Single cell transcriptome profiling of developing chick retinal cells

Lauren A Laboissonniere[1], Gregory M Martin[2], Jillian J Goetz[3], Ran Bi[4], Brock Pope[1], Kallie Weinand[1], Laura Ellson[1], Diane Fru[1], Miranda Lee[1], Andrea K Wester[1], Peng Liu[4] and Jeffrey M Trimarchi[1]*

[1] Department of Genetics, Development and Cell Biology

2114 Molecular Biology, Iowa State University, Ames, IA 50011

[2] Current address: Oregon Health and Science University, Portland, OR 97239

[3] Current address: Department of Ophthalmology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

[4] Department of Statistics, 2117 Snedecor Hall, Iowa State University, Ames IA 50011

*Corresponding author: Jeffrey M. Trimarchi

Email: jtrimarc@iastate.edu

Telephone: (515) 294-7059

Fax: (515) 294-7629

ABSTRACT

The vertebrate retina is a specialized photosensitive tissue comprised of six neuronal and one glial cell types, each of which develops in prescribed proportions at overlapping timepoints from a common progenitor pool. While each of these cells has a specific function contributing to proper vision in the mature animal, their differential representation in the retina as well as the presence of distinctive cellular subtypes makes identifying the transcriptomic signatures that lead to each retinal cell's fate determination and development challenging. We have analyzed transcriptomes from individual cells isolated from the chick retina throughout retinogenesis. While we focused our efforts on the retinal ganglion cells, our transcriptomes of developing chick cells also contained representation from multiple retinal cell types, including photoreceptors and interneurons at different stages of development. Most interesting was the identification of transcriptomes from individual mixed lineage progenitor cells in the chick as these cells offer a window into the cell fate decision-making process. Taken together, these datasets will enable us to uncover the most critical genes acting in the steps of cell fate determination and early differentiation of various retinal cell types.

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INTRODUCTION

Cellular diversity among mature neurons, including functional traits as well as connectivity with other cells, arises during development and is undoubtedly tied to both a cell's genetic makeup and environmental influences. Through a combination of intrinsic and extrinsic signaling, specific neuronal types with distinct functionalities are generated in the proper numbers and at the correct timepoints from neural progenitor cells. Identifying the exact combinations of these different signals that drive the formation of a fully operational central nervous system (CNS) is a daunting task. Fortunately, the intricacies of CNS development can be modeled using the genesis of the neural retina.

The vertebrate retina is an extension of the CNS, with a simple organization and relatively fewer populations of cells. The retina, therefore, is a highly valued tissue when studying neuronal diversity. Comprised of 6 neuronal and 1 glial cell types, the adult retina consists of rod and cone photoreceptors within the outer nuclear layer (ONL), bipolar, horizontal, amacrine interneurons, as well as Müller glia, in the inner nuclear layer (INL), and ganglion and displaced amacrine cells in the ganglion cell layer (GCL) (Cepko et al. 1996). Each retinal cell is generated from a common population of retinal progenitor cells (RPCs) in distinct yet overlapping periods during retinogenesis (Sidman 1961; Young 1985). These timespans can be variable in duration, however, as observed in the developing chick retina where RPCs have been reported to be capable of generating some fates for anywhere from a few days to over a week (Calaza Kda & Gardino 2010). To add to this complexity, RPCs have been shown through lineage analyses to be multipotent, with some cell divisions capable of generating two distinct retinal cell types (Holt et al. 1988; Turner & Cepko 1987; Turner et al. 1990).

The development of the chick retina follows that of well-studied mammals, with retinal ganglion cells (RGCs) generated in the first wave of cell birth (Snow & Robson 1994; Young 1985). Multiple studies examining RGC birth have yielded a window for RGC birthdates that spans from embryonic day 3 (E3) through E8 (Spence & Robson 1989; Mey & Thanos 2000; Snow & Robson 1994; Sakagami et al. 2003) with full RGC differentiation not completed until E16 (Mey & Thanos 2000). The majority of these cells are born before the plexiform layers begin to form, as the GCL and inner plexiform layer (IPL) cannot be observed until E7, and the outer plexiform layer (OPL) does not begin to develop until E9 (Spence & Robson 1989; Rodrigues et al. 2016). This first birth wave also sees production of amacrine cells (ACs) from E3-E14, depending upon their subtype and location in the retina (Spence & Robson 1989; Mey & Thanos 2000; Snow & Robson 1994). This birth window remains large due to the fact that separate subtypes of ACs are born at different times. For example, dopaminergic ACs are born between E3 and E7, but their distinct morphologies do not appear until E14, while serotonergic ACs develop specific wiring patterns between E12 and post-natal day 7 (P7) (Mey & Thanos 2000). Alongside the development of ACs, horizontal cells (HCs) begin to form after E3 and continue to develop until E6 (Kahn 1974; Edqvist et al. 2006). Closely following these birth dates, the photoreceptors are generated from E4-E17, and reach their full functionality by P3 (Adler 2000; Mey & Thanos 2000). Finally, bipolar cells (BCs) are the last neuron to be produced, with terminal divisions beginning after E8 (Spence & Robson 1989), immediately following the birth of Müller glia (Mey & Thanos 2000). Many cells remain plastic for some time after their terminal division before they are irreversibly committed to their cell fate (Adler 2000), but by the time the eye opens, all cells are functional.

For these reasons, the retina is an excellent model tissue to study cell development, especially as it relates to the central nervous system. Many studies of this tissue take place in the mouse, though we decided to examine the intrinsic factors present during the development of the chick retina. The diurnal chick has larger eyes than the nocturnal mouse, with which it relies more heavily on eyesight for sensory intake (Vergara & Canto-Soler 2012). While the murine retina contains two cones with opsins excitable at 360 nm (S-opsin) and 508 nm (M-opsin) (Fu & Yau 2007), the chick retina has four types of cones with excitability ranges between 350-700 nm (Wilby et al. 2015), allowing for excitation at higher wavelengths than both the mouse and the human, whose three cones experience maximum excitability at wavelengths between 420-562 nm (Bowmaker & Dartnall 1980). The convenience of the developing chick retina is also a major advantage for studying early-born retinal neurons as one can access the embryo *in ovo* for genetic manipulation, whereas the mouse is more difficult to modify as it develops *in utero*. Both of these model systems have sequenced genomes and multiple tools for transcriptomic analysis available, and the similar development of both the mouse and chick retinas makes this tissue a great tool for understanding cell fate acquisition. Furthermore, by comparing multiple vertebrate models it is possible to determine conserved gene programs, perhaps suggesting that these same programs will be conserved in other organisms as well, including humans.

A recent study examining the whole chick retina by RNA-Seq enabled the identification of genes expressed at a few specific timepoints during development (Langouet-Astrie et al. 2016). Since the developing retina is composed of many different cell types at various stages of maturation, single cell studies allow for a more precise examination of the transcriptomes of cells as they vary throughout development. Our study in particular details the transcriptomes of individual chick retinal cells at different developmental timepoints. We have identified and

characterized a large cohort of single RGC transcriptomes. In addition, during our characterization of these ganglion cells we also profiled the transcriptomes of single developing ACs, a cone photoreceptor cell, and other interneurons. In each case, we identified a large number of genes which were previously uncharacterized in the chick for each cell type. Finally, this project has yielded clusters of genes, which mark a transitional RPC that we believe is in the process of deciding between a horizontal and cone photoreceptor fate. From these transcriptomes, we have learned much about the gene expression of developing chick retinal cells, and also identified a set of transcription factors that may serve as combinatorial determinants for cell fate acquisition.

MATERIALS AND METHODS

Single cell microarrays

Tissue dissociation and cell isolation

The single cell profiling was performed as previously described (Goetz & Trimarchi 2012). For all steps in the single cell protocol requiring trituration or addition of reagents, filter tips were employed to avoid potential contamination. Briefly, retinas were dissected and dissociated with papain in Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM HEPES and 25 mM Cysteine in 5 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0. Chick retinas were incubated for 5 minutes at 37°C and the tissue was agitated by gentle trituration 10-15 times using a p1000 pipettor to ensure complete dissociation. DNase I (Roche) was added to remove the genomic DNA and the samples were incubated for 5 minutes at 37°C. After further trituration, the samples were centrifuged for 3 minutes at 3000 rpm. The supernatant was carefully removed and the pellet of dissociated cells dislodged by firm tapping. HBSS was used to wash the pellet and the samples were resuspended in 1X phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA).

Depending upon the developmental stage of the retina, a portion of the suspended cell solution was then plated in a 6 cm dish with 5 ml of fresh PBS containing 0.1% BSA, being cautious to not overcrowd the plate with cells. As a rule, we estimated the concentration of the dissociated retinas and aimed to plate 100,000 – 300,000 cells. A second 6 cm dish was prepared, also containing 0.1% BSA/PBS, to be used as a wash plate. The cells were allowed to settle for 5 minutes before isolation. Cells were isolated on an Olympus IMT-2 inverted microscope using pulled-glass micropipettes. While cells readily entered the micropipette through capillary action, they were gently expelled using pressure on the aspirator tube. After selecting one or more cells

from the first plate, the solution was expelled onto the wash plate to minimize contamination. From there, we were able to definitively select an individual cell to then be placed into its own PCR tube. A fresh micropipette was always employed to move an individual cell from the wash plate to a 0.2 ml PCR tube containing cell lysis buffer (1X PCR Buffer [10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.2 mM $MgCl_2$], 0.1 M DTT, a cocktail of RNase inhibitors [Applied Biosystems AM2694 and AM2682], 2.5 mM dNTPs, 10% NP-40 and 0.5 µM modified Oligo d(T) primer [TATAGAATTCGCGGCCGCTCGCGAT$_{24}$]). In an attempt to maximize the number of developing RGCs isolated, cells were selected based upon their size (i.e. the largest cells were targeted for isolation).

