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Analysis of gene expression in wild type and Notch1 mutant retinal cells by single cell profiling

Karolina Mizeracka[1], Jeffrey M. Trimarchi[1,2], Michael B. Stadler[3], Constance L. Cepko[1,4]
[1] Department of Genetics, Department of Ophthalmology, Harvard Medical School, Boston, MA 02115
[2] Current Address: Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50014
[3] Friedrich Miescher Institute for Biomedical Research, 4058 Basel, Switzerland
[4] Howard Hughes Medical Institute, Department of Genetics, Department of Ophthalmology, Harvard Medical School, Boston, MA 02115

Correspondence:
Constance Cepko
77 Avenue Louis Pasteur, Boston, MA 02115
Phone: (617) 432-7618
Fax: (617) 432-7595

Running title: Single cell profiling of Notch1 mutant retinal cells

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Summary:

- Profiling of individual Notch1 deficient and wild type postnatal retinal cells on microarrays reveals changes in gene expression obscured by whole tissue analysis
- Notch1 deficient cells downregulate progenitor and cell cycle markers with a concomitant upregulation in early rod photoreceptor markers
- Based on classification, single Notch1 deficient and wild type cells represent transition from progenitor to postmitotic cell
- Individual wild type retinal cells express cell type markers of both photoreceptors and interneurons

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Abstract

Background: The vertebrate retina comprises sensory neurons, the photoreceptors, as well as many other types of neurons and one type of glial cell. These cells are generated by multipotent and restricted retinal progenitor cells (RPCs) which express *Notch1*. Loss of *Notch1* in RPCs late during retinal development results in the overproduction of rod photoreceptors at the expense of interneurons and glia.

Results: To examine the molecular underpinnings of this observation, microarray analysis of single retinal cells from wild type or *Notch1* conditional knockout retinas was performed. *In situ* hybridization was carried out to validate some of the findings.

Conclusions: The majority of *Notch1* deficient cells lost expression of known Notch target genes. These cells also had low levels of RPC and cell cycle genes, and robustly upregulated rod precursor genes. In addition, single wild type cells, in which cell cycle marker genes were downregulated, expressed markers of both rod photoreceptors and interneurons.

Introduction

The vertebrate retina is an excellent model system for understanding how regulatory pathways control gene expression during development. It consists of six major neuronal cell types and one glial cell type that can be readily identified by molecular markers, gene expression, and morphology. These cell types arise in a temporal, overlapping order from a pool of multipotent RPCs (Livesey and Cepko, 2001), as well as from terminal divisions from some more restricted RPCs (Rompani and Cepko, 2008; Godhinho et al. 2007; Hafler et al. 2011) . During retinal neurogenesis, ganglion cells are generated first, followed by horizontal cells, cone photoreceptors, and amacrine cells. Rod photoreceptors, bipolar cells, and Müller glial cells are the last cell types to be produced (Wong and Rapaport, 2009; Young, 1985b).

Previous studies have determined that the Notch signaling pathway regulates both cell cycle exit and cell fate specification during retinal development (Austin et al., 1995; Dorsky et al., 1995; Furukawa et al., 2000; Henrique et al., 1997; Hojo et al., 2000; Jadhav et al., 2006a, 2006b; Nelson et al., 2006, 2007; Riesenberg et al., 2009; Satow et al., 2001; Scheer et al., 2001; Silva et al., 2003; Tomita et al., 1996; Yaron et al., 2006; Zheng et al., 2009). Genetic removal of a *Notch1* conditional allele from early RPCs resulted in cell cycle exit and the premature onset of neurogenesis (Jadhav et al., 2006a; Yaron et al., 2006). Furthermore, overproduction of cone photoreceptors at the expense of other cell types was observed in these *Notch1* mutant retinas. Deletion of *Notch1* by viral delivery of Cre during later, postnatal stages of retinal development led to the overproduction of rod photoreceptors (Jadhav et al., 2006a), in keeping with the birth order of rod and cone photoreceptor cells. Furthermore, *Notch1* mutant cells generated in the postnatal environment acquired their phenotype in a cell autonomous manner. Therefore, Notch signaling is crucial for maintenance of the progenitor state, as well as for the repression of the photoreceptor fate.

Despite our knowledge of a number of factors involved in cell fate specification, it is currently unknown when and how cells become locked into their respective identities. Lineage analyses in the postnatal retina have shown that individual RPCs can give rise to two very different cell types: a rod and Müller glial cell, a rod and a bipolar cell, or a rod and an amacrine cell, as demonstrated by the composition of two cell clones (Turner and Cepko, 1987). These cells may be sorting out their fates as they exit the cell cycle, or perhaps after entering a newly postmitotic state. Previous single cell transcriptional profiling showed that cycling cells are very heterogeneous in terms of gene expression (Trimarchi et al., 2007, 2008). They must lose this heterogeneity as they transition into differentiated neurons, since even in the wild type (WT) case, most take on the rod fate. We wished to further explore the newly postmitotic state where these processes were taking place, and exploit the differences among WT and *Notch1* conditional knockout (N1-CKO) cells for insight into these events. To this end, we examined the individual transcriptional profiles of 13 WT cells and 13 N1-CKO cells by single cell microarray analysis. Comparisons between the two sets of cells led to the identification of a large number of genes that were either up or down regulated in the absence of *Notch1*. From this dataset, we identified Notch dependent genes that may regulate or be markers of cell cycle, progenitor state, and cell fate determination. By post hoc classification, we were able to identify WT and N1-CKO individual cells at different stages of the progenitor to postmitotic neuron continuum, revealing the transcriptional profile of cells during this transition. Finally, we observed that single WT cells expressed early differentiation genes of both interneurons and photoreceptors. These expression profiles may indicate that there is plasticity regarding cell fate, and/or that certain types of genes are de-repressed transiently during this phase of retinal development.

