 4,6) (Deiner et al., 1997; Livesey and Hunt, 1997). These data are consistent with *Neurog2* activity residing sitting at the top of the early RGC genetic hierarchy.

### **Ascl1 Rescue Transcriptome**

Another overt goal was to use transcriptomics to investigate the underlying basis of *Ascl1* rescue of the wave of neurogenesis in *Neurog2* mutants. Both *Neurog2<sup>GFP/GFP</sup>* and *Neurog2<sup>Ascl1KI/GFP</sup>* genotypes lacked functional *Neurog2*, but also showed upregulated endogenous *Ascl1* (Figures 4–6). The specificity of the latter outcome was also validated by qPCR, using primers specific for an endogenous *Ascl1* gene amplicon (Figure 6). Among the gene expression lists with highly significant level changes ( $q < 0.05$ ), we asked if there was a subset both downregulated in *Neurog2* mutants and upregulated in the *Ascl1* rescue. By these criteria we found just three genes with known roles in developmental neurobiology: *Neurod4*, *Pou4f2* and *Onecut2* (gray shading in Figure 4). We further confirmed these outcomes by qPCR, as well as verified expression level changes for other factors with less robust, but potentially meaningful alterations ( $p < 0.05$ ) (Figures 4,6). We also noted four bHLH factors, *Atoh7*, *Neurod1*, *Neurod4* and *Olig2*, were each significantly increased in the



*Neurog2*<sup>Ascl11KI/GFP</sup> GFP lineage, although none were restored to the level of *Neurog2* heterozygotes (Figure 6). The simultaneous upregulation of four bHLH factors was quite striking, particularly when combined with the upregulation of *Pou4f2*, *Isl1* and *Onecut2* (Figure 6). Intriguingly, *Notch3* and *Hes5* levels were significantly altered in the *Neurog2*<sup>GFP/GFP</sup> versus *Neurog2*<sup>Ascl11KI/GFP</sup> transcriptomes (Figure 4), but only the changes in *Hes5* mRNA levels could be validated in a real-time PCR assay (Figure 6). Overall, we conclude that the loss of *Neurog2* stalls retinal neurogenesis, thereby stimulating upregulation of *Ascl1* to rescue RGC genesis, within a lineage where it is otherwise not normally active.

## Discussion

Retinal neurogenesis is a dynamic process that requires the coordination of multiple cellular activities. One intrinsic, temporal regulator of neurogenesis is the proneural bHLH transcription factor *Neurog2* (Hufnagel et al., 2010). Here we demonstrate that *Neurog2* simultaneously regulates RPC cell cycle exit and early RGC differentiation. Transcriptomic analyses confirmed that although *Neurog2* activity is required for *Atoh7* expression, it also impacted a broader transcription factor network underlying RGC development. We also found that ectopic *Ascl1* drove this same network to a sufficient threshold that correlates with a rescue of RGC development.

### ***Neurog2* regulation of cell cycle exit, and its rescue by *Ascl1***

*Neurog2*-mutant RPCs do not exit mitosis appropriately, relative to controls. This is consistent with *Neurog2* promotion of cell cycle exit in the spinal cord and neuronal culture, where it stabilizes the cyclin-dependent kinase inhibitor *Cdkn1b/p27<sup>kip1</sup>* (Farah et al., 2000; Nguyen et al., 2006). By examining retinal cell cycle length via window labeling, we found an abnormal accumulation of mitotic RPCs in *Neurog2* mutants. We interpret this as a delay in cell cycle exit, since the proportion of S-phase RPCs is unaffected in E12.5 *Neurog2*<sup>GFP/GFP</sup> retinas (Hufnagel et al., 2010). We further propose that without *Neurog2*, RPCs accumulate at the G<sub>1</sub>/G<sub>0</sub> checkpoint. *Neurog2* regulation of *Cdkn1b* in other developmental contexts make it an appealing molecule to be affected here, which could also explain the delay in retinal neurogenesis. However, no significant changes in *Cdkn1b* mRNA levels were found in *Neurog2*<sup>GFP/GFP</sup> versus *Neurog2*<sup>GFP/+</sup> transcriptomes. Nonetheless, loss of *Neurog2* might affect some other type of *Cdkn1b* regulation in the retina, and/or occur indirectly via *Atoh7*, since Cdkn1b+ cells are reduced in *Atoh7* mutant retinas (Le et al., 2006). Regardless of which gene controls *Cdkn1b* expression, the regulation presumably occurs post-transcriptionally. It would be interesting to explore these mechanisms in the future, by using *Cdkn1b* phosphorylation site-specific mutations, to test for temporal retinal neurogenesis phenotypes (Besson et al., 2006).

The mechanism by which *Ascl1* misexpression rescued the cell cycle phenotype of *Neurog2* mutant RPCs unfortunately remains unresolved. Throughout the nervous system, *Ascl1* was originally thought to promote cell cycle exit and neuronal differentiation (Ahmad et al., 1998; Cai et al., 2000; Tomita et al., 2000; Farah and Easter, 2005). Yet, genomic profiling studies in the brain demonstrated that *Ascl1* can activate the expression of cell cycle

progression genes, including canonical cell cycle regulators and oncogenic transcription factors (Castro et al., 2011). Although, we overtly searched for upregulated expression in these gene families, within the *Neurog2*<sup>Ascl11KI/GFP</sup> dataset, no such candidates were identified further evaluation. This would suggest that transcriptomic profiling to identify genes affected in temporal mutants may require tighter control of developmental time than is technically feasible during mouse embryogenesis.