Library preparation

After isolation, samples were briefly spun in a table-top microcentrifuge to ensure submersion of cells into the lysis buffer. Lysis was promoted by incubating the samples for 90 seconds at 70°C. Reverse transcription mixture (SuperScript III [200 U/µl], RNase Inhibitor [40 U/µl, Applied Biosystems AM2682], and T4 gene 32 protein [NEB]) was added, and the mixture was incubated at 50°C for 50 minutes and inactivated at 70°C for 15 minutes before replacement on ice. Free primer removal was accomplished using Exonuclease I (NEB) at 37°C for 30 minutes and was inactivated at 80°C for 25 minutes. Tailing reaction mixture (10X PCR Buffer, 100 mM dATP, 10 U/µl Terminal Transferase [Roche], 0.05 U/µl RNase H [Invitrogen]) was added to the samples, which were incubated at 37°C for 20 minutes and inactivated at 70°C for 10 minutes. Finally, to perform the single-cell PCR, the following components (10X Ex-Taq Buffer, 0.25 mM dNTPs, 100 µM Oligo d(T) primer, 0.05 U/µl TaKaRa Ex Taq Hot Start [Takara]) were added to the samples. The PCR reaction used to amplify single-cell cDNA was as follows: 95°C for 2 minutes; 37°C for 5 minutes; 72°C for 16 minutes; then 34 cycles of 93°C

for 40 seconds, 67°C for 1 minute, and 72°C for 6 minutes plus 6 seconds more per cycle; finally, after incubating at 72°C for 10 minutes, the samples were held overnight at 4°C. To assess the initial quality of the cDNA library, it was analyzed on a 1% agarose gel along with a DNA size ladder. Ideal libraries exhibited a robust smear most brightly between 500-2000 bp. These were compared to blank media samples that were run through the entire washing and amplification process to determine whether any contamination exists throughout the process. Further quality tests to pre-screen samples before microarray hybridization included PCR screens for specific marker genes.

Microarray hybridization

15 µg of (1 µg/µl) amplified cDNA samples was fragmented in a solution containing 1X One-Phor-All Buffer (500 mM Potassium Acetate, 0.1 M Tris Acetate, 0.1 M Magnesium Acetate) and 1 U DNase I. Samples were incubated at 37°C for 13 minutes and inactivated at 99°C for 15 minutes. Labeling was accomplished by adding 1X TdT buffer, 25 µM Biotin N6-ddATP (Enzo Biosciences), and 4 U/µl TdT, then samples were incubated at 37°C for 90 minutes and inactivated at 65°C for 5 minutes, before storage at -20°C or immediate hybridization to Affymetrix microarrays according to standard Affymetrix protocols.

Clustering/heatmaps

Clusters of co-expressed genes were determined using hierarchical clustering through the Gene Cluster Software package (Eisen et al. 1998). The data were filtered by signal such that any gene not achieving a signal of 1000 in at least one single cell was removed. In our experience signals above this level are robust in that they are always denoted as "present" using the Affymetrix algorithm and they routinely validate by *in situ* hybridization. The remaining data was log-transformed and normalized using the standard methods included in the software

instructions (Eisen et al. 1998). Heatmaps were generated using Genesis software (Sturn et al. 2002).

RGC Correlation Analysis

To produce a list of genes which were highly co-expressed among our developing RGCs, we performed a correlation analysis to identify genes which were most highly correlated in expression level with that of NF-L. The sample correlation of NF-L with each of the 38534 genes was calculated and transformed using Fisher's Z-Transformation, where $Z = \frac{1}{2}\ln\left(\frac{1+r}{1-r}\right)$, is the transformed value of *r*, the sample correlation, and *ln* is the natural logarithm. Fisher's Z-Transformation is a function of *r* whose sampling distribution of the transformed value is close to normal. Following transformation, we tested whether the sample correlation is larger than zero or not for each genes, and controlled false discover rate (FDR) by Benjamini-Hochberg method (Benjamini & Hochberg 1995), allowing for the selection of genes which are highly correlated to our population indicator, NF-L. The output list of genes while FDR was controlled at level 0.05 contained 194 genes highly correlated with NF-L, while the list at FDR level 0.01 contained 115 genes. The list of 115 genes was used to define what we are labeling as the RGC "character" of each cell. This "character" was calculated by determining the percentage of genes from this output that were expressed by our isolated cells. This percentage was then normalized on a scale of 0-10, and those resulting scores were used to determine which cells were more likely to be developing RGCs depending upon their proximity to 10 on this scale.

***In Situ* Hybridizations**

Probe synthesis

Sequences for probe templates were amplified from chick cDNA (primer sequences in Table 1) and cloned into the pGEM-T vector (Promega) before confirmation via sequencing. Depending on the direction of insertion, antisense riboprobes were then transcribed. Approximately 1 µg of DNA was added to a solution containing 5X Optizyme Buffer (Fisher), 1X DIG RNA labeling mix (Roche), RNase Inhibitor (Roche), and either T7 or Sp6 RNA polymerase (Fisher). The reaction took place at 37°C for 1 hour. After a 15-minute DNase I (RNase free, Roche) treatment at 37°C, probes were precipitated overnight at -20°C using 10 mM Tris-1 mM EDTA pH8 (TE), 4 M lithium chloride (LiCl) and 100% Ethanol (EtOH). The next day, probes were spun at 4°C for 10 minutes at 13,000 rpm. After removal of the supernatant, probes were washed with 70% EtOH, then spun again for 2 minutes at 4°C. Following removal of ethanol, the probes were allowed to air dry for 5-10 minutes and were resuspended in TE (pH8).

Section in situ hybridization

In situ hybridizations (ISH) were performed on chick retinal cryosections. Eyes were fixed in 4% paraformaldehyde (PFA) in 1X PBS overnight at 4°C. The following day, the eyes were washed 3 times in PBS for 10 minutes at room temperature. Retinas were dissected and rocked in 30% sucrose in PBS for 30 minutes. After this wash, retinas were placed in a solution of 50% optimal cutting temperature (OCT)/50% sucrose in PBS and equilibrated for about 1 hour. Retinas were then frozen in this mixture at -80°C overnight. Blocks were cut into 20 µm sections on a cryostat, placed on Superfrost coated slides (Fisher), and fixed for 10 minutes in 4% PFA. Slides were washed 3 times in 1X PBS with 0.1% Tween (PBT), then tissue was permeabilized in 1 µg/ml proteinase K in PBS. After 2 PBT washes, slides were again fixed in 4% PFA for 5 minutes, then once again washed 3 times in PBT. These slides were then

incubated in acetic anhydride in 0.1 M triethanolamine hydrochloride (TEA) for 10 minutes. Slides were rinsed with PBT and probes were placed on individual slides in a solution of hybridization buffer (1 M Tris pH 7.5, 5 M NaCl, 0.5 M EDTA, 10% sodium dodecyl sulfate, 10 ml dextran sulfate, 1X Denhardt's solution, yeast tRNA [Sigma], 50% formamide) following a 95°C denaturing step of the probes. Probes were hybridized to the tissue overnight at 65°C.

Coverslips were removed in 5X sodium saline citrate (SSC) and slides were washed for 30 minutes in 1X SSC/50% formamide at 65°C. This step was followed by a 10-minute wash in 1 M Tris-5 M NaCl-0.5 M EDTA pH7.5 (TNE) at 37°C. RNase A (20 µg/ml, Roche) was added to a wash of TNE and the slides were exposed to this solution for 30 minutes, then re-washed in the original TNE for an additional 10 minutes. Slides were then placed in a series of 20-minute washes at 65°C: one wash in 2X SSC, then two washes in 0.2X SSC. This was followed by two 5-minute washes in 1 M Tris-5 M NaCl-Tween 20 pH7.5 (TNT) at room temperature. Slides were blocked for 1 hour in 20% heat-inactivated sheep serum (HISS) in TNT. Next, slides were blocked overnight in a solution of anti-Digoxigenin-Alkaline-Phosphatase (α-DIG-AP, Roche, RRID: AB_514497) antibody in 5% HISS/TNT at 4°C.

The next day, slides were washed in 5 M NaCl-1 M Tris-1 M $MgCl_2$ pH 9.5 (NTM) for 10 minutes, following a series of washes in TNT. Development took place at room temperature in the dark due to a solution of NTM containing Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). After visual inspection of signal, slides were individually placed into NTM to stop development and washed for 10 minutes. Slides were then washed twice for 5 minutes in PBS, fixed in 4% PFA for 30 minutes, then washed twice more in PBS. Just prior to mounting with Fluoromount-G, slides were rinsed quickly in dH_2O then allowed to air dry for 10 minutes. Images were acquired using a Nikon Eclipse 55i microscope at

10x and 20x. Adobe Photoshop was used to crop and lighten the background of photos such that the signal is clear. No other manipulations were performed.