Results

Profiling single WT and N1-CKO retinal cells

We aimed to generate cells that had lost *Notch1* by electroporation, and thus first sought to confirm that electroporation into the *Notch1*$^{fl/fl}$ background recapitulated the phenotype observed in the previous viral experiments (Jadhav et al., 2006a). Retinas of *Notch1*$^{fl/fl}$ P0 pups were electroporated *in vivo* with plasmids encoding Cre driven by a broadly active promoter, CAG, along with a Cre-responsive GFP reporter, also driven by the CAG promoter (CALNL-GFP) (Matsuda and Cepko, 2004) to generate GFP+ N1-CKO cells. For controls, the retinas of sibling P0 *Notch1*$^{fl/fl}$ pups were electroporated with CAG:GFP to generate WT GFP+ cells. The animals were sacrificed after the maturation of the retina (>P21) and the fate of GFP+ cells was assessed by morphology and location in the retinal layers. For example, rod photoreceptors are exclusively localized in the outer nuclear layer, which is distinct from the location of the other neurons and Müller glial cells. In accord with previous studies, the majority of cells that lost *Notch1* took on a rod photoreceptor fate, whereas WT cells took on a variety of fates (Figure 1A, B).

After confirming that Cre electroporation into the *Notch1*$^{fl/fl}$ background produces a *Notch1* loss of function phenotype, we profiled individual P3 N1-CKO and WT cells on microarrays in order to examine their transcriptomes at earlier stages of development. For single cell profiling, *Notch1*$^{fl/fl}$ P0 pups were electroporated as described above, but the retinas were harvested at P3 to allow time for *Notch1* to be deleted by Cre and downstream gene expression changes to occur, but before the electroporated cells could fully differentiate into mature cell types. At this time point, the majority of electroporated cells was either exiting the cell cycle or had recently exited the cell cycle. We know this for several reasons. First, our previous studies of cell fates marked by electroporation at P0 showed that cell types born in the embryonic period were only rarely labeled, with the vast majority instead being cell types generated in the postnatal period (Matsuda and Cepko, 2004). Our interpretation of this finding is that the cells facing the subretinal space where the DNA is introduced are the most likely to be electroporated, and are RPCs or cells that are exiting cell cycle. By P3, Young showed that proliferation is almost over in the center of the retina and waning in the periphery (Young, 1985a, 1985b), so these P3 electroporated cells are likely RPCs or newly exited cells. In corroboration of these results are the clone sizes of retrovirally marked clones from P0 infections in the mouse. Gammaretroviruses can only mark mitotic cells and their progeny, and marking is initiated after the first or second M phase following viral infection (Roe et al., 1997). In previous work from our lab, we measured the clone size for two different retroviruses, one encoding alkaline phosphatase (AP) and one encoding lacZ. The average clone sizes for these two vectors were 1.8 cells/clone and 1.9 cells/clone, respectively, following infection at P0 (Fields-Berry et al., 1992). As well, the clone size distribution indicates that most RPCs produce postmitotic daughters or RPCs with very limited proliferation capacity. Taken together, all of these data indicate that the majority of cells electroporated at P0, and analyzed at P3, will be cells in transition from cell cycle to the newly postmitotic state.

Retinas electroporated with either CAG:Cre and CALNL-GFP or CAG:GFP alone were dissected and dissociated to individual cells, which were then harvested under a dissecting microscope on the basis of their GFP signal. In total, 13 N1-CKO cells and 13 WT cells were harvested and profiled on Affymetrix microarrays. These methods have been used previously for profiling individual retinal cells, with the results validated by several methods, primarily *in situ* hybridization (Brady and Iscove, 1993; Tietjen et al., 2003; Trimarchi et al., 2007, 2008).

In order to confirm that Notch1 signaling was indeed depleted in N1-CKO cells, the average levels of the direct Notch target genes, *Hes1*, *Hes5*, and *Nrarp* were assessed in N1-CKO vs. WT cells (Ohtsuka et al., 1999; Krebs et al., 2001). Expression levels of these genes were markedly lower in N1-CKO cells as compared to WT cells (Figure 1C). As RPCs divide to generate neurons, these newly born cells turn off genes associated with cell cycle and progenitor status and begin to express genes that regulate neuronal identity. Some early transcription factors that influence neuronal fate include *Math3*, *NeuroD1*, and *Blimp1* (Brzezinski et al., 2010; Chang et al., 2002; Inoue et al., 2002; Morrow et al., 1999). These genes were found to be strongly upregulated in N1-CKO cells as compared to WT cells (Figure 1C).