### ***Atoh7* as a direct downstream target gene for *Neurog2***

A key initiation step for retinal neurogenesis is *Neurog2* direct activation of *Atoh7* expression (Skowronska-Krawczyk et al., 2009). Lineage tracing and protein colocalization experiments show that in the embryonic retina, virtually all *Atoh7*<sup>LacZ</sup> cells are also *Neurog2*<sup>+</sup>, but not the reverse situation (Hufnagel et al., 2010; Miesfeld et al., 2018). Thus, at any given point, the *Neurog2* lineage should include more RPCs, than the *Atoh7* lineage. Additionally, *Neurog2* and *Atoh7* are expressed during distinct cell cycle phases, with *Neurog2* largely found in mitotic cells, and *Atoh7* predominantly present in post-mitotic RPCs (Brown et al., 1998; Yang et al., 2003; Le et al., 2006; Ma and Wang, 2006; Brzezinski et al., 2012; Miesfeld et al., 2018). Together this suggests that a subset of *Neurog2*<sup>+</sup> RPCs transit into *Atoh7*<sup>+</sup> cells, complete cell cycle exit and differentiate. The *Atoh7* mRNA expression domain was clearly smaller in E11.5 *Neurog2* mutant retinas versus controls (Hufnagel et al., 2010), implying there would be a significant loss of *Atoh7* mRNA levels in mutant eyes. But, this was not the case for the *Neurog2* mutant transcriptome dataset, although we did find a significant loss by qPCR. These differing outcomes might be attributed to variability in the precise age of the samples collected for each assay. Another confounding variable could be a more limited sequencing efficiency at the *Atoh7* locus, due to high guanine-cytosine (GC) content. RNA-seq efficiency is reduced if GC content is either too high or too low (Risso et al., 2011; Zheng et al., 2011; Hansen et al., 2012; Filloux et al., 2014). The 5' end of the *Atoh7* transcript contains a 185 nucleotide stretch with 85% GC content (Prasov et al., 2010), which could introduce a negative bias for sequence read-depth.

### **How does *Ascl1* “rescue” the *Neurog2* temporal phenotype?**

In the developing mouse retina, *Ascl1* expression initiates about two days later than *Neurog2*, with its activity required for bipolar interneuron development and suppression of Müller glia (Jasoni and Reh, 1996; Tomita et al., 1996; Tomita et al., 2000; Brzezinski et al., 2011). Interestingly, the *Ascl1* lineage includes all major retinal cell classes, except for RGCs, and the loss of *Ascl1* does not impact RGC differentiation. Yet, one subset of *Ascl1*<sup>+</sup> RPCs normally gives rise to *Atoh7*-expressing cells (Brzezinski et al., 2011). It is possible that in the *Neurog2* mutant lineage ectopic *Ascl1* could directly activate *Atoh7* transcription, given the multiple E-box binding sites (CANNTG) in conserved *Atoh7* regulatory DNA (Murre et al., 1989; Hutcheson et al., 2005; Hufnagel et al., 2007; Skowronska-Krawczyk et al., 2009). The binding specificity between bHLH factors largely relies on variations in the central NN nucleotides, but in particular contexts sequences immediately surrounding the E-box are also influential (Powell et al., 2004; Seo et al., 2007; Gohlke et al., 2008; Gordan et al., 2013). In general, *Ascl1* has high affinity for CAGCTG consensus sequences, whereas *Neurog2* binds to CAGATC sequences (McNeill et al., 2012; Borromeo et al., 2014).

Alternatively, we propose that rather than inappropriate binding of ectopic *Ascl1* to a preferred *Neurog2* consensus site, *Atoh7* transcription was prematurely stimulated via the same indirect regulatory mechanism normally employed within the endogenous *Ascl1* lineage (Brzezinski et al., 2011). Thus, the recovery of key genes in the RGC network in the *Neurog2<sup>Ascl1KI/GFP</sup>* dataset could be attributed to *Ascl1* stimulation of *Atoh7* expression, which in turn activated the other genes. However simultaneous upregulation of four bHLH factors (along with *Pou4f2*, *Isl1* and *Onecut2*) suggests that ectopic *Ascl1* induced the transcription of multiple genes. *Ascl1* was previously shown to control genetic cascades, which give rise to particular neuronal fates in the brain, and maintain the right size RPC pool for the late, postnatal retinal fates (Jasoni and Reh, 1996; Tomita et al., 1996; Tomita et al., 2000; Castro et al., 2011). *Ascl1* activity is also critical during retinal regeneration in multiple organisms (Wilken and Reh, 2016; Jorstad et al., 2017), and more recently implicated in mechanisms of tumorigenesis (Ma et al., 2017; Park et al., 2017). Thus, much more work is needed to tease apart the mode by which this factor successfully rescued RGC neurogenesis in the absence of *Neurog2*. Did *Ascl1* behave ‘normally’ but in a new context to activate *Atoh7*, or by virtue of its interactions with chromatin remodeling proteins, rapidly stimulate the transcription of an array of genes, above the minimum threshold necessary for RGC development?

Finally, we propose that *Neurog2* also occupies the critical node for RGC development, by virtue of its activation of *Atoh7*, plus other early bHLH factors. This is consistent with the significant downregulation of *Pou4f1,2*, *Isl1*, *Onecut2*, and *Ebf3* in *Neurog2* mutants (Xiang et al., 1995; Erkman et al., 1996; Gan et al., 1999; Mu et al., 2008; Jin et al., 2010; Wu et al., 2012; Shi et al., 2013). Arguably many of these same genes act downstream of and require *Atoh7*, but *Ascl1* only substitutes for *Neurog2*, and not *Atoh7* (Hufnagel et al., 2010; Hufnagel et al., 2013; Gao et al., 2014). Given that each bHLH factor has a “salt-and-pepper” pattern throughout retinogenesis, it provokes the question of how many bHLH factors an individual RPC must express at distinct timepoints, and whether particular combinations are sufficient to provide robustness for producing an RGC neuron. Although static co-expression pattern comparisons for all relevant transcription factors will be informative, we advocate single cell genomics to gain the most accurate understanding of the complex and important question of which early factors drive RGC genesis.

## Experimental Procedures

### Animals

Two gene replacement allele mouse strains were used in this study: *Neurog2<sup>GFP</sup>* (*Neurog2<sup>tm4Fgu</sup>*) (Seibt et al., 2003) and *Neurog2<sup>Ascl1KI</sup>* (*Neurog2<sup>tm3(Ascl1)Fgu</sup>*) (Fode et al., 2000), both maintained on an ICR background. PCR genotyping was as previously described (Fode et al., 2000; Seibt et al., 2003). Embryonic age was determined through timed matings, with the date of the vaginal plug as E0.5. All mice were housed and cared for in accordance with the guidelines provided by the National Institutes of Health, Bethesda, Maryland, and the Association for Research in Vision and Ophthalmology, and conducted with approval and oversight from the CCHRF and UC Davis Institutional Animal Care and Use Committees.