RESULTS

Our goal was to gain insight into cell fate determination and cellular differentiation of early-generated chick retinal neurons using single cell transcriptomics. To achieve this goal, chick retinas were harvested from multiple stages of development, between E3 and E14, as these timepoints reflect the complete window of cell birth for RGCs in the chick (Spence & Robson 1989; Mey & Thanos 2000; Snow & Robson 1994; Sakagami et al. 2003). In order to maximize the number of developing RGCs, we isolated the largest cells in different fields of the plate as we have found that these tend to be mainly RGCs and occasionally HCs. Using fine-pulled pipettes, individual cells were collected from papain dissociated retinas at each of the timepoints and placed into a wash plate of PBS/BSA. Each cell was then re-aspirated from the wash plate and expelled into lysis buffer. After lysis, the mRNA was reverse transcribed and free primers were removed with Exonuclease I. Following a brief tailing step with TdT, cDNA libraries were amplified using a PCR program containing 34 cycles. The quality of the libraries, as determined by examining the size range of the resultant cDNA, was assessed on an agarose gel (data not shown). We have found that high quality libraries (those with a consistently successful microarray hybridization) are those with smears between 500-2000bp. To test our success at isolating RGCs, we employed a PCR-based screen using known marker genes. Neurofilament light (NF-L) primers allowed us to focus on isolated cells which were developing RGCs based on their expression of this intermediate filament gene (Liu et al. 2013) (data not shown). While expression of NF-L has been observed in a few non-RGC retinal neurons (Chien & Liem 1995), the majority of cells that express this marker are RGCs, allowing for its utilization in the enrichment of single ganglion cells profiles. Once we had identified a number of cells that we believed to be good candidates for developing RGCs, we prepared our samples for microarray

hybridization by DNase treating 15µg of each sample, followed by biotinylation. The labeled cDNA was hybridized to Affymetrix chick microarray chips and the resulting array data was normalized and log transformed using Affymetrix Microarray software (MAS 5.0). The full data set can be accessed through the Gene Expression Omnibus (GEO) at NCBI (GSE87663).

Gene expression in developing retinal ganglion cells – clusters of co-expressed genes

Since we isolated larger cells and pre-screened their cDNAs using NF-L primers, we strongly believed we had profiled many developing RGCs. To confirm this prediction and to assess how well conserved RGC gene expression is between mouse and chick, we mined our dataset for the presence of genes previously identified through single cell transcriptomics of developing mouse RGCs (Trimarchi et al. 2007). Genes such as synuclein gamma (Sncg) and the neurofilaments, both light (NF-L) and medium (NF-M) chain, were examined for expression in our dataset as they are expressed by RGCs during development (Surgucheva et al. 2008; Liu et al. 2013; Torelli et al. 1989; Trimarchi et al. 2007). As demonstrated through our microarray data, both NF-L and NF-M were expressed at high levels among our isolated cells, and were detected as early as E3 in a subset of our cells (Fig. 1). While Sncg was expressed by many of our cells, this gene was found in fewer developing RGCs than the neurofilaments (Fig. 1). This differs from the expression of Sncg in the mouse, where this gene marks the entire RGC population from their initial birthdate (Trimarchi et al. 2007; Soto et al. 2008).

We next wished to identify clusters of additional genes that were also highly expressed in our developing chick RGCs. Since NF-L and NF-M were highly enriched in developing RGCs in the chick retina, we used these genes as landmarks for the clustering. To determine RGC gene clusters, the data was filtered such that only genes with at least one signal about 1000 were used,

gene Cluster software (Eisen et al. 1998) was employed and the resulting heatmaps visualized using Genesis software (Sturn et al. 2002). Specifically, we focused on those genes that were strongly associated (correlation coefficient = 0.75) with NF-L/NF-M in hierarchical clustering. Through this analysis we found ~400 genes (Table 2), many of which had been observed in developing mouse RGCs, e.g. ubiquitin C-terminal hydrolase L1 (Uchl1) and early B-cell factor 3 (Ebf3) (Trimarchi et al. 2007). Other genes in this cluster included many synapse formation-related genes such as calmodulin 2, synaptosomal associated protein, 25kDa, calcium dependent secretion activator, and synaptotagmin 4 (Table 2). Some of these genes were expressed as early as E3, as determined by our microarray data, with strong expression of many of these genes between E5 and E14 (Fig. 1). Of these 39 cells, 27 showed significant expression of these genes and were believed to be developing RGCs (Fig. 1).

To provide additional evidence in support of our hypothesis that these cells were developing RGCs, we attempted to classify each of our isolated cells into their respective cell types. We employed a statistical method for the generation of gene sets that corresponded to 5 specific cell types we were interested in investigating. Those cell types were RGCs, ACs, cones, HCs, and RPCs. Gene correlates were determined for each population, using known marker genes as the standard by which to compare the other genes. For example, NF-L was used as the RGC population marker, while cyclin D1 and cell division cycle 20 (cdc20) were used together as markers for the RPC population. Due to the fact that very few of our single cell profiles are believed to be from cones, HCs, and RPCs, we were unable to generate statistically significant gene correlate lists for these populations. We were also unable to obtain reliable correlate lists for the AC population, even though some of our isolated cells appear to express a number of AC genes. The markers we chose for this population, Tac1 and Cbln2, produced 0 and 11 gene

correlates, respectively. Though we would like to examine the AC "character" of our cells, the difficulty in generating a gene correlate list probably lies within the fact that this cell population is quite diverse, not unlike the AC population of the mouse (Cherry et al. 2009), and we were not able to perform such an analysis. However, the gene correlate analysis for NF-L produced a list of 194 genes that were highly correlated with this gene [false discovery rate (FDR) was controlled at 0.05]. When the FDR was instead held at 0.01, this list was reduced to 115 genes whose expression patterns mirrored that of NF-L. Comparing this list of 115 to our initial list of ~400 genes produced using hierarchical clustering (Table 2), we found that 105 of these 115 were in both sets, suggesting these are very efficient means of correlation analysis.

With this list of highly correlated genes in hand, we devised a method to classify the RGC "character" of each single cell profile. First, we determined how many of these 115 genes were expressed by each of our cells and divided that number by the total number of genes expressed above our cut-off by each cell. The resulting numbers were then normalized to one another and equated to a 0-10 scale (Table 3), as had similarly been done in an analysis of mouse single cells (Trimarchi et al. 2008a). Cells that scored greater than 7 from this analysis were found to be expressing the majority of these RGC-associated genes and, therefore, have been labeled as developing RGCs. We found a total of 6 such cells in our data set that met this criterion. Cells that scored between 4 and 7 express somewhat fewer of these RGC- associated genes, but we are still confident in their potential to develop into RGCs. We identified 21 of these cells in our data set. Finally cells that scored less than 4 we believe to be other developing retina cells. In fact, a closer look at their expression profiles showed that these cells were more likely to be RPCs or, in one case, a mature cone photoreceptor.

One potential reason for the variety of RGC- character" scores might be that fact that the cells we transcriptomically profiled were from various stages of development. Given this, we hypothesized that some RGC markers might display a dynamic expression pattern. In fact, we found that certain RGC markers showed an onset of expression early in development, while others were not detected until days later. To further define if there were different clusters of RGC-expressed genes separated in a temporal manner, we examined our hierarchical clustering in more detail. We found that there were actually two temporal clusters of genes specific to our RGCs. The first cluster, described above, revolved around the expression of NF-L and NF-M, which were detected first at early timepoints with their expression maintained throughout development. A second RGC-expressed cluster of genes, whose expression appeared to be somewhat delayed when compared to the first cluster, was strongly associated with the gene neurofilament heavy (NF-H). This cluster includes ion channels and other genes that may play a role in later stages of RGC maturation (Table 4).

Gene expression in developing retinal ganglion cells – *in situ* hybridization

Since the number of cells we could profile using single cell transcriptomics was limited, we wanted to explore the expression patterns of a subset of the genes that appeared in our gene clusters across the entire chick retina. Therefore, we employed *in situ* hybridization (ISH) at various developmental timepoints. The expression pattern of each probe examined in this study during developmental timepoints E4-E14 is summarized (Table 5). We wished to examine a wide variety of genes from our different gene clusters and also genes that emerged from a visual inspection of the different transcriptome profiles. We focused on initial attention on genes from the profiles of cells that had the highest RGC "character" (Table 3). During early retinal development, the retina is comprised of predominantly cycling RPCs and a small population of

differentiating neurons. We investigated the expression of three early-RGC markers at E4 throughout the entire retina in order to get a better understanding of where these genes were being expressed in a complete section of tissue, whether among RPCs or cells which have recently exited the cell cycle. Images were taken and compiled to construct the whole section observed at this timepoint (Fig. 2). Depictions of retinal layers at developmental timepoints are diagrammed for the whole retina at E5 (Fig. 2A), retinal sections between E3-E8 (Fig. 2B), and retinal sections after E9 (Fig. 2C). The genes observed were Uchl1 (Fig. 2D), Protein phosphatase 3, Catalytic Subunit, Alpha Isozyme (Ppp3ca) (Fig. 2E), and Tagln3 (Fig. 2F). Both Ppp3ca and Tagln3 had not previously been found in retinal cells, so this was the first evidence that both genes are expressed by RGCs. The expression pattern of these three genes begins in the center of the retina and then extends outward through the tissue in a fan-like pattern. This pattern follows the generation of the first neurons in the retina, much like the development of the mouse retina. In addition, Tagln3 was detected in a subset of cells in the outer neuroblastic layer (ONBL) (Fig. 2F). The ONBL is where cycling cells reside together with newly postmitotic neurons, indicating that Tagln3 may be expressed in developing RGCs even before they migrated from the apical side of the retina, where mitosis occurs to their final position in the basal side of the retina (Fig. 2F). The other two genes were only detected in the inner neuroblastic layer (INBL), the layer of the developing retina adjacent to the developing vitreous where maturing neurons reside (Blackshaw et al. 2004). These three genes were among some of the earliest genes expressed in our isolated cells, denoting an early-expressed set of RGC genes. Other early-expressed genes (found as early as E3) included microtubule-associated protein 6 (Map6), two of the Iroquois homeobox transcription factors (Irx1, Irx2), as well as both NF-L and NF-M (Fig. 1).