In order to validate the single cell profiling method as a means to assess changes in gene expression after the removal of *Notch1*, we performed a qPCR assay on populations of N1-CKO and WT cells. Retinas of *Notch1*$^{fl/fl}$ P0 pups were electroporated *in vitro* with plasmids encoding CAG:Cre, along with a Cre-responsive GFP reporter

(CALNL-GFP). For controls, the retinas of sibling *Notch1*$^{fl/fl}$ pups were electroporated with CAG:GFP. Electroporated retinas were cultured for three days and dissociated to single cells. GFP+ cells (pooled from two retinas for each sample) were sorted by flow cytometry and collected. RNA was extracted from each sample and cDNA was generated. Samples were subjected to quantitative real time PCR in order to detect expression of *actin* (as a control), *Hes1*, *Nrarp*, *Math3*, *NeuroD1*, and *Blimp1* (Figure 1D). In accord with the changes observed by microarray analysis, *Hes1* and *Nrarp* were downregulated in N1-CKO cells, as compared to WT cells (Figure 1D). Additionally, *Math3*, *Blimp1*, and *NeuroD1* were upregulated in N1-CKO cells as compared to WT cells (Figure 1D). Changes in expression of these key genes in a population of N1-CKO cells as compared to WT were highly similar to those observed in individual cells.

Classification of cells using their molecular signatures

We wanted to understand the transitions that cells undergo as they exit the cell cycle and choose their fate, both in the WT and N1-CKO cells. In order to investigate if there are indications of an eventual fate choice during this transition, we classified each N1-CKO and WT cell according to its expression of cell type-specific markers. The classification scheme, as devised previously, is based upon the normalized values of genes coexpressed with known markers of each of the retinal cell types (Trimarchi et al., 2007, 2008). As an example, to determine if a cell has characteristics of an amacrine cell, we used the expression levels of genes coregulated with well-validated markers of the amacrine fate. These coregulated genes had been identified using microarray data from single cells profiled previously, as genes whose expression was strongly associated with the expression of the known amacrine-specific genes, *Tcfap-2β, Gad1,* and *Glyt1*. The associations were derived from 194 single retinal cells that were profiled in our lab and encompassed most retinal cell types (Cherry et al., 2009; Kim et al., 2008; Roesch et al., 2008; Trimarchi et al., 2007, 2008). A Fisher's exact test was used to determine the p-value for correlations between any given gene and *Tcfap-2β, Gad1,* and *Glyt1*. Only associated genes that had p-values of <0.01 were considered to be highly associated. The relative expression level for each associated gene in each P3 N1-CKO or WT cell was calculated by dividing a cell's signal level by the maximum signal level found in all of the single cells within the entire dataset of 194 cells. These scaled values for all of the amacrine associated genes in each cell were summed, and then the sums were scaled, such that the maximum score was 10. This classification procedure was repeated with markers of other retinal cell types to generate scores for each cell type. For an RPC score, genes associated with *FGF15, Sfrp2,* and *μ-crystallin* were used; for retinal ganglion cells, those associated with *NF68* and *Ebf3*; for Müller glia, those associated with *Apoe*; and for bipolar cells, those associated with *Og9x* (Trimarchi et al., 2008). In order to generate a developing photoreceptor score, the gene *Blimp1* was used to find associated genes. This gene has been shown to be expressed early in photoreceptor development and its expression tapers off as these cells mature (Brzezinski et al., 2010; Chang et al., 2002; Katoh et al., 2010). This was considered a more appropriate marker for newly postmitotic cells that would likely achieve the rod fate, instead of a more typical rod photoreceptor marker, such as *rhodopsin*, whose expression is later in development.

Using this classification scheme, the majority of the N1-CKO cells scored highly as incipient rod photoreceptors (Figure 2, see N1-CKO cells 1-10). Three of the N1-CKO cells scored highly as amacrine precursor cells (Figure 2, see N1-CKO cells 11-13). Most of the WT cells were classified as RPCs (Figure 2, see WT cells 2-6, 8, 12), while some cells had high rod (Figure 2, see rod WT cells 9-11, 13) or amacrine scores (Figure 2, see amacrine WT cell 7). Some WT cells had intermediate scores for RPC, rod, and amacrine cell types (discussed below) (Figure 2, see WT cells 1, 2, 6). These outcomes are in keeping with *Notch1* depletion in N1-CKO cells, as none of these mutant cells scored as RPCs and the majority were classified as rod precursor cells. For comparison, the classification scores of postnatal WT cells which were picked for other studies and classified as either amacrine precursors (cells P0 A4, P0 B1, P0 D1, P0 G3)(Cherry et al., 2009) or rod precursors (cells P0 E1, P5 C4, P5 D2, P5 C2) (unpublished), are depicted.

Additionally, it is worth noting that none of these postnatal cells had a ganglion cell signature. This gives confidence in the classification scheme, and is in keeping with the idea that primarily mitotic or newly postmitotic cells adjacent to the subretinal space were electroporated (Matsuda and Cepko, 2004). Ganglion cells would have already been produced and would have migrated away from this surface by P0 when the retina was electroporated. Overall, these molecular data support the observed cell fate changes, as well as provide a source of gene expression changes that are likely informative with regards to the network that is regulated by *Notch1*.