## Immunohistochemistry and Cell Quantification

Embryos were fixed in 4% paraformaldehyde/PBS for 40-50 minutes at 4°C, cryoprotected in 5% and 15% sucrose/PBS, embedded in TissueTek OCT, and 10µm cryosections immuno-labeled as in (Mastick and Andrews, 2001). Primary antibodies used were rat anti-BrdU (AbD Serotec, Cat#:OBT0030, 1:100), chick anti-GFP (Abcam, 1:1000, AB13970), rabbit anti-Ki67 (Vector Labs, 1:1000, VP-K451), and rabbit anti-PH3 (Millipore-Sigma, 1:200, 06-570), goat anti-Pou4f (Santa Cruz 1:50, sc-6026), mouse anti-Is11 IgG<sub>2B</sub> (DSHB, 1:50, AB2314683). Secondary antibodies were conjugated to Alexa Fluor488 or Alexa Fluor594 (Invitrogen/Life Science, Grand Island, NY 1:500); or biotinylated (1:500) and sequentially labeled with streptavidin AMCA350 (Jackson ImmunoResearch, West Grove, PA, 1:200). Nuclear staining was performed with DAPI (1:1000 dilution of 10 mg/ml solution, Sigma, Cat#:28718-90-3).

Microscopy was performed with either a Zeiss fluorescent microscope, Zeiss camera and Apotome deconvolution device or a Leica DM5500 microscope, equipped with a SPEII solid state confocal. Images were processed using Zeiss Axiovision (v6.0), Leica LASAF and/or Adobe Photoshop (CS4) software programs. All digital micrographs were equivalently adjusted for brightness, contrast and pseudocoloring. Pou4f+GFP+ or Is11+GFP+ cells were quantified using the count tool in Adobe Photoshop, CS4 and one-way ANOVA, plus a Bonferroni post-hoc test used to determine p-values (GraphPad Prism software (v6)). In all experiments 3 individuals per genotype were analyzed, using at least 2 sections per individual. Equivalent anatomical depth in the retina was determined by proximity to the optic nerve.

## Mitotic Window Labeling

BrdU was injected into pregnant dams (0.1 mg/g body weight of 10 mg/mL BrdU in 0.9 M NaCl) at either 1.5 or 18 hours prior to embryo harvest. For all analyses, 3 biologic replicate embryos per age and genotype were analyzed. Ten micron cryosections were labeled as in (Le et al., 2006) and BrdU+, Ki67+, and BrdU+Ki67+ populations quantified within the *Neurog2<sup>GFP</sup>* lineage, using the Axiovision measurements module. The percentage of GFP+BrdU+Ki67+ per GFP+BrdU+ cells ± standard error of the mean (SEM) was calculated within a 100× field, and one-way ANOVA, plus Tukey post-hoc test used to determine p values (Instat Software, v3).

## Flow cytometry and RNA preparations

Pairs of E12.5 GFP+ optic cups were dissected and dissociated into single cell suspensions using TrypLE Select (Invitrogen, 12563). The eBioscience 7-AAD viability marker (ThermoFisher, 1:250, 00-6993-50) was added to all samples, and GFP+7AAD-neg cells purified with a Becton Dickinson FACS-Aria machine. Total RNA was immediately extracted using the RNeasy micro kit (Qiagen, Cat No 74004), and stored at -80°C. All samples were submitted to the CCHMC Gene Expression for quality assessment using an Agilent Bioanalyzer. Three biologic replicates per genotype were selected for RNA-seq analyses, through a combination of matched somites counts, average total RNA concentration ( 1.97 ng/µl) and RIN score ( 8.7). The selected samples were then submitted for transcriptome analysis.

## RNA-seq and qPCR analyses

Sequencing libraries were generated using the TruSeq RNA library kit (Illumina, RS-122-2001), and analyzed on the Illumina Hi-Seq 2000, using single-end 50-bp read specifications with a read depth of 25 million (Illumina, San Diego, CA). Following removal of primers and barcodes, sequence reads were aligned to the mm10 mouse genome assembly with the BWA and Bowtie programs. Aligned reads were analyzed for differentially expressed transcripts using the CuffDiff program in the Galaxy bioinformatics package ([www.usegalaxy.org](http://www.usegalaxy.org)). Differentially expressed transcripts were initially evaluated with an adjusted p-value cutoff of  $q < 0.05$ . For some transcripts, significance was broadened to  $p < 0.05$ , with the requirement of validation. Transcripts were grouped by ontology using PANTHER ([www.geneontology.org](http://www.geneontology.org)) and ranked by fold enrichment. The sequence reads (RPKM) for particular genes were visualized with the Integrative Genomics Viewer (IGV) browser (v. 2.3)(Robinson et al., 2011; Thorvaldsdottir et al., 2013). RNA-seq datasets (see Supplemental Tables) were deposited in NCBI Gene Expression Omnibus (Edgar et al., 2002) and assigned accession number GSE111666 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111666>).

Real-time PCR was performed by reverse transcribing E12.5 total retinal RNA into cDNA using Superscript III (ThermoFisher, 18080093) and performing qPCR with primer sets listed below, Fast Sybr Green Master Mix (Applied BioSystems, Cat#:4385614) and an Applied BioSystems StepOnePlus machine. Relative quantification (RQ) values were calculated using the  $2^{-CT}$  method (Livak and Schmittgen, 2001) with GAPDH as endogenous control. Statistical significance was determined using IBM SPSS Statistics (v. 24), with an unpaired 2-sample T-Test and Welsh correction.