To gain a better understanding of gene expression throughout the development of the chick retina, we performed ISH at multiple timepoints on chick retinal tissue sections. First, we examined the expression of the two neurofilament genes, NF-L and NF-M, which we used in our clustering analysis. The neurofilament proteins have been previously shown to mark RGCs as well as other cells of the INL and ONL; in particular, NF-L marks RGCs at all stages of chick development and protein levels have been detected among ACs at E17 and later (Liu et al. 2013). Using our probes, we found NF-L and NF-M were strongly expressed in the basal retina (Fig. 3A, E), where the GCL will eventually form. Additionally, we observed expression across the retina at E4 in a significant number of cells in the ONBL. These cells may be cycling cells or newly exited RGCs that were migrating to their appropriate layer. By E6, both genes were primarily found in the GCL and, aside from the occasional cell in the INL, remained that way for the remainder of development (Fig. 3B-D, F-H).

The expression of Uchl1 protein in the mature chick retina was found to be present at low levels among RGCs (Bonfanti et al. 1992), so we aimed to correlate the RNA localization with these findings and extend the studies across development. ISH staining showed strong expression in the developing ganglion cell layer (GCL) beginning at E4 and continuing through E8 (Fig. 3I-K), with detection observed in the RGCs and some cells within the INL at E14 (Fig. 3L). We believe that the Uchl1+ cells in the INL were likely displaced RGCs due to their large size (Prada et al. 1989), though we cannot rule out the possibility that there may be a small subset of ACs expressing this gene.

During our ISH analysis of genes identified in the clustering analysis, we observed the Affymetrix microarray probe Gga.19620.1.S1_at, which showed strong expression in our single cell profiles and was highly correlated with NF-L in our clustering analysis (Fig. 1). Using the

NCBI basic local alignment search tool (BLAST), we examined the sequences with the highest alignment similarity in order to make an RNA probe to this gene. We found chick clone ChEST382l11 was a 100% match to this probe sequence and decided to investigate its signal by ISH. Around E4, very few cells in the INBL showed expression of ChEST382l11 (Fig. 3M), but by E6 and later, this gene was widely expressed by a high number of cells in the INBL (Fig. 3N-P). Based on these results, we further characterized the gene associated with ChEST382l11 and found it was a 97% match for chick POU class 4 homeobox 1, or Brn3a. POU class 4 transcription factors play an important role in the development of chick RGCs (Liu et al. 2000) as well as in other animals. The identification of this previously unnamed probe also demonstrates the effectiveness of our gene search and our ability to identify important RGC-specific genes.

In our previous studies in the mouse (Trimarchi et al. 2007), we found that RGCs and ACs share a substantial amount of gene expression in common. During our ISH investigation of genes in our RGC clusters , we also found a number of genes expressed by both RGCs and ACs. For example, Early B-cell factor 3 (Ebf3) showed strong expression in our single cell profiles (Fig. 1). By ISH, Ebf3 was expressed among cells of the INBL (Fig. 3Q-R). This signal persisted at later timepoints, and also appeared in a few INL cells (Fig. 3S-T), suggesting this gene is also expressed by a subset of ACs, which would remain consistent with the discovery of this gene in a small subset of ACs in the mouse (Kay, Voinescu, et al. 2011).

Previous studies in the mouse have shown the guidance receptor, Roundabout guidance receptor 2 (Robo2), is exclusively expressed by RGCs and is necessary for bundling of RGC axons towards the optic fiber layer (Thompson et al. 2009). We also found the expression of this receptor among our isolated cells and chose to investigate the expression of this gene further

during chick retinal development. Through ISH we found Robo2 expression showed a different staining pattern than was found in the mouse study. In the developing chick retina, Robo2 signal was first detected in the INBL at E4 along with a few cells stained in the ONBL (Fig. 3U). This pattern continued at E6 (Fig. 3V), but by E8 and later, Robo2 was expressed strongly in cells located in three distinct regions of the retina (Fig. 3W-X), consistent with expression of Robo2 in HCs, RGCs and at least a subset of ACs.

Using the gene clusters from our single cell microarray data, we began to look for novel genes expressed in developing RGCs and ACs. Employing ISH, Ppp3ca and Map6 were both found to be expressed by RGCs at E6 and E8 (Fig. 4A-D). Among the RGC genes discovered, one of them was Delta/Notch-Like EGF Repeat Containing (DNER), which acts as a Notch ligand for maturation of cerebellar glial cells (Saito & Takeshima 2006). Though expression has been suggested in subsets of cortical neurons (Saito & Takeshima 2006), the expression of this gene has not previously been reported in the retina. Through ISH, we found DNER was expressed weakly by a subset of cells in the INBL at E6 (Fig. 4G) and E8 (Fig. 4H). By E8, we also observed weak expression of DNER in developing ACs and HCs (Fig. 4H). We found C17h9orf7 expressed in our isolated RGCs and through ISH observed expression was enriched in the INBL at E6 (Fig. 4I) and increased in the developing GCL by E8 (Fig. 4J). Finally, Tmem163, a putative zinc transporter, was expressed in our isolated RGCs and was found enriched in the INBL at both E6 and E8 in our chick retinas (Fig. 4K-L). These newly identified RGC genes may play an important role in the development of RGCs and will need to be studied through gain and loss of function experiments in the future.

Finally, through the NF-L/NF-M clustering analysis, we also identified an Affymetrix probe that was expressed highly among our RGCs, GgaAffx.21667.1.S1_s_at. Using BLAST,

the probe sequence was compared to available databases and reported a 100% sequence homology with chick clone ChEST742d11. We investigated this gene's expression at these developmental timepoints and found strong expression of ChEST742D11 in the INBL at E4 and E6 (Fig. 3Y-Z). By E8, expression was now within the GCL but was accompanied by staining in the INL, which continued through late development (Fig. 3A'-B').

Upon further analysis, this sequence was found to match one known gene in the Japanese quail, at 93% homology. That gene is stathmin 3, which produces an important microtubule stabilizing protein (Nair et al. 2014). In our clustering analyses, we observed that two related genes, Stathmin-like 2 and Stathmin-like 4, were also highly correlated with NF-L. To ask whether our genes with related functions were also present in our gene clusters, we used a GO analysis tool and found that many of the correlated genes produce proteins with functions related to microtubule stabilization and polymerization as well as receptors and proteins important for neuronal processes, such as SNARE binding proteins and axon guidance receptors. Among the microtubule stabilization and polymerization group, the stathmin proteins have been shown to play an important role in the transition from neural progenitor to differentiated neuron in both the mouse and the chick (Boekhoorn et al. 2014; Ratié et al. 2014).

Taken together our clustering and in situ hybridization results identified a significant number of RGC expressed genes, some of which were known from work in other species and some of which had not been previously shown to be expressed in these cells.

Genes expressed only in a subset of our profiled chick retinal cells

Our investigation into RGC markers also uncovered some genes that were expressed only by a subset of RGCs. One of those genes was Sncg, whose expression is seen among the

majority of the ganglion cell population of the mouse (Trimarchi et al. 2007; Soto et al. 2008) and the rat (Surgucheva et al. 2008). Demonstrating a conservation of expression of this gene between mouse and chick, we found Sncg expressed in the INBL at all early timepoints examined and then in the GCL at later timepoints (Fig. 5A-D). However, unlike in rodents, Sncg was not detected in the profiles of all RGCs in the chick, but rather only in a large subset. Similarly, the expression patterns of two transcription factors, Irx1 and Irx2 appeared in a large subset of developing RGC single cell profiles (Fig. 1). Using ISH, these two transcription factors showed weaker expression at early timepoints, E4 and E6, among cells in the INBL (Fig. 5E-F, I-J), yet their signal increased during late development, E8 and E14, and was confined to the GCL (Fig. 5G-H, K-L). We next examined the expression of Tagln3 at these four timepoints. Tagln3 was found to mark a subset of cells in the INBL during early development (Fig. 5M-O), as well as RGCs and a subset of ACs by E14 (Fig. 5P). Finally, as a comparison, we sought to examine the expression of Islet1 (Isl1), a gene which has been shown to play an important role in both chick HCs and RGCs (Suga et al. 2009; Boije et al. 2009; Okamoto et al. 2009). In the mouse, Isl1 is expressed by the majority of RGCs and is known to play a role in RGC cell fate determination together with Brn3b (Pan et al. 2008; Wu et al. 2015). We found this gene was expressed in a subset of RGCs throughout development of the chick retina (Fig. 5Q-S) and could be seen in a large population of RGCs at E14 (Fig. 5T). Isl1 was also expressed among cells of the INL at E14, likely within a subset of ACs and HCs, and potentially demonstrating expression among a small subset of BCs (Fig. 5T).