Changes in RPC and cell cycle gene expression in single cells that have lost *Notch1*

In contrast to whole tissue microarray analysis, single cell profiling affords the ability to examine changes in gene expression at the resolution of individual cells. Cell-by-cell analysis is especially important in the retina, as RPCs and the neurons they produce are highly heterogeneous in the types of genes they express (Cherry et al., 2009; Trimarchi et al., 2007, 2008). We anticipated that the downstream changes in gene expression in WT cells vs. N1-CKO cells would include genes relevant to Notch signaling, the progenitor state, and cell fate choices. We visually inspected the microarray data for changes in key genes that represent these stages and whose expression patterns may be different from cell to cell.

Although on average N1-CKO cells lost expression of Notch target genes as compared to WT cells (Figure 1C), inspection of gene expression in individual cells showed that this group of cells was in various stages of maturation, as suggested by the classification scheme (Figure 2). For example, a few N1-CKO cells still expressed some, but not all, downstream target genes, suggesting that they had not lost all of their Notch signal and were in the process of downregulating Notch signaling (Figure 3, see N1-CKO cells 1-3). Some WT cells expressed high levels of Notch targets, indicating that they still had active Notch signaling (Figure 3, see WT cells 1-6, 13). As discussed above, most of these cells were classified as RPCs (Figure 2, see WT cells 2-6). Similar to the majority of the N1-CKO cells, several of the WT cells exhibited low levels of Notch target genes (Figure 3, see WT cells 7-12). These WT cells most likely were in a transitional state during which they were downregulating Notch activity to exit the cell cycle, as occurs normally, especially at this time in development (Young, 1985b).

We also examined genes predicted to regulate or drive cell cycle, as loss of *Notch1* has been reported to lead to cell cycle exit (Jadhav et al., 2006a; Yaron et al., 2006). The levels of cell cycle genes, *Geminin, Ccna2, CyclinB1, Cdc20,* and *CyclinB2* (Trimarchi et al., 2008), were thus assessed in N1-CKO and WT cells (Figure 3). Indeed, the N1-CKO cells showed a reduction in the expression levels of these cell cycle genes (Figure 3, see N1-CKO cells 4-13). Moreover, the cells with the most comprehensive reduction in cell cycle genes were the same cells that showed the most significant loss of Notch target genes (Figure 3, see N1-CKO cells 4-13). The WT cells with low levels of Notch targets, also had reduced levels of these same cell cycle genes (Figure 3, see WT cells 7-13). In contrast, WT and N1-CKO cells that expressed Notch target genes had high levels of cell cycle genes (Figure 3, see WT cells 1-6 and N1-CKO cells 1-3).

In addition to cell cycle genes, expression of other previously identified RPC genes was assessed. Some of these genes are in all or most RPCs, but are also expressed in subsets of neurons (e.g. *Pax6*). We chose to analyze expression of genes that are expressed in most RPCs, but are not expressed in many neurons. These included *Lhx2, Mik67, Cdca8, Cdc2a, Fgf15, Ttyh1*, and *μ-crystallin* (Figure 3) (Blackshaw et al., 2004; Trimarchi et al., 2008). Most N1-CKO cells had low levels of these genes and were the same cells that had scored highly as neurons in the classification scheme described above (Figure 2 and 3, see N1-CKO cells 4-13). Interestingly, the WT cells that showed low levels of Notch target genes and cell cycle genes retained expression of some RPC genes (Figure 3, see WT cells 7-13), even though they were classified as neurons when the wider range of marker genes was scored in the classification scheme (Figure 2, see WT cells 7-13). This observation may indicate that these WT cells had a slower pace of exiting the RPC state and executing their differentiation process. In contrast, a number of WT cells that expressed high levels of Notch target genes and cell cycle genes, also expressed high levels of most RPC genes (Figure 3, see WT cells 1-6). This group of cells was classified as RPCs, with the exception of WT cell 1, which had an intermediate

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RPC and rod score (Figure 2). These data reveal which genes are sensitive to Notch1 signaling and provide examples of single cells at various stages in the transition between RPC and determined states.

Unbiased search for genes with expression changes following loss of *Notch1*

An unbiased search for significantly downregulated genes was conducted by comparing gene expression levels in cells classified as RPCs (WT cells 2-6, 8, 12) to those in cells classified as rod precursor cells (N1-CKO cells 1-10). These particular cells were selected, because it was anticipated that RPCs express different sets of genes than rod precursor cells. T-test analysis with a cutoff p-value of <0.05 was performed to find significantly downregulated genes (Table S1). We observed that Notch target genes such as *Hes1* and *Hes5* were found to be significantly downregulated (Table S1), in accord with the trend observed for the average signal levels across all the profiled cells (Figure 1).