### qPCR Primer Pairs

Gene	Primer 1	Primer 2
<i>Ascl1</i>	TTGAACTCTATGGCGGGTTC	CAAAGTCCATTCCCAGGAGA
<i>Atoh7</i>	ATCACCCCTACCTCCCTTTC	CGAAGAGCCTCTGCCATA
<i>Dcc</i>	CAAGCTGGCTTTTGTACTCTTCG	GAATCCTCGGTCGGACTCT
<i>Ebf3</i>	TCACCCTCCCTTCAAACCTGTA	GTTTCACTGCGGAGATGACAT
<i>Hes5</i>	AGTCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
<i>Gapdh</i>	TGAAGGGGTCGTTGATGG	AAAATGGTGAAGTCGGTGT
<i>Isl1</i>	TATCCAGGGGATGACAGGAAC	GCTGTTGGGTGTATCTGGGAG
<i>Neurod1</i>	ATGACCAAATCATAACAGCAGAG	TCTGCCTCGTGTTCCTCTGT
<i>Neurod4</i>	AGCTGGTCACACCACAATCCT	GTTCCGAGCATTCCATAAGAGC
<i>Neurog2</i> exon1	AAGCAGCTCGGCTTTAACT	GTGTGTGTCCGGGAATGT
<i>Neurog2</i> exon2	AACTCCACGTCCCCATACAG	GAGGCGCATAACGATGCTTCT
<i>Notch3</i>	AAGCGTCTCCTGGATGCTG	GAATCTGGAAGACAGCCTGG
<i>Olig2</i>	TCCCCAGAACCCGATGATCTT	CGTGGACGAGGACGCAGTC
<i>Onecut1</i>	GGCAACGTGAGCGGTAGTTT	TTGCTGGGAGTTGTGAATGCT
<i>Onecut2</i>	AGAGGGTCTATGCCGTCT	GGGATTTCTTCTGCGAGTTG
<i>Pou4f1</i>	AGGCCTATTTTGCCGTACAA	CGTCTCACACCCTCCTCAGT

Gene	Primer 1	Primer 2
<i>Pou4f2</i>	ATGGTGGTGGTGGCTCTTAC	CGGAGAGCTTGTCTTCCAAC
<i>Prdm1 Exon6</i>	TGCTCACTACCCCAAGTTCC	TGGGATAAGCACCTCTTTGG
<i>Prdm1 3'UTR</i>	GAACCTGCTTTTCAAGTATGCTG	AGTGTAGACTTCACCGATGAGG
<i>Tubb3</i>	TAGACCCCAGCGGCAACTAT	GTTCCAGGTTCCAAGTCCACC