The chick retina is comprised of at least 26 RGC subtypes, many of which have functionally and morphologically similar characteristics to subtypes in mammals (Naito & Chen 2004). We began looking for subtypes of cells in our population by using known molecular

markers to initially identify these subpopulations. Very few RGCs types in the mouse have known molecular identifiers, though direction-selective cells have begun to demonstrate uniform gene expression within their populations (Kay et al. 2011). One of the most widely used molecular identifiers of an RGC subtype in the mouse is junction adhesion molecule 2 (Jam2), also referred to as JAM-B (Kim et al. 2008). In the mouse, this molecular marker is used to identify subtype of direction-selective RGCs, but the localization of Jam2 in the chick retina has not been characterized. We examined the expression pattern of this gene in our isolated cells and observed signal in five of our cells, E5 Cells #1 and #3, and E14 Cells #2, #3, and #4. All of these cells, except for E5 Cell #3 appeared to be developing RGCs, due to their expression of many RGC-specific genes (Fig. 1) and RGC "character" scores (Table 3). E5 Cell #3, however, failed to express any of the early-expressed RGC markers including the neurofilaments, suggesting that perhaps Jam2 is playing a role in another type of chick retinal cell, and may not be restricted to only RGCs. Among the E14 cells, we found that #2 and #4 also expressed Tachykinin 1 (Tac1) (Fig. 6A), while the other Jam2+ cell, E14 Cell #3, expressed Tachykinin receptor 2 (data not shown). The expression of the Tac1 neuropeptide has been demonstrated to mark ACs in a number of vertebrates (Bagnoli et al. 2003), and one study suggested Tac1 is utilized among a small subset of RGCs that selectively innervate the tectum (Yamagata et al. 2006). We found another Jam2+ cell also expresses Dopamine receptor D3, an inhibitory dopamine receptor whose expression pattern in the retina has not been characterized. Among these Jam2+ cells we also observed the expression of Cerebellin-1 (Cbln1) and 2 (Cbln2). These two proteins have been shown to play a role in synaptic organization within the brain and Cbln2 has been found in ACs and a small subset of RGCs in the retina (Reiner et al. 2011; Trimarchi et al. 2007). Cells such as these are interesting in that they express many typical RGC genes, but

the expression of some AC related genes also appeared transiently within them. This points to the possibility that these Jam2+ cells arose from a population of progenitors that can generate either ACs or RGCs, which could explain the co-expression of many cell type markers. However, it is also possible that Jam2 does not serve only as a marker for RGCs in the chick, as new evidence has been found for expression of this gene among RGCs and ACs of the mouse (Martersteck et al. 2017).

We continued to examine our single cell transcriptomes for the identification of other cells potentially at this decision point between RGC and AC by looking for neurotransmitter specific genes among our isolated cells. This investigation began with choline acetyltransferase (ChAT), the synthesizing enzyme for acetylcholine (Oda 1999), which has been shown to be expressed by a subset of ACs (Stanke et al. 2008). We found two E9 cells expressing ChAT (E9 Cell #2 and E9 Cell #3), which we had previously deemed RGCs based on their expression of genes such as Sncg, Map6, and Uchl1, coupled with their RGC scores (Table 3). One possibility is that this cell was still in the process of making a final decision regarding fate. If this were the case, one might expect a number of regulatory factors playing roles at this time, specifically transcription factors. We found several transcription factors expressed in the two ChAT+ cells, including Atoh8, members of the Forkhead and Iroquois families, and NeuroD6. The presence of these transcription factors within the ChAT+ cells might indicate that these cells are potentially still plastic when it comes to an ultimate cell fate. Uncovering the precise roles for these genes, however, will require functional studies in the future.

Gene expression of developing amacrine cells

In addition to developing RGCs, we also found some cells that did not have as strong an RGC "character" score as others (Table 3). ACs are a very diverse group of cells with a multitude of subtypes (Masland 2012), some of which have similar expression to RGCs (Trimarchi et al. 2007). Therefore, we decided to ask if this was also the case in the chick by looking first at the expression of AC genes. We performed PCR for known AC markers such as Tac1 and transcription factor AP-2β (Tfap2b) on cDNA from our isolated single cells prior to microarray hybridization. We found multiple cells at both E9 and E14 expressing Tac1, which suggested these cells may be ACs (Bagnoli et al. 2003), though a small population of RGCs in the chick retina have been shown to use this neuropeptide (Yamagata et al. 2006). These cells were considered strong candidates to be developing amacrine cells, so we proceeded with microarray hybridization. We generated a heatmap of AC genes and their expression intensity in our isolated cells (Fig. 6A). While many of these genes showed some expression in our cells in various combinations, there did not appear to be uniform AC-gene expression across a multitude of cells. These findings are reminiscent of AC expression in the mouse retina, where no AC marker gene was found to be present in the entire population (Cherry et al. 2009).

However, E6 Cell #1 showed high levels of expression for many RGC-associated and AC-associated genes (Fig. 1A, 6A). ISH using RNA probes for four of these genes showed the expression patterns during development. We began by looking at Tfap2b, whose expression has been characterized previously among HCs and ACs in the developing and mature chick (Bisgrove & Godbout 1999) and mouse (Bassett et al. 2012) retinas. Tfap2b showed high levels of expression in only 3 of the single cell profiles (Fig. 6A), but appeared to be strongly expressed by a subset of ACs between E4 through E14 (Fig. 6B-D). By E14, the signal of this gene was strong among HCs and ACs, within both the INL and GCL (Fig. 6E). Slit guidance ligand 2

(Slit2), unlike its counterpart Slit1, is typically expressed by ACs in the mouse retina during later developmental timepoints (Erskine et al. 2000). In our experiments, Slit2 was not robustly detectable until E8 (Fig. 6F-I), though a few isolated cells showed expression of this gene at E6 (Fig. 6A). By E14, Slit2 was expressed exclusively in the INL among a subset of ACs (Fig. 6I). We also chose to examine Tac1, an AC marker in the mouse and AC/RGC marker in the rat and primate (Bagnoli et al. 2003). The expression of this gene began at E8 (Fig. 6J-L) and remained confined to a subset of cells in the GCL and INL at later timepoints (Fig. 6M). Finally, we looked at Cbln2 at these timepoints due to the fact that it has been shown to be present in ACs and RGCs in both the chick and mouse (Reiner et al. 2011; Siegert et al. 2009) . We found faint expression in the INBL at E4 (Fig. 6N) and at E6 (Fig. 6O), which then increased between E8 and E14 (Fig. 6P-Q), to be expressed solely among a subset of cells in the INL whose position was consistent with ACs.

As with developing RGCs, we wished to use our data from these developing ACs to uncover novel markers of this population. Since we had so few cells in this class, we had to rely on visual inspection of the data rather than clustering analysis. In our single cell profiles, we asked which genes were expressed preferentially in the putative ACs and found a few genes to investigate further using ISH. First, we examined Fibroblast growth factor 13 (Fgf13) and found that by E14, this gene was expressed in the INL and GCL, marking a subset of ACs and possibly some RGCs (Fig. 7A). We also investigated a TF from this population, Pre-B-Cell Leukemia Homeobox 1 (Pbx1). At E14, we found the mRNA for this gene only in the INL in an area consistent with expression by ACs (Fig. 7B). Another unknown probe, ChEST32g3, arose in this dataset and we found this gene expressed by a subset of ACs (Fig. 7C). Similar to ChEST32g3, Septin 11 (Sept11) was expressed by a subset of ACs at E14 (Fig. 7D), demonstrating these two

genes are novel AC markers in the chick. Additionally, we decided to examine the expression of melanopsin in the retina after finding this gene expressed in a number of our cells. It should be noted that the chick has two different genes for melanopsin (Bellingham et al. 2006), and the one we investigated was the mammalian gene homolog (Opn4m), coinciding with the probe on the microarray chip. While the expression of this gene has been reported among a large proportion of cells in the INL, likely BCs, our investigation found this gene only in a small subset of cells at E14 (Fig. 7E). This directly contrasts with findings in mammals, where Opn4 is localized to a small subset of RGCs (Provencio et al. 2000). Finally, this investigation highlighted another transcription factor, POU class 6 homeobox 2 (Pou6f2) due to its expression in our isolated ACs. When examining the expression of Pou6f2 at E14, we found this gene expressed by a small population of cells in the GCL as well as the INL, likely in ACs and HCs (Fig. 7F). The expression pattern of this gene varies somewhat from the observed localization in the mouse retina, where it was expressed among a large subset of RGCs (Zhou et al. 1996).

Single cell profiling of the cell fate transition process

We were also interested in potentially identifying genes involved in the process of early born retinal cells selecting a particular cell fate. As single cell profiling is an excellent technique to address this question, we began by looking for any cells in our dataset that were expressing chick atonal homolog 7 (Cath5). The murine homolog of this gene, Math5, is known to play a crucial role in retinogenesis, and the loss of this TF has been shown to lead to a near complete loss of RGCs (Feng et al. 2010; Wang et al. 2001; Brown et al. 2001; Le et al. 2006). In addition, our lab and others have observed the expression of Math5 during the G2/M transition portion of the cell cycle, during the crucial timepoint just prior to cell cycle exit (Feng et al. 2010). Therefore, we decided to examine the transcriptomes of cells expressing Cath5 in further detail,

with the hopes that we would uncover other factors playing a role in cell fate acquisition. One of our chick cells, E4 Cell #1, did not appear to be either an RGC or an AC by both gene expression and by RGC "character" score (Table 3). By visual inspection, we identified a number of HC genes expressed by E4 Cell #1 and generated a heatmap to look more closely at the expression of these genes across our entire population of cells. This heatmap shows HC expressed genes, fibroblast growth factor 19 (Fgf19) (Okamoto et al. 2009; Francisco-Morcillo et al. 2005; Kurose et al. 2004), and LIM homeobox 1 (Lhx1) (Suga et al. 2009), as well as the TF, Cath5, all expressed in E4 Cell #1 (Fig. 8A). Based on the expression of these genes, we hypothesized that E4 Cell #1 was a developing HC (Fig. 8A). No other cells showed similar expression patterns, although some single cells expressed some of these genes at varying levels and in different combinations, e.g. E6 Cell #3 and E7 Cell #1 (Fig. 8A).

Using ISH, we wanted to examine if the expression of these genes was consistent with developing HCs in the chick retina. Cath5 expression was observed in a subset of cells in the ONBL from E4-E8 (Fig. 8B-F). Fgf19 was also expressed in a subset of cells in the ONBL across the retina at E4 and E5 (Fig. 8G-H), but by E6 and E7 this gene showed increased staining in a layer of cells just above the developing GCL (Fig. 8I-J). As HCs migrate bi-directionally through the retina during development (Edqvist & Hallböök 2004; Boije et al. 2009), this expression pattern of Fgf19 was consistent with it being present in a population of developing HCs, although we cannot rule out expression in other cells as well. Finally, by E8, Fgf19 was confined to cells abutting the developing OPL, a position also consistent with HCs (Fig. 8K).