As loss of Notch signaling leads to the overproduction of rod photoreceptors at this stage of retinal development, it was anticipated that genes involved in photoreceptor development would be upregulated in N1-CKO cells. Again, gene expression levels were compared between WT RPC cells and N1-CKO rod precursor cells. T-test analysis with a cutoff p-value of <0.05 was performed to find significantly upregulated genes (Table S2). *NeuroD1*, *Math3,* and *Blimp1* are three such genes that were significantly upregulated in rod precursor N1-CKO cells (Figure 1, 3, Table S2), similar to the trends observed when all the single cells were taken under consideration (Figure 1C). *NeuroD1* and *Math3* encode pro-neurogenic bHLH transcription factors that can lead to overproduction of rods when misexpressed (Inoue et al., 2002; Morrow et al., 1999, Cherry et al. 2011). Interestingly, *Math3* was upregulated in the N1-CKO cells that were classified as incipient rods, but not in the cells classified as amacrine precursor cells (Figure 2, 3 see rod N1-CKO cells 1-10 and amacrine N1-CKO cells 11-13), while *NeuroD1* was upregulated in N1-CKO cells classified as amacrine cells, as well as in those classified as rods (Figure 2 and 3, see N1-CKO cells 1-13). This is in keeping with the expression of *NeuroD1* in amacrine cells and the induction of amacrine cells, along with rods, following *NeuroD1* misexpression (Cherry et al., 2011; Inoue et al., 2002; Morrow et al. 1999).

Blimp1, a gene that has been demonstrated to positively regulate the production of photoreceptor cells through repression of the bipolar cell fate (Brzezinski et al., 2010; Katoh et al., 2010), was also upregulated in N1-CKO cells (Figure 4, 5, discussed below). Because its expression is transient in retinal development (Brzezinski et al., 2010; Chang et al., 2002), *Blimp1* is thought to demarcate the early period of photoreceptor formation. The robust upregulation of these key photoreceptor genes provides additional support for the validity of the single cell microarray approach in defining the genes responding to the loss of *Notch1* and inducing the rod fate.

Identification of genes associated with *Blimp1*

Blimp1 is expressed during embryonic time points to early postnatal stages in the developing retina in a temporal and spatial pattern highly correlated with incipient photoreceptors (Brzezinski et al., 2010; Chang et al., 2002; Katoh et al., 2010). Double immunohistochemistry experiments showed that *Blimp1*+ cells do not express RPC markers, but do coexpress *NeuroD1* (Brzezinski et al., 2010). Since cells expressing *Blimp1* have the molecular characteristics of early rods, the genes that are coregulated with *Blimp1* are candidates for genes involved in photoreceptor differentiation. Using the pairwise comparison described above to find coregulated genes, genes with expression patterns similar to *Blimp1* with p-values of <0.01 were identified (Figure 4A). Some known factors that closely tracked with *Blimp1* included *Rax*, *Math3*, and *Rbp3*. These genes are either markers of developing rods (*Rbp3*) or have been shown to play a role during photoreceptor genesis (*Rax*, *Math3*) (Chen and Cepko, 2002; Inoue et al., 2002; Jin et al., 2009; Muranishi et al., 2011)(Figure 4A). In addition, *Epha8*, a gene not previously identified as associated with rod development, was identified. This may be a novel marker of rod photoreceptors, perhaps playing a functional role during rod specification and/or differentiation (Figure 4A).

In order to validate if *Epha8* has a similar expression pattern to *Blimp1*, *in situ* hybridization (ISH) was performed on retinal sections at P3, P9, and adult stages. Detection of *Blimp1* expression by ISH matched previous reports of Blimp1 expression by ISH, immunohistochemistry, and transgene expression (Brzezinski et al., 2010; Chang et al., 2002; Katoh et al., 2010). At P3, *Blimp1* expression was expressed in the scleral outer neuroblastic layer (ONBL), where incipient photoreceptors are located (Figure 4B). A similar expression pattern was observed at P9 (Figure 4C). Very faint staining was detected at adult stages (Figure 4D). The expression pattern of *Epha8* was investigated at the same stages. At P3, staining in the ONBL was observed, similar to the pattern of *Blimp1* expression (Figure 4B, E). *Epha8* expression was at a very low level throughout the retinal layers at P9 and adult stages (Figure 4F, G). These results corroborated the microarray analysis, as *Epha8* expression was very similar to *Blimp1* expression at postnatal stages (Figure 4). Further study is necessary to identify a functional role for *Epha8* during retinal development.

Markers of cell types expressed by profiled single cells

In addition to learning about genes involved in rod development, it was of interest to mine the microarray data from WT and N1-CKO cells for the expression of genes that are markers of amacrine cells, bipolar cells, and Müller glia. These fates are the ones normally taken by approximately 30% of the postnatally generated cells, and which are greatly reduced in the N1-CKO population. The expression patterns of known amacrine markers were compared to rod marker expression in WT and N1-CKO cells. They were also compared to the values in cells previously analyzed by our lab, which had been classified as either rod or amacrine precursor cells (Figure 5). As expected from the classification results, P3 N1-CKO cells, which had been classified as rod precursor cells, expressed rod marker genes robustly, but did not express amacrine marker genes (Figure 2 and 5, see N1-CKO cells 1-10). The transcriptional profiles of these cells resembled those of previously profiled rod precursor cells (Figure 5, see P0 cell E1, P5 cell C4, P5 cell D2, P5 cell C2) (Trimarchi et al., 2007). Conversely, N1-CKO cells classified as developing amacrine cells expressed amacrine marker genes and not rod marker genes (Figure 2, 5, see N1-CKO cells 11-13). The expression profiles of these cells were similar to cells classified as amacrine precursor cells in our previous studies (Figure 5, see P0 cell A4, P0 cell B1, P0 cell D1, P0 cell G3)(Cherry et al., 2009; Trimarchi et al., 2007).