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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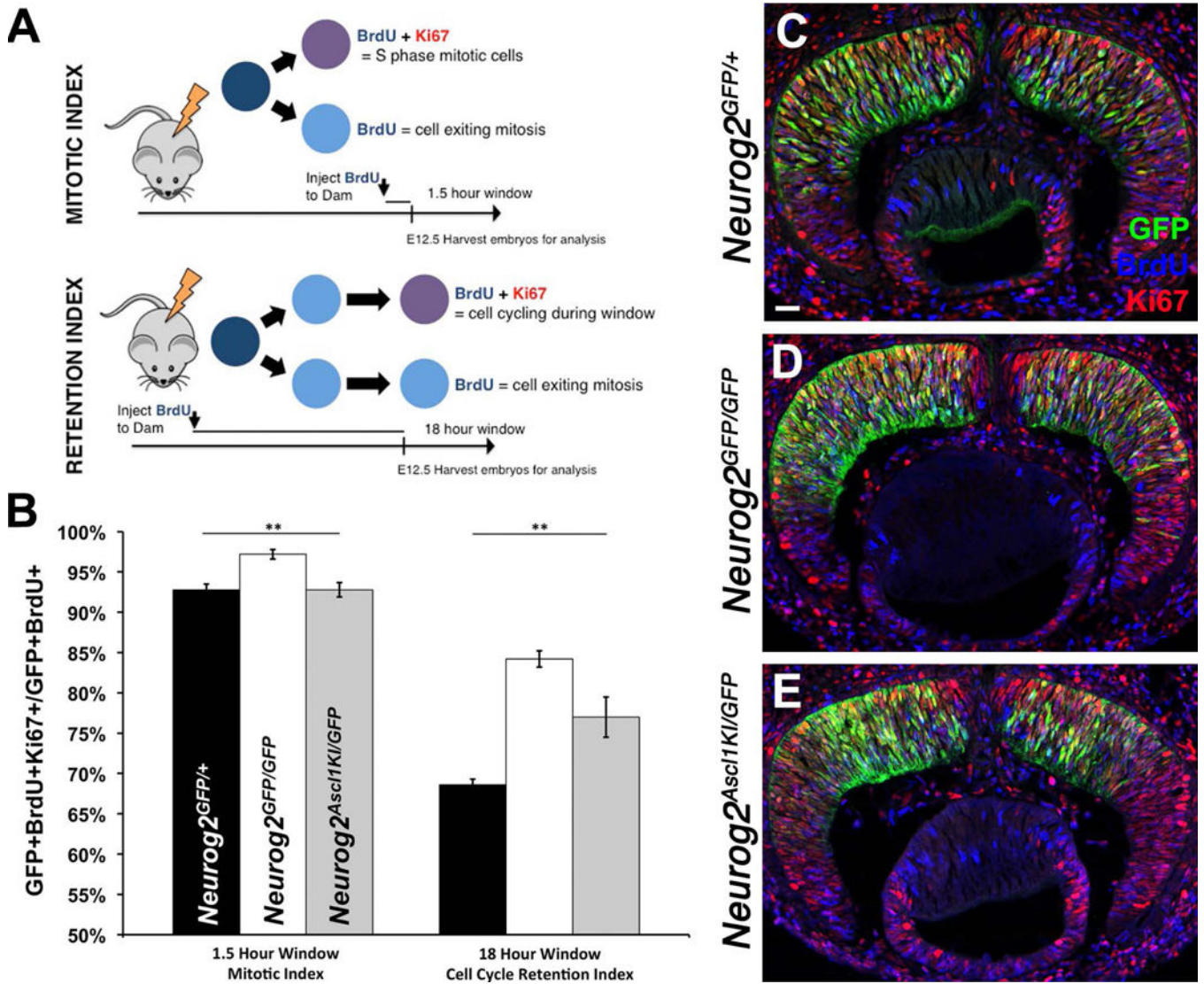
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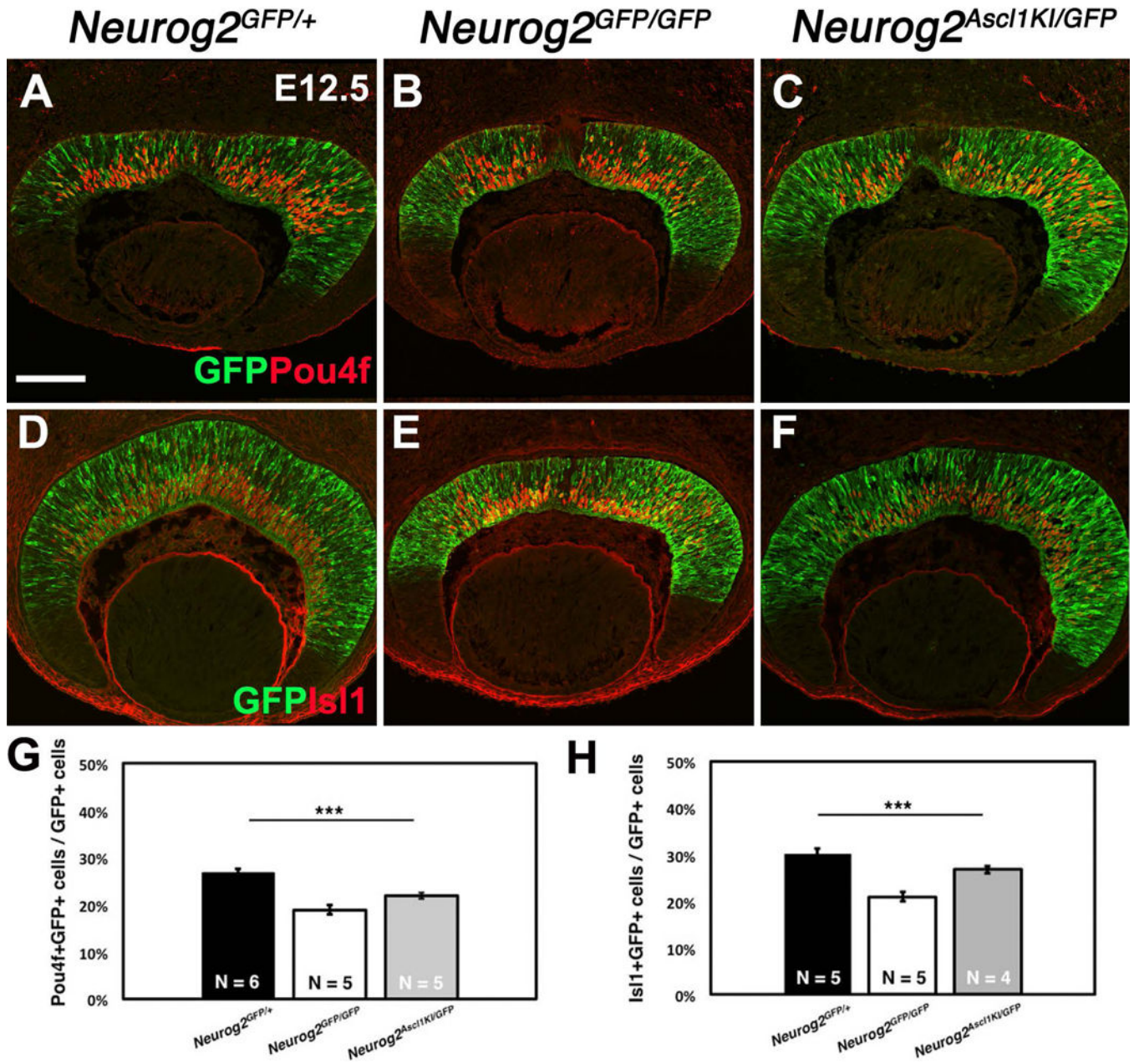
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**Figure 1. *Neurog2* is required for retinal progenitor cell cycle exit**

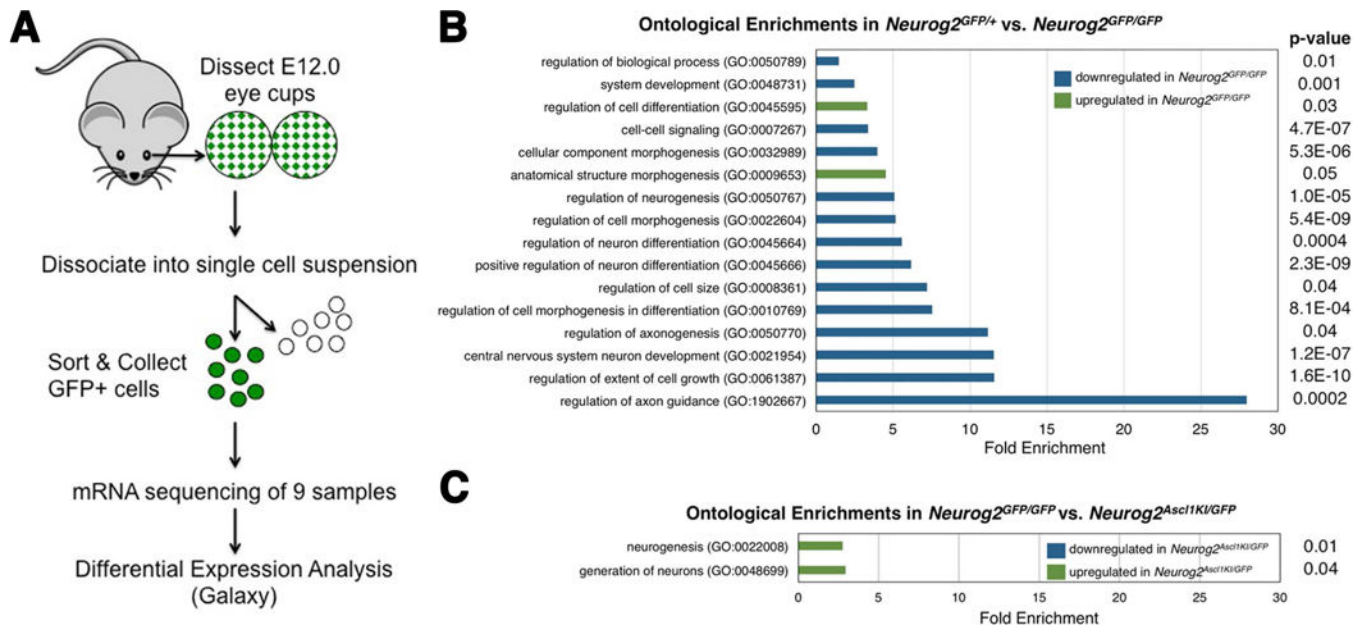
**A)** Experimental strategy to label RPCs with BrdU for 1.5 or 18 hours prior to embryo harvest at E12.0. **B)** Percentages of *Neurog2* lineage cells (GFP+) in the cell cycle. There was a significant increase in mitotic RPCs in *Neurog2*<sup>GFP/GFP</sup> retinas, which was more apparent with a longer labeling window. *Asc11* recombined into the *Neurog2* locus rescued this phenotype. **C-E)** Representative triple-labeled retinal images for *Neurog2*<sup>GFP/+</sup>, *Neurog2*<sup>GFP/GFP</sup>, and *Neurog2*<sup>Asc11KI/GFP</sup> embryos after 18 hour BrdU labeling. A one-way ANOVA, plus Tukey post-hoc test was used to determine p-values. \*\*p < 0.01; error bars = SEM; n = 3 embryos/genotype; apical is up; scale bar = 50µm.