Since the expression of Fgf19 by ISH appeared among migrating cells during early development, we wanted to perform an experiment to see how many Fgf19+ cells are RPCs. To determine this proportion, we introduced ^{3}H-thymidine *in ovo* at E4 for 1 hour before isolating

the retina. ISH for Fgf19 performed in conjunction with ^{3}H-thymidine detection was performed on 3 independent chick eyes, with 5 random fields sampled. This analysis demonstrated a 21.9% +/- 4.2% overlap at E4 after a 1-hour pulse (data not shown). We further examined the overlap of Fgf19 and ^{3}H-thymidine following an 8-hour pulse, using the same sampling technique, and found that only 67% +/- 6% of cells were co-labeled, suggesting the remaining 33% of cells began to express Fgf19 after they had exited the cell cycle. This is interesting since it had been suggested that Fgf19 plays a similar role in the chick as Fgf15 plays in the mouse (Kurose et al. 2004). However, Fgf15 has been shown to overlap with ^{3}H-thymidine in 50% of cells after a 1-hour pulse (Trimarchi et al. 2008b), which is a much larger population of cycling cells than were labeled with Fgf19.

During our investigation into the genes highly expressed by E4 Cell #1, we came across a probe, Gga.19701.1.S1_at, which was not assigned to a specific gene. This probe was not only highly expressed, it was exclusively expressed by this cell and no other cells in our data set. We decided to investigate further to see if this probe was a HC specific marker, or if it might lend insight into the development of E4 Cell #1. Using the NCBI BLAST tool, this probe sequence produced a 99% alignment to predicted chick sequence ChEST736n4, as well as high alignment scores (>90%) for the gene Onecut2 (OC2) in a variety of animals. Onecut2, which has been shown to be expressed by HCs in the mouse, results in a loss of 50% of the HCs when removed from mice (Sapkota et al. 2014; Goetz et al. 2014). To examine the expression pattern further in the chick retina, we completed ISH and found expression of ChEST736n4 within a subset of cells in the ONBL at E4 (Fig. 8L), much like the expression of Cath5 and Fgf19 at that timepoint. From E5-E7, this probe was confined to cells in the basal retina, which we suspected were cycling HCs at those timepoints (Fig. 8M-O). Ultimately, ChEST736n4 signal was found

within the apical INL by E8 (Fig. 8P), suggesting a conserved role for this gene in the development of HCs of multiple organisms.

During our examination of E4 Cell #1, we noticed high expression of LIM-Homeobox 1 (Lhx1) and Orthodenticle Homeobox 2 (Otx2). Lhx1 is specific to a population of mature HCs in the chick (Suga et al. 2009), while Otx2 has been shown to be expressed by mature cones (Nishida et al. 2003). The co-expression of these TFs suggests that E4 Cell #1 had not committed to a cell fate, and that this cell was likely deciding between a HC or a cone photoreceptor fate. A population of RPCs has been demonstrated to give rise specifically to HCs and cones, with respect to the variation of genetic factors which instruct the decision of cell fate (Emerson et al. 2013). RPCs which express the Onecut TFs at high levels were likely to pursue a horizontal fate, while RPCs that expressed Otx2 in greater amounts typically developed into cones (Emerson et al. 2013). E4 Cell #1 became a cell of increased interest due to its expression of ChEST736n4 (OC2), Lhx1, and Otx2, suggesting this cell was in the process of committing to a cell fate, either a HC or a cone. Therefore, we can use the data from this cell to identify other genes, specifically TFs, which may play a role in this fate decision process. For example, we found expression of Beta3, Zeb2, Olig3, and Pbx4 in E4 Cell #1 (Fig. 8A).

In our search for additional genes expressed in E4 Cell #1, we also identified Znf488. This gene was found in a small number of our single cell profiles, including E4 Cell #1, so we used ISH to determine the expression pattern of this gene in the retina. In retinal sections, this gene was not detected at E4 but was found by E6 on the apical side on the INBL (Fig. 9A-B), in a position consistent with the location of ACs. At later timepoints (E8-E14), Znf488 expression was localized to a subset of cells in the basal INL, likely among a subset of ACs (Fig. 9C-F). We hypothesized that E4 Cell #1 was deciding between a HC and a cone fate, yet it was also

expressing Znf488, suggesting that this cell may also express genes indicative of developing ACs. However, since we could not detect Znf488 by ISH at the E4 timepoint it is possible that its expression pattern is different this early and the single cell profiling method is just more sensitive than ISH. Alternatively, the expression of this later AC marker could point to a state of potential "confusion" encountered by RPCs prior to cell fate acquisition, which has also been observed in other single cell studies from different tissues (Olsson et al. 2016).

Gene profile of cone photoreceptors

To examine more mature cells, we also examined the transcriptomes of a few cells isolated at E15. An initial analysis of the data in Excel revealed that E15 Cell #1 expressed several genes characteristic of cone photoreceptors. To identify more cone-expressed transcripts, we performed hierarchical clustering using the entire data set from E3-E15. An examination of the genes highly correlated to guanine nucleotide binding protein, alpha transducing activity polypeptide 2 (Gnat2) (correlation coefficient=0.60) revealed more than 90 genes, many of which were known cone markers but also many of which were novel genes or not known to be expressed in cones (Table 6). We evaluated the expression of some of these genes within our isolated cells and generated a heatmap to represent this data (Fig. 10A). To determine the photoreceptive capabilities of E15 Cell #1, we studied the expression of the various opsins and determined that this cell possessed low but significant expression of both violet opsin and the UV opsin Opn5 (data not shown). These findings indicate that the isolated cell may be a specialized avian double-cone cell. An additional cell, E15 Cell #3, displayed expression of many these genes in different combinations as well (Fig. 10A). It should again be noted that E4 Cell #1 was observed to express a number of these genes (Fig. 10A), further suggesting this cell is potentially deciding between multiple fates (Arendt et al. 2016).

We employed ISH to examine the expression of these cone genes in the developing chick retina. Gnat2 was clearly expressed among cells in the developing ONL as early as E6 (Fig. 10B). This signal is maintained and intensifies throughout development (Fig. 10C-E). Retinoid X receptor gamma (Rxrg) also showed signal as early as E6 among developing ONL neurons (Fig. 10F), and this expression pattern persisted through E14 (Fig. 10G-I). This gene also showed some signal among developing ACs at E8 and E14 (Fig. 10G, I). Both spalt-like transcription factor 1 (Sall1) and Purpurin mimic the expression of Gnat2 and were confined to cone photoreceptors in the mature retina (Fig. 10J-Q). The expression patterns of these genes found in E15 Cell #1 further validates that this cell is a cone photoreceptor.

A small number of these cone-expressed genes are also found in the transcriptome of E15 Cell #3, though we do not believe this cell is a cone photoreceptor. We decided to investigate this cell further and found that it was expressing Opn4, an indicator of photosensitive cells in the chick (Verra et al. 2011). The chick is among the vertebrates which possess two separate genes coding for the melanopsin protein; this is in stark contrast to both humans and mice, both of which only utilize one Opn4 gene (Bellingham et al. 2006; Morera et al. 2016). The Opn4 gene we found expressed in this cell is the shared gene among all vertebrates, commonly referred to as Opn4m (Bellingham et al. 2006). Opn4m has been shown to be expressed by intrinsically photosensitive RGCs in the mouse (Provencio et al. 2000), but its expression in the chick appears widespread throughout the INL, likely expressed by chick ACs or BCs (Bellingham et al. 2006). We decided to examine this cell more to uncover which type of interneuron it was and found multiple TFs expressed, including Otx2 and visual system homeobox 1 and 2 (Vsx1 and Vsx2), which led us to conclude that E15 Cell #3 was a BC.

DISCUSSION

Single developing RGC profiles

In the current study, we have isolated developing retinal cells from multiple timepoints, ranging from E3-E15 and have completed transcriptome analyses on 42 individual cells. We focused primarily on larger cells that were positive for a handful of RGC markers in our PCR-based screen to allow for the identification of gene clusters involved in RGC development specifically. Our profiling experiments led to the observation of at least two phases of gene expression that were most likely correlated with different phases of RGC differentiation. Our data showed that a few RGC markers were detected as early as E3, while the later timepoints saw a significant increase in the number of RGC genes observed, both in the profiled cells and within the whole retina by ISH. However, while we observed markers of RGCs at early timepoints, we did not see many robust differences among the cells, perhaps indicating that the RGC fate is acquired first and then RGC subtype diversification takes place much later in development. We previously observed a very similar phenomenon in developing mouse RGCs (Trimarchi et al. 2007), perhaps pointing to the generality of this method of subtype diversification among RGCs. One potential model is that an RPC, which decides on a ganglion cell fate early during development, requires an interaction with the environment (i.e. extracellular signals from the other cells that will connect to that RGC) to fully commit to a specific RGC subtype. Consistent with this possible model is the observation that genes such as Jam2 increase in their expression later in chick retinal development.

Another goal of this study was to uncover genes related to cell fate acquisition and differentiation. Therefore, we were surprised when our analysis revealed that transcription factors (TFs) were under-represented in the clusters of RGC associated genes. One possible

explanation is that the specification of RGC cell fate only requires a very limited number of transcription factors, as has been suggested (Wu et al. 2015). In this study, the expression of Brn3b and Isl1 in place of Math5 led to the production of RGCs, demonstrating that the expression of these two TFs in conjunction is sufficient to specify the RGC cell fate in mice (Wu et al. 2015). The same may be true in chicks, as we observe both a Brn3 factor and Isl1 in our developing cells. However, since over 30 different subtypes of RGCs have been identified in the mouse (Sanes & Masland 2014), and it is not clear how many types are present in the mature chick, it is still possible that the further specification of a specific RGC subtype will require additional TFs later in development (Rousso et al. 2016; Sanes & Masland 2014). In this scenario, RPCs determine a general RGC cell fate and sometime thereafter the specific subtype is determined so that appropriate wiring and circuitry can form throughout the retina, as has been shown to occur for subtypes of mammalian ACs (Kay, Voinescu, et al. 2011). The acquisition of a specific subtype identity may even vary from type to type as other studies have demonstrated that at least for two types of direction-selective RGCs, the subtype decision can be made by a cell prior to cell cycle exit (De la Huerta et al. 2012).