Interestingly, some WT cells expressed marker genes specific to mature amacrine cells, as well as genes specific to rod photoreceptors. This is in keeping with the scores on the classification scheme, as some WT cells did not exhibit scores indicating clear cell type identities. Examples of "mixed identity" cells that expressed several amacrine marker genes (such as *Tcfap-2β*, *Fgf13*, *Nhlh2*) and rod marker genes (such as *Crx*, *Otx2*, *Nrl*) include WT cells 1, 2, and 6 (Figure 5). These cells did not score highly in the classification scheme for any of the potential cell types (Figure 2, see WT cells 1, 2, and 6) and retained expression of cell cycle and RPC marker genes (Figure 3, see WT cells 1, 2, and 6).

In order to independently validate the observation that WT cells can coexpress marker genes of two different cell types, we performed two-color fluorescent dissociated cell *in situ* hybridization. This was done on dissociated cells to remove any ambiguity of signals overlying more than one cell. The probes were applied simultaneously to detect expression of an amacrine marker (Tcfap-2β) and a rod marker (Crx) in individual P3 retinal cells (Figure 6A). We found that 47.9+/-1.3% of all cells were Crx+, 10.3+/-1.0% were Tcfap-2β+, and 2.2+/-0.5% of cells were positive for both markers (Figure 6C). In addition, we probed for the expression of the ganglion cell marker, NF68, and the rod marker, Crx, in dissociated P3 cells (Figure 6B). Because ganglion cells are not produced postnatally, we did not anticipate that any cells would be in a transitional state in which they would be double positive for Crx and NF68. In this experiment, 54.2+/-1.5% of all cells were Crx+ and 1.3+/-0.15% of all cells were NF68+ (Figure 6C). We found that a negligible amount of cells (0.03+/-0.02%) were double positive for both Crx and NF68

(Figure 6C). The coexpression of an amacrine marker gene and a rod marker gene in a subset of individual P3 WT cells supported our microarray findings.

The profiled cells were also examined for expression of Müller glial and bipolar cell marker genes. Previous studies have shown that a number of genes expressed by mature Müller glial cells, such as *Sox2*, *μ-crystallin*, and *Dkk3,* were also expressed in WT late RPCs (Blackshaw et al., 2004; Roesch et al., 2008; Trimarchi et al., 2008). Some of these shared genes were downregulated in N1-CKO cells (Figure 3, Table S1). Additionally, marker genes thought to be specific for Müller glial cells, such as *Apoe* and *clusterin* (Blackshaw et al., 2004; Roesch et al., 2008), were not expressed above detectable levels in almost any of the profiled cells (Figure S1). For these reasons, it was difficult to determine by direct inspection of the heatmaps if cells were becoming Müller glia. In addition, using the classification scheme, which relies on a large number of Müller glial genes, none of the P3 cells were classified as Müller glial cells (Figure 2). Inspection of the profiled cells for expression of bipolar genes did not yield many positives, which may not be surprising, as known bipolar marker genes are not robustly expressed at P3 *(e.g. Lhx3*, *Car8, Car10*, and *Nfasc)* (Figure S2) (Kim et al., 2008). The absence of these marker genes does not preclude the possibility that some of these cells may later express bipolar genes or be on the path to becoming bipolar cells.

Discussion

In this study, we profiled single N1-CKO and WT retinal cells on Affymetrix microarrays to examine changes in gene expression that occur in the absence of *Notch1* and as cells transition from the progenitor to the neuronal state. At early postnatal stages, the majority of RPCs produce postmitotic cells, which then differentiate into functional neurons over the course of several weeks (Young, 1985b). Levels of Notch1 signaling are likely being read out during the time period when mitotic cells are producing postmitotic cells, and the fates of these cells are being established. This expectation is based on the expression of *Notch1* RNA using *in situ* hybridization (Bao and Cepko, 1997; Lindsell et al., 1996), SAGE (Blackshaw et al., 2004), and single cell microarrays (Trimarchi et al., 2008). Furthermore, the loss of function studies performed in several labs support a role for Notch1 in cycling cells (Dorsky et al., 1995; Henrique et al., 1997; Jadhav et al., 2006a; Nelson et al., 2007; Yaron et al., 2006). Our previous studies of electroporated cells in the P0 retina have shown that mitotic cells or cells exiting mitosis take up plasmids (Matsuda and Cepko, 2004, 2007). These observations, coupled with the analysis of retroviral clone sizes (Fields-Berry et al. 1992) and Young's birthdating studies (Young, 1985b), lead to the conclusion that the electroporation of Cre into *Notch1*$^{fl/fl}$ mice at P0, with harvest at P3, resulted in the removal of *Notch1* from this transitioning population of cells. Because the Notch signaling pathway is a potent regulator of many cellular processes, it is tightly regulated to prevent sustained activation (Kopan and Ilagan, 2009). For example, it is known that the activated form of the receptor, NICD, does not accumulate or linger in the cell, due to a PEST sequence that targets it for degradation (Öberg et al., 2001). From these observations, we anticipated that the rate of Notch protein turnover in retinal cells was relatively rapid, likely faster than the rate at which retinal cells take on their various fates. Indeed, the transcriptomes of the 13 N1-CKO cells that were profiled supported this expectation. The majority of these cells lost expression of Notch target, cell cycle, and progenitor genes, while a few cells appeared to be in the process of downregulating these genes. Furthermore, these cells expressed early marker genes of rods (such as *Blimp1*, *Crx*, *Otx2*), but not markers of differentiated rods (such as *rhodopsin*), providing evidence that the loss of *Notch1* did not dramatically accelerate their differentiation program. However, the state of these cells did appear further advanced down the rod development pathway as compared to the WT cells that had also turned down Notch target genes, but which had retained expression of some cell cycle and progenitor genes.