### Figure 2. The impact of *Neurog2* loss on RGC differentiation

**A-C,G)** E12.5 Pou4f+ RGCs in the *Neurog2<sup>GFP</sup>* lineage are significantly reduced in *Neurog2* mutants but rescued by *Ascl1* misexpression in the *Neurog2* locus. **D-F,H)** E12.5 Isl1+ cells (most of which are RGCs) are analogously reduced in *Neurog2* mutants and rescued by *Ascl1*. A one-way ANOVA, plus Bonferroni post-hoc test were used to determine p-values. \*\*\*p < 0.001; error bars = SEM; apical retina is up; n = 3 embryos/genotype; scale bar = 100µm.



**Figure 3. High-throughput analysis of transcriptional changes in E12.5 *Neurog2*-GFP+ RPCs among three genotypes**

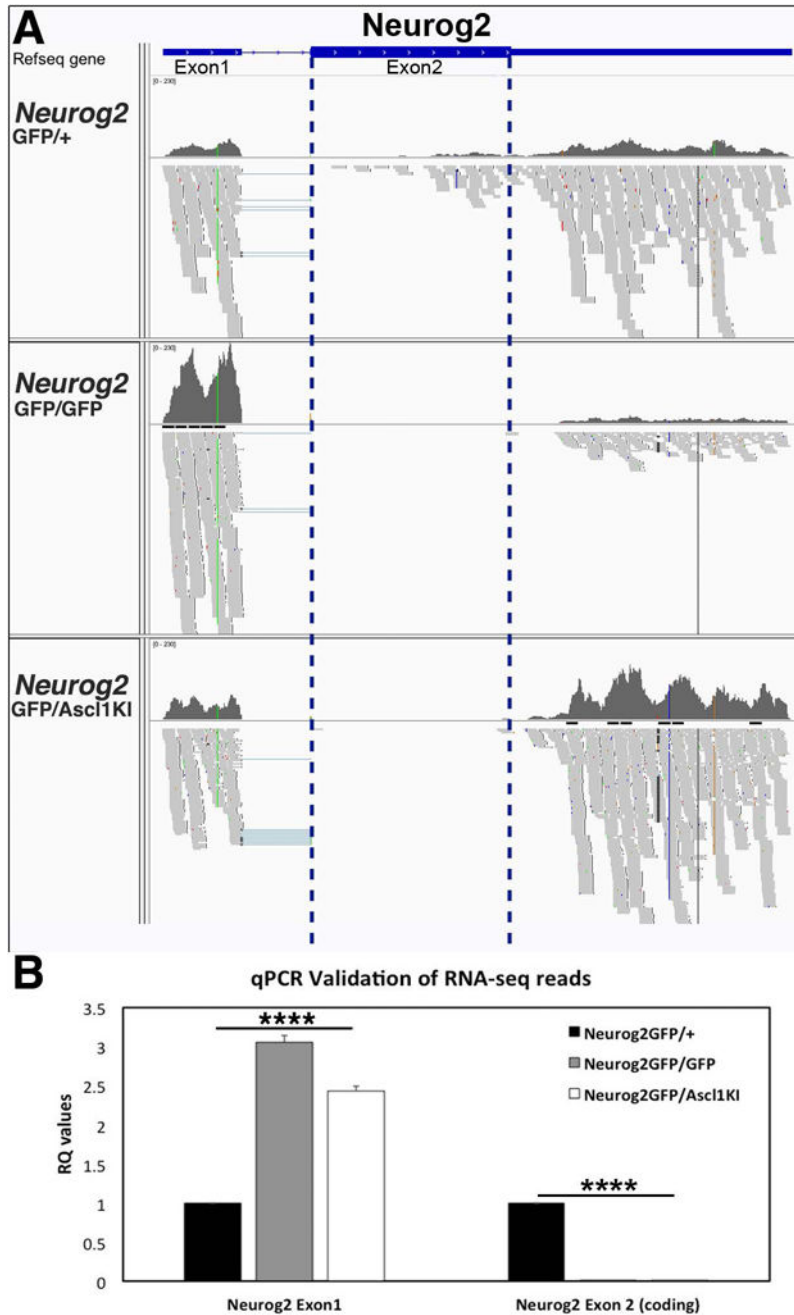
**A)** Work flow diagram of optic cup collection, dissociation and flow-sorting of GFP+;7-AAD-neg populations from *Neurog2*<sup>GFP/+</sup>, *Neurog2*<sup>GFP/GFP</sup>, and *Neurog2*<sup>Asc1KI/GFP</sup> embryos, followed by RNA-seq analyses. **B)** Gene ontology analyses among the genotypes, with statistically significant functional groups ranked by fold enrichment. Blue bars indicate GO categories with significant downregulated gene expression; green bars denote categories with significant upregulated expression.