Single developing AC profiles

We were also able to transcriptionally profile other developing neurons, such as amacrine cells, during retinogenesis. Amacrine cells are an even more diverse population than RGCs and subtype determination timepoints have been identified in the chick for very few AC populations (Spence & Robson 1989). Despite the variety of amacrine cells present in the mouse retina, it was still surprising that no single marker was found that marked all amacrine cells (Cherry et al. 2009). Using Tfap2b as our best AC identifier (Bisgrove & Godbout 1999), we were able to identify E6 Cell #1, E6 Cell #8, and E9 Cell #1 as ACs. We also looked for ChAT among our

cells as a marker for cholinergic ACs, and found a few cells expressing this gene. Interestingly, we found the ChAT+ cells contained more RGC associated genes, even though previous studies suggested that this gene was used only by cholinergic ACs in the chick (Stanke et al. 2008). Specifically, E9 Cell #2 and E9 Cell #3 both express ChAT at high levels, though both of these cells had been deemed RGCs due to their expression of Uchl1, Brn3a (ChEST382l11), and Map6, among other genes. Interestingly though, neither of these cells were positive for Sncg, but neither were expressing Tfap2b, both of which are often used to classify RGCs and ACs, respectively. There are several reasons why these cells may have appeared to express both RGC and AC markers. First, we may be examining a novel type of RGC which utilizes acetylcholine in the chick retina, as opposed to glutamate which is typically the neurotransmitter used by all RGCs. ChAT-positive RGCs have been documented in the rat retina (Yasuhara et al. 2003), but their presence in other species has proven elusive. Second, it is possible that these cells were still sorting out their final cell fate and, therefore, still showing transcriptional hallmarks of both RGCs and ACs. When we examined the transcriptomes of these cells in more detail, we found many genes expressed by both RGCs and ACs, but more importantly we found an abundance of RGC specific genes. This observation may indicate that the balance is tipping toward an RGC, but further experimentation will be required in the chick retina to know for certain.

Through the use of ISH, we examined a few genes that may mark different subsets of ACs and may lead to information regarding amacrine subtypes in the chick. For example, Slit2 was one of few genes with expression strictly limited to subsets of cells within the INL, but it did not mark all amacrine cells. This stands in contrast to Slit1, whose expression has been found specifically among RGCs in the chick (Jin et al. 2003). Slit proteins, the secreted ligands responsible for binding to Robo receptors (Kidd et al. 1999), have been shown to act as

regulators of growth cone guidance (Brose et al. 1999). Though we cannot conclusively determine its function without functional experimentation, it is tempting to speculate that Slit2 expression in an AC subset may indicate a role in the neurite outgrowth of particular chick ACs (Fig. 6I). While the AC population is rather large and diverse among vertebrates (Masland 2012), we were able to use our dataset to uncover multiple genes expressed by varying subsets of ACs. Among these genes were Tac1 (Fig. 6M), Fgf13, Pbx1, ChEST32g3, Sept11, Opn4, and Pou6f2 (Fig. 7A-F). These genes stood out as we were expecting to find Opn4 and Pou6f2 among RGCs, but were able to observe their expression in a subset of ACs as well. This expression pattern of Opn4 is interesting but may not be the first occasion which points to putative photoreceptive ACs. A study examining the expression of chick TMT (cTMT) opsin and Opn3 found a very small subset of ACs expressing cTMT in the INL (Kato et al. 2016). Perhaps TFs such as Pou6f2 and Pbx1 are playing a role in regulating gene expression of distinct AC subtypes in the chick. Furthermore, Sept11 has been found expressed among GABAergic neurons in the rat cortex (Li et al. 2009), which may suggest a role for this gene in the function of GABAergic ACs. Finally, ChEST32g3 arose in our dataset and was expressed by a relatively small subset of ACs at E14 (Fig. 7C). The identity of this gene remains unknown, however it appeared in only a fraction of these interneurons, which may be indicative of an AC subtype.

It is important to note, that it can be difficult to make definitive conclusions regarding RGC versus AC expression based upon observing gene expression by ISH. The reason for that is that about 50% of cells in the GCL are amacrine cells in most vertebrates (Génis-Gálvez et al. 1977). Furthermore, it should be noted that the neurofilament genes have been reported to be expressed in non-RGCs such as ACs and HCs (Stanke et al. 2008; Liu et al. 2013). The presence of NF-L in some of our potential ACs is consistent with these previous observations. During

development, the neurofilaments are not the only genes RGCs and ACs share as previous studies investigating the transcriptomes of developing RGCs and ACs in the mouse showed significant overlap for marker gene expression in these two cell types (Cherry et al. 2009). Many of our developing ACs displayed the expression of some RGC-associated genes at lower levels and, while this might suggest that these cells were still developing neurons, it could also just be that these two types of neurons share gene expression in common. Along these lines, a few of our suspected ACs were isolated at E9, a time believed to be outside of the RGC birth window. Additionally, it could be that in the retinas of post-hatch chicks, mature ACs may no longer express these RGC-associated genes.

Single cell profiles of potentially neurogenic progenitor cells

Of the 42 cells isolated for this study, one cell in particular, E4 Cell #1, stood out as being Cath5-positive, indicating its identity as a progenitor cell around the time of its final division. This TF plays a necessary role in the development of ganglion cells in various vertebrates, and Cath5 has been demonstrated to regulate the transcription of Brn3c and an increase in differentiation of RGCs (Liu et al. 2001). Upon further examination, we found that E4 Cell #1 also expressed Fgf19, a marker of horizontal cells in the chick (Kurose et al. 2004). The expression of these two genes together at E4 is consistent with this cell being a newborn HC. Looking further at the transcriptome of E4 Cell #1 revealed additional HC marker genes (OC2 and Lhx1), but also genes that are expressed in developing cone photoreceptors (Otx2, NeuroD1, and Sall1). While initially surprising, this finding strongly suggests that this RPC was either about to divide or had just divided and given rise to a HC/cone precursor cell (Emerson et al. 2013).

Initially discovered in subsets of developing murine single cells, studies of OC1- and OC2-deficient mice revealed that these genes are required for the generation of horizontal cells (Sapkota et al. 2014; Wu et al. 2013; Goetz et al. 2014). However, the Onecut factors are not purely limited to expression in mature horizontal cells, as further investigation showed that OC1 is a part of a regulatory network that biases Olig2+ retinal progenitor cells towards a horizontal cell fate (Emerson et al. 2013; Hafler et al. 2012). In these RPCs, greater combinatorial expression of the transcription factors OC1 or Otx2 weigh the progenitor either towards a horizontal or cone cell fate, respectively (Emerson et al. 2013). These findings point to the identity of E4 Cell #1 as a progenitor deciding between the fate of a HC or a cone photoreceptor, and underscore the importance of transcriptomic analysis at the single cell resolution. Furthermore, given where this cell stands in terms of cell fate determination, it is interesting to note the abundance of transcription factors (Nr6a1, Pbx4, Olig3, Stat3, Beta3, and Zic1) found in this single cell transcriptome. Determining how all these transcription factors work together to produce a specific progeny cell will be a challenge for future studies, but it may lead to a greater understanding of the decision that is crucial to forming a specific cell type.

Cone photoreceptor single cell profile

During our investigation into identifying other cell types among our isolated population, we came upon a cone photoreceptor in the dataset: E15 Cell #1. This cell expressed cone photoreceptor-specific genes, such as Purpurin, Gnat2, Rxrg, and Pde6h (Berman et al. 1987; Ying et al. 1998; Hoover et al. 1998; Corbo et al. 2007; Blixt & Hallböök 2016). The transcription factor Sall3 was also differentially expressed in cone populations both during development and in the more mature retina (de Melo et al. 2011). Through the identification of these clusters of genes, there now exists a more expansive list of marker genes for different cell

populations of the chick retina and there are new targets to examine functionally to determine the unique combinations of genes that produce each cell type.

Conservation of gene expression between mouse and chick retinal development

Part of our interest in expanding the study of single-cell transcriptomics to the chick retina was to compare and contrast our findings with data from isolated cells of the mouse retina. Through our analysis of over 200 cells from the mouse at various stages of development, we were able to identify novel markers of RPCs and maturing retinal cells as well as to differentiate between developing subsets of retinal cell types (Trimarchi et al. 2008b). For many of our cells, we found a strong overlap of genetic expression between the two models. For example, RGC/AC genes such as Sncg, NF-L, Brn3a, and Ebf3 remain consistent for both animals (Trimarchi et al. 2007). We also show members of the Iroquois homeobox family as expressed among chick RGCs, confirming findings of Irx signal in the mouse (Trimarchi et al. 2007). Regarding ACs, Tcfap2b and ChAT are conserved among this population in both the mouse (Trimarchi et al. 2007) and the chick, while genes such as Uchl1 and Stathmin-like 3 (ChEST742d11) are expressed by both ACs and RGCs in both models. Interestingly, Brn3b and Isl1 have been demonstrated to play an important role in regulating the development of RGCs in the mouse, while the chick retina has been shown to utilize Brn3a, Brn3b, and Brn3c among RGCs (Liu et al. 2000). Isl1 is also a well characterized marker of a subset of HCs in the chick (Boije et al. 2009), and this gene can be found in ACs in both models (Trimarchi et al. 2007). While we see overlap in gene expression among cell types between the two model organisms, it is interesting to note the differences among some of these cell type specific genes. Isl1, for example, is conserved in its expression among both RGCs and ACs in the chick and the mouse, however this gene is an important marker of a HC population in the chick (Fischer et al. 2007). Perhaps this

TF plays a role in the development of a subset of RPCs, which give rise to RGCs, ACs, and HCs in the chick, but this function is lost in mouse HCs because that subset of interneuron does not exist..