Using this unbiased method of single cell profiling, we identified a large number of genes that were either up or downregulated in the absence of *Notch1*. The cohort of downregulated genes included cell cycle regulators or progenitor markers, some of which were not yet appreciated to be *Notch1* sensitive (e.g. *Fgf15*, *Cdc20*, *Crym*). Upregulated genes included known regulators or markers of rod photoreceptor development, such as *NeuroD1*, *Math3*, *Rbp3* and *Blimp1*. Future experiments need to be performed to test the novel Notch responsive genes identified by this study for their roles in retinal development.

The majority of the profiled N1-CKO cells were classified as incipient photoreceptors using *Blimp1* as a marker. As described above, *Blimp1* is expressed in rod precursor cells. Genes whose expression patterns were highly correlated with *Blimp1* included genes known to be expressed in rods, such as *Math3*, *Rbp3*, and *Rax*, in addition to the newly identified gene, *Epha8*. The expression pattern of *Epha8* at P3 suggests that this gene is a good marker of early rod photoreceptors, similar to *Blimp1*. Future experiments will determine this specificity, as well as elucidate whether *Epha8* plays a functional role in retinal development.

Single cell microarray analysis and *in situ* hybridization on dissociated retinal cells simultaneously using probes for two genes revealed that newly postmitotic retinal cells coexpressed amacrine and rod marker genes. This result is consistent with the idea that cells may transition through a plastic phase shortly after exiting the cell cycle during which they can express marker genes of different cell types. Alternatively, the coexpression of markers of two cell types may indicate that certain loci are de-repressed, independently of whether a cell is still plastic enough to choose more than one cell fate. It is important to note that the coexpression of rod and amacrine markers does not appear to be an artifact of the single cell profiling method. Only certain genes were coexpressed, and the same ones were seen in multiple cells. If, for example, coexpression was the result of contamination of a single cell's RNA preparation with RNAs from another cell, one might predict random patterns, as opposed to consistent genes found in such profiles. In addition, many cells that appeared more mature, as assessed by higher levels of specific genes and classification scores that indicated a more definitive fate, did not coexpress genes of two cell types (Figure 2).

It is unclear what population of cells is represented by the cells that coexpress these markers. If the majority of RPCs are determined to give rise to stereotyped progeny, and coexpression of amacrine and rod genes occurs in the RPCs that will give rise to a rod and an amacrine, then only a few single cells should coexpress amacrine and rod marker genes. This prediction is based upon Young's birthdating data, as amacrine cells are only a small percentage (1-2%) of the progeny of P0 RPC (Young, 1985b). However, most of the profiled WT cells in this study coexpressed amacrine and rod marker genes. If there are determined subsets of RPCs that produce a rod and an amacrine, a rod and a bipolar, or a rod and Müller glial cell, then single cells coexpressing rod and bipolar genes, as well as single cells coexpressing rod and Müller genes would have been predicted. In fact, Müller glial genes are expressed in the majority of P0 RPCs, though only a small percentage of the progeny of postnatal RPCs (<10%) will be Müller glial cells (Blackshaw et al., 2004; Roesch et al., 2008; Trimarchi et al., 2008; Young, 1985b). Single WT cells did coexpress marker genes shared by late RPCs and Müller glial cells, but not marker genes exclusive to Müller glial cells. In addition, the single cells did not express most known markers of bipolar cells, which is likely because these genes are not expressed as early as P3 (Kim et al., 2008). There may be active repression of most bipolar genes as a result of *Blimp1* activity, which is expressed in these cells and has been shown to inhibit bipolar fate, likely via Chx10-mediated represssion (Brzezinski et al., 2010; Katoh et al., 2010). This repression may be transient, as *Blimp1* expression wanes late in the first postnatal week, when many bipolar cells are born and/or begin to differentiate (Kim et al., 2008; Young, 1985b). Despite this, however, there was expression of *Gnb3*, a bipolar gene in the mature retina, in the majority of N1-CKO cells. Further analysis of these genetic relationships will be required to understand how these cells sort out their fates, and to understand the meaning of the coexpression of two different cell type marker genes.