Gene Name	GFP/+ vs. GFP/GFP			GFP/GFP vs. <i>Ascl1KI/GFP</i>			Encoded protein, proposed function			
	GFP/+ RPKM Value	GFP/GFP RPKM Value	<i>Ascl1KI/GFP</i> RPKM Value	Log2 (Fold Change)	p-value	Adj. p-value		Log2 (Fold Change)	p-value	Adj. p-value
<i>Ascl1</i> **	7.4	14.9	15.7	1	5 X10 <sup>-5</sup>	0.01	0.11	NS	NS	Transcription factor, Neurogenesis
<i>Neurog2</i> **	30.2	35.5	112.3	0.3	NS	NS	1.6	5X10 <sup>-5</sup>	0.01	Transcription factor, Neurogenesis
<i>Atoh7</i> **	195.3	145	206	-0.4	0.01	NS	0.5	0.0009	NS	Transcription factor, RGC development
<i>Pou4f1</i> **	7.5	3.1	3.8	-1.5	5 X10 <sup>-5</sup>	0.01	0.3	NS	NS	Transcription factor, RGC development
<i>Pou4f2</i> **	53.1	30.5	70.9	-0.8	5X10 <sup>-5</sup>	0.01	1.2	5X10 <sup>-5</sup>	0.01	Transcription factor, RGC development
<i>Isl1</i> **	16.3	12.8	24.1	-0.4	NS	NS	0.63	0.01	NS	Transcription factor, Neurogenesis
<i>Onecut1</i>	0.76	1.1	5.1	0.6	NS	NS	0.4	NS	NS	Transcription factor, Cell Fate Determination
<i>Onecut2</i> **	16.6	7.7	25.3	-1	0.0002	0.04	0.7	5X10 <sup>-5</sup>	0.01	Transcription factor, Cell Fate Determination
<i>Onecut3</i>	3.8	1.2	1	-1.6	5X10 <sup>-5</sup>	0.01	-0.2	NS	NS	Transcription factor, Cell Fate Determination
<i>Ebf3</i>	28.9	11.6	18	-1.3	0.0001	0.02	0.6	NS	NS	Transcription factor, Differentiation
<i>Olig2</i> **	4.6	2.5	2.8	-0.9	0.0003	0.04	0.2	NS	NS	Transcription factor, Neurogenesis/Gliogenesis
<i>Neurod1</i> **	41.8	29	32.3	-0.5	0.02	NS	0.14	NS	NS	Transcription factor, Cell Differentiation
<i>Neurod4</i> **	18.3	9.4	15.1	-1	5X10 <sup>-5</sup>	0.01	0.7	5X10 <sup>-5</sup>	0.01	Transcription factor, Neural Development
<i>Notch3</i> **	5.4	5	8	-0.08	NS	NS	0.7	5X10 <sup>-5</sup>	0.01	Notch receptor, Signal Transduction
<i>Hes5</i> **	64.1	42.3	21.3	-0.6	0.001	NS	-1	5X10 <sup>-5</sup>	0.01	Transcription factor, Signal Transduction
<i>Hey1</i>	61.4	47	51.2	-0.4	0.009	NS	0.14	NS	NS	Transcription factor, Signal Transduction
<i>Dcc</i> **	53.8	32.6	37.8	-0.7	5X10 <sup>-5</sup>	0.01	0.23	NS	NS	Transmembrane protein, Axon Guidance
<i>Prdm1</i> #	17.8	10.7	11	-0.7	5X10 <sup>-5</sup>	0.01	0.05	NS	NS	Transcription factor, Differentiation
<i>Prdm13</i>	2.3	0.7	1.1	-1.7	5X10 <sup>-5</sup>	0.01	0.65	0.04	NS	Transcription factor, Differentiation
<i>Acsl6</i>	9.7	3.8	10.3	-1.3	0.0001	0.02	0.24	NS	NS	Acyl-CoA synthetase long-chain fam member 6
<i>Camk2n1</i>	24	13.9	22.9	-0.8	5X10 <sup>-5</sup>	0.01	0.7	5X10 <sup>-5</sup>	0.01	Calcium/calmodulin-dependent protein kinase II inhibitor 1
<i>Cd59a</i>	21.7	13.3	23.2	-0.7	0.0003	0.04	0.8	5X10 <sup>-5</sup>	0.01	Complement protein
<i>Gje1</i>	20.7	52	34	1.3	5X10 <sup>-5</sup>	0.01	-0.6	0.0003	0.04	Gap junction protein epsilon 1
<i>Insm2</i>	16.8	10.7	16	-0.6	0.0002	0.03	0.6	2.5 X10 <sup>-5</sup>	0.04	Insulinoma-associated protein
<i>Nova2</i>	12.7	6.1	9.9	-1	5X10 <sup>-5</sup>	0.01	0.7	5X10 <sup>-5</sup>	0.01	Neuro-oncological ventral antigen
<i>Nsg2</i>	16	7.6	11.8	-1.1	5X10 <sup>-5</sup>	0.01	0.7	0.0002	0.04	Neuron specific gene family member 2
<i>Rab3c</i>	2.6	1.3	2.2	-1	5X10 <sup>-5</sup>	0.01	-0.7	0.0002	0.03	GTPase
<i>Tmem60</i>	42.4	74	29	0.8	5X10 <sup>-5</sup>	0.01	-1.3	5X10 <sup>-5</sup>	0.01	Transmembrane protein 60
<i>Trappc6b</i>	73	46	71	-0.7	5X10 <sup>-5</sup>	0.01	0.6	0.0002	0.03	Trafficking protein particle complex 6b

**Figure 4. Retinal development genes displaying significant expression changes among E12.5 *Neurog2*<sup>GFP/+</sup>, *Neurog2*<sup>GFP/GFP</sup> *Neurog2*<sup>*Ascl1KI/GFP*</sup> cells**

Two, selected alphabetical lists of genes with significant changes in gene expression. The first group are those known to act during early retinogenesis, the second group are largely active in the CNS. Columns 2-4 are reads per kilobase of transcript per million mapped reads (RPKM) values. The log-fold change between two different genotypes are listed in columns 5 and 8, followed by statistical significance in columns 6 and 9 = p-values; columns 7 and 10 = q-values. Some transcripts had significant q-values in one genotypic comparison, but not the other. Those genes validated by qPCR are denoted with \*\* or #. (# *Prdm1* validation can be found in Kowalchuk et al 2018# *Prdm1* validation can be found in Kowalchuk et al in press). Three genes in the top group (gray shading) were significantly downregulated in *Neurog2* mutants and upregulated by ectopic *Ascl1*.