To compare a more substantial number of genes found in developing RGCs of both species, we compared our list of NF-L correlated genes (Table 2) with NF-L correlated genes from a published mouse analysis (Trimarchi et al. 2007). Working with members of the Affymetrix team, we were able to obtain a list of 11535 probesets that are comparable between the Affymetrix Mouse Array 430 2.0 and the Affymetrix Chicken Genome Array. We extracted those probesets, which were found in our NF-L correlate gene list, and found that only 259 of our total 434 genes in that table have equivalent mouse probesets. Using the gene lists from developing mouse RGCs, we could find that 71, 146, and 140 of these genes had equivalent chick probesets (Trimarchi et al. 2007). The chick gene list of 259 was then compared to each of the lists from the mouse and we found that approximately 20% of the genes in the mouse lists were also in our chick table. It is important to note that about half of the genes in each table were discarded, as those genes do not have equivalent probesets in the other animal.

To better understand why we found only a 20% overlap between the two animal models, we performed a visual examination of the gene lists from both the current study and the mouse study (Trimarchi et al. 2007). We discovered there were many genes expressed by developing mouse RGCs that had family members or functionally related genes expressed by our developing chick RGCs. For example, neuron specific family member 2 and sodium channel subunit 3 beta were found in the mouse, while neuron specific family member 1 and sodium channel subunit 3 alpha were in our chick gene list. Taken together, this attempted chick/mouse comparison highlights the challenges in performing cross-species comparisons, but also suggests that there

may be conservation at least at the level of gene family in multiple organisms. By comparing the genes present in subsets of retinal cells throughout development, we hope to improve our understanding of the conserved factors that have maintained similar influences across evolutionary history. As we expand our datasets to include more retinal cells in the future, we hope to be able to improve our understanding of the most important factors that lead to the specification of distinct retinal cells among vertebrates.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all of the data in the manuscript and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceived and designed the study: GMM, JMT. Performed the experiments and acquired the data: LAL, GMM, JJG, BP, KW, LE, DF, ML, AKW. Data analysis: LAL, JJG, RB, PL, JMT. Writing the manuscript: LAL, JJG, JMT. Supervised the study: JMT

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FIGURE LEGENDS

Figure 1. Gene expression in single developing retinal ganglion cells. A heatmap generated using Genesis software showing the expression of designated RGC-associated genes from 39 single chick cell transcriptomes. The cells and timepoints are listed as individual columns, while that data for each gene is displayed in the individual rows. Increasingly bright red color indicates high expression, black demonstrates no expression, and the varying shades of red indicate expression levels of varying degrees.

Figure 2. *In situ* hybridization of selected chick retinal ganglion cell-associated genes. Diagrams of retinal layers throughout development are shown in A-C. (A) The chick retina at E5 with the ONBL and INBL labeled. (B) A section of chick retina between timepoints E3-E8 with the ONBL and INBL labeled. (C) A section of chick retina after E9 with the ONL, OPL, INL, IPL, and GCL labeled. (D-F) *In situ* hybridization was performed on chick retinal cryosections at E5. Photos were taken across the retina sections and images assembled in Photoshop to display the entire distribution of signal for each probe. The probes investigated were Uchl1 (D), Ppp3ca (E), and Tagln3 (F). Scale bars represent 100 μm. Abbreviations: outer neuroblastic layer (ONBL); inner neuroblastic layer (INBL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL).

Figure 3. *In situ* hybridization of chick retinal ganglion cell-associated genes. *In situ* hybridization was performed on chick retinal cryosections at the timepoints E4, E6, E8, and E14. The probes examined were: NF-L (A-D), NF-M (E-H), Uchl1 (I-L), ChEST382l11 (M-P), Ebf3 (Q-T), Robo2 (U-X), and ChEST742d11 (Y-B'). Scale bars represent 100 μm.

Figure 4. The developmental expression patterns of newly characterized retinal ganglion cell markers. Previously uncharacterized RGC specific genes were investigated for their expression in retinal sections at E6 and E8. The probes used were: Ppp3ca (A-B), Map6 (C-D), DNER (E-F), C17h9orf7 (G-H), and Tmem163 (I-J). Arrowheads indicate areas of expression enrichment for selected probes. Scale bars represent 100 μm.

Figure 5. Expression of retinal ganglion cell-subset markers. *In situ* hybridization was performed on chick retinal cryosections at the timepoints E4, E6, E8, and E14 with a focus on genes that were found only among subsets of the single cell transcriptomes. The specific probes examined were: Sncg (A-D), Irx1 (E-H), Irx2 (I-L), Tagln3 (M-P), and Isl1 (Q-T). Scale bars represent 100 μm.

Figure 6. Developing amacrine cell gene expression. (A). A heatmap generated in Genesis software showing the expression of AC-associated genes from 39 single chick cell transcriptomes. The cells and timepoints are listed as individual columns, while that data for each gene is displayed in the individual rows. Increasingly bright red color indicates high expression, black demonstrates no expression, and the varying shades of red indicate expression levels of varying degrees. Four of these AC-associated genes were examined further using *in situ* hybridization on retinal cryosections at E4, E6, E8, and E14. The specific probes used were: Tfap2b (B-E), Slit2 (F-I), Tac1 (J-M), and Cbln2 (N-Q). Scale bars represent 100 μm.

Figure 7. Analysis of new amacrine cell markers by *in situ* hybridization. Genes in the dataset that were identified as potential markers of AC subsets were investigated by ISH on

cryosections at E14. The probes utilized were Fgf13 (A), Pbx1 (B), ChEST32g3 (C), Sept11 (D), Opn4 (E), and Pou6f2 (F). Scale bars represent 100 μm.

Figure 8. Gene expression in an exiting progenitor cell. (A). A heatmap generated in Genesis software showing the expression of genes enriched in E4 cell #1 across 39 single chick cell transcriptomes. The cells and timepoints are listed as individual columns, while that data for each gene is displayed in the individual rows. Increasingly bright red color indicates high expression, black demonstrates no expression, and the varying shades of red indicate expression levels of varying degrees. Each column corresponds to the individual cells isolated in our dataset and the rows are individual genes. The box at the intersection of column and row demonstrates high expression when red, and no expression when black, with varying expression levels indicated by different shades of red. The retina expression patterns of three genes from E4 cell #1 were further investigated during mid-development, at E4, E5, E6, E7, and E8. The probes used were Cath5 (B-F), Fgf19 (G-K), and ChEST726n4 (L-P). Scale bars represent 100 μm.

Figure 9. Znf488 expression during development. The expression of Znf488 was examined throughout development in retinal sections at E4 (A), E6 (B), E8 (C), E10 (D), E12 (E), and E14 (F). Scale bars represent 100 μm.

Figure 10. Markers of cone photoreceptors observed in chick retinal cells. (A) A heatmap generated in Genesis software showing the expression of cone-associated genes present at significant levels in E15 cell #1 in all 42 single cell transcriptomes. The cells and timepoints are listed as individual columns, while that data for each gene is displayed in the individual rows. Increasingly bright red color indicates high expression, black demonstrates no expression, and

the varying shades of red indicate expression levels of varying degrees. To further determine the retinal expression patterns of selected cone marker genes, we examined expression at E6, E8, E10, and E14. The genes examined were Gnat2 (B-E), Rxrg (F-L), Sall1 (K-M), and Purpurin (N-Q). Scale bars represent 100 μm.

Table 1. *In situ* hybridization probe primer sequences. The sequences of the oligonucleotides used to amplify specific genes from chicken retinal cDNA for generating RNA probes are shown in this table.

Table 2. Genes highly correlated with NF-L and NF-M. Hierarchical clustering was used to reveal the genes that were most highly correlated with NF-L and NF-M. The table contains the genes along with the corresponding Affy Probe ID, gene symbol, and gene title. Correlation coefficient = 0.75.

Table 3. Single cell scores for NF-L gene cluster expression. A list of 115 genes with highest expression correlation to that of NF-L, following the application of a false discovery rate of 0.01,was generated. The expression of these genes among each of the isolated cells was examined and used to determine a score, on the range of 0-10, for these cells. Cells with a score greater than 4 are considered as having significant RGC “character”, while those scoring below 4 are a different retinal cell type.

Table 4. Genes highly correlated with NF-H. Hierarchical clustering was used to reveal the genes that were most highly correlated with NF-H in our single cell dataset. The table lists Affy Probe ID, gene symbol, and gene title. Correlation coefficient = 0.67.

Table 5. Expression summary of in situ probes. For each probe examined, the location of expression is summarized here. Timepoints range from E4-E14 and empty spaces indicate that an analysis was not performed for that gene at that particular timepoint. Abbreviations: INBL (inner neuroblastic layer), GCL (ganglion cell layer), INL (inner nuclear layer), ONBL (outer neuroblastic layer), ND (not detected), HC (horizontal cells), ONL (outer nuclear layer).

Table 6. Genes highly correlated with Gnat2. Hierarchical clustering was used to reveal the genes that were most highly correlated with the expression of Gnat2 in our dataset. The table lists Affy Probe ID, gene symbol, and gene title. Correlation coefficient = 0.60.