**Figure 5. *Neurog2* RNA-seq transcription profiles for gene replacement mutations**  
 IGV browser view of high-throughput sequences aligned to the *Neurog2* genomic locus (mm10). **A**) In all three genotypes, many reads mapped to 5' or 3' UTR. Blue dotted lines demarcate the ORF within Exon2 and better highlight the lack of sequence reads for both mutants. These alleles were made through precise replacement of *Neurog2* coding sequences with GFP or *Ascl1* cDNA, but retention of both endogenous UTRs (Fode et al., 2000; Seibt et al., 2003). **B**) Exon-specific qPCR using optic cup cDNA from E12.5 littermates of those used for RNA-seq libraries. Exon1 mRNA is elevated over control in both mutants, but both

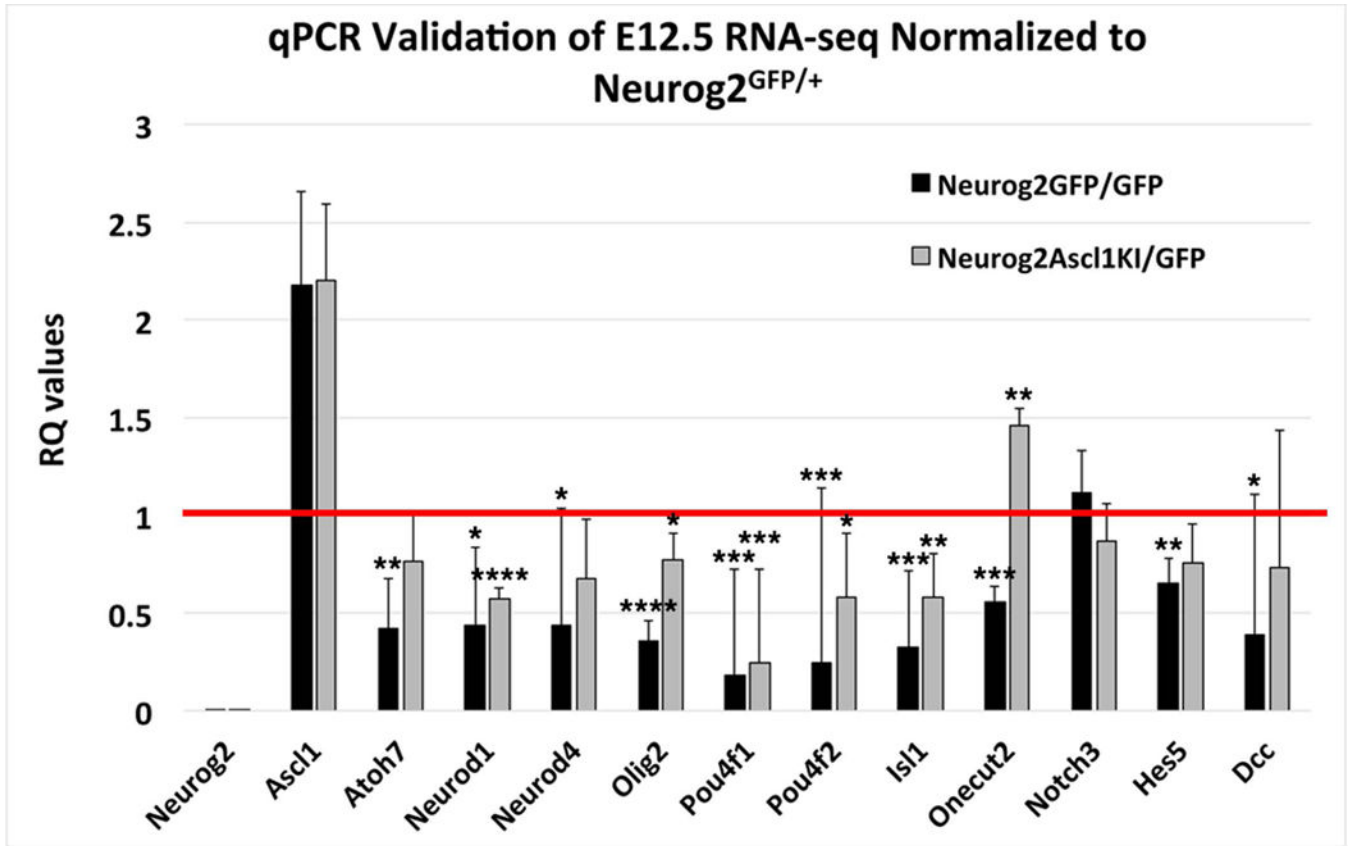
mutants lack Exon2-ORF mRNA. n = 3 biologic replicates/genotype; \*\*\*\* p < 0.0001; error bars = SEM.

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**Figure 6. Validation of gene expression changes in a *Neurog2* allelic series**  
qPCR outcomes for thirteen genes with significant q and/or p-values among the transcriptomic datasets. Elevated *Ascl1* levels solely reflect endogenous transcripts, since one primer resides in *Ascl1* 3' UTR that is not present in this replacement allele. *Neurog2*<sup>GFP/+</sup> cell transcript levels were normalized to 1. The *Neurog2* Exon2-ORF data from Fig 5B was also included here for comparison. n = 3 biologic replicates/genotype; \*p 0.05; \*\*p 0.01; \*\*\*p 0.001; \*\*\*\*p 0.0001; error bars = SEM.