An intriguing possibility is that coexpression of different marker genes represents a plastic stage through which cells transition after cell cycle exit as they take on their identities. Although newly postmitotic cells likely receive fate determining factors from progenitor cells, it is currently unknown whether cell fate determination occurs in cycling progenitor cells or their postmitotic daughter cells. Interestingly, removal of *Notch1* from newly postmitotic cells results in the overproduction of rods at the expense of other cell types at postnatal stages, demonstrating that input of this signaling pathway is necessary for specification of non-rod fates even after cell cycle exit (Mizeracka et al., In Press). Together with the microarray data presented here, these findings point to the idea that some newly postmitotic cells are not locked in their fate choices, and that fate acquisition in non-rod cells is a Notch1-dependent process that may occur over the course of several days.

Experimental Procedures

Animals

Notch1$^{fl/fl}$ were maintained as homozygotes (Radtke et al., 1999). WT CD-1 mice were obtained from Charles River Laboratories. All experiments were approved by the Institutional Animal Care and Use Committee at Harvard University.

In vivo electroporation

In vivo electroporation were performed as previously described (Matsuda and Cepko, 2004, 2007). DNA constructs used include CAG:GFP, CAG:Cre, CALNL-GFP (Matsuda and Cepko, 2007).

Single Cell Probe Preparation and Affymetrix Array Hybridization

Single cells were isolated and profiled as described previously (Trimarchi et al., 2007, 2008). Cells were chosen based on GFP expression. Probe reactions were performed as described previously, and Affymetrix microarrays (Mouse 430 2.0 arrays) were hybridized and processed using standard Affymetrix protocols (Cherry et al., 2009; Roesch et al., 2008; Trimarchi et al., 2007, 2008). Global scaling was performed using the Affymetrix Microarray Software (MAS 5.0) and the target intensity was set to 500. The signal data for each probe set was exported for further analyses in Microsoft Excel. To eliminate probesets called marginal or absent and to reduce the false-positive rate, only probesets with a RS > 1000, as determined by MAS 5.0 were considered in this analysis. Previous reports suggest that this threshold corresponds to transcripts that are present at between 10 and 100 copies per cell (Tietjen et al., 2003). Treeview software was utilized to view the microarray signal data. Previously profiled cells that were classified as either amacrines or rod photoreceptors were chosen to provide examples of WT cells that were further along in their differentiation (from a previous study, Trimarchi and Cepko, unpublished, and Cherry et al., 2009). The raw and processed Affymetrix data files have been deposited in the NCBI Gene Expression Omnibus (GEO). GEO submission: GSE35682.

FACS purification and semi-quantitative PCR

FACS was performed on BD Aria II sorter, gated for GFP detection. 3-5 x 10^5 GFP+ cells were collected from two dissociated retinas for each sample. After sorting, GFP+ cells were lysed in Trizol (Invitrogen) and stored at -80°C. Phenol-chloroform extractions were performed to isolate total RNA. cDNA was generated using Accuscript High Fidelity (Agilent Technologies) according to manufacturer's guidelines. Semi-quantitative real time PCR was performed and gene expression was normalized according to *actin* expression in each sample. Primers used included: *actin* –

accaactgggacgacatggagaa, tacgaccagaggcatacagggac; *Nrarp* - agggccagacagcactacac, cttggccttggtgatgagat; *Hes1* - acaccggacaaaccaaagac, atgccgggagctatctttct; *Blimp1* - cacacaggagagaagccaca, ttgtgacactgggcacactt; *Math3* – attcagggctcgaagagtca, gttccttgccagtcgaagag; *NeuroD1* – gtgtcccgaggctccagggt, gggaccttggggctgaggct.

Immunohistochemistry and *in situ* hybridization

Retinas were fixed either as wholemounts for 30 minutes or as eyeballs for 2 hours in 4% PFA at room temperature in 1X PBS. Retinas were equilibrated in sucrose/PBS solutions of increasing sucrose concentrations (5, 20, 30%), a 1:1 solution of OCT (Tissue-Tek) and 30% sucrose/PBS, and frozen on dry ice. 20 μm cryosections were cut using a disposable blade on a Leica CM3050S cryostat.

For immunohistochemistry, retinal cryosections were blocked for 1 hour in 0.1% Triton, 0.02% SDS, 1% BSA in 1X PBS. Sections were then incubated in a humidified chamber at 4°C overnight with chicken anti-GFP (1:2000; Abcam) diluted in blocking solution. Sections were washed in 1X PBS and incubated for 2 hours with fluorescently coupled secondary antibodies (Jackson ImmunoResearch) and DAPI (Sigma-Aldrich). Slides were mounted in Fluoromount-G (Southern Biotechnology Associates).

Retinas were collected at various developmental time points for *in situ* hybridization. Section *in situ* hybridization and double fluorescent *in situ* hybridization on dissociated cells was performed as previously described (Trimarchi et al., 2007).

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Figure 1. Removal of *Notch1* from the postnatal retina by electroporation

Plasmids encoding CAG:GFP or CAG:Cre with a Cre reporter (CALNL-GFP) were electroporated *in vivo* into *Notch1*$^{fl/fl}$ P0 mouse retinas. The fates of electroporated cells were analyzed in the mature retina after P21 by histology. Electroporation of CAG:GFP alone (WT) into *Notch1*$^{fl/fl}$ retinas labeled GFP+ photoreceptors (located in the ONL), and interneurons and Müller glial cells (located in the INL) (A). Electroporation of CAG:Cre and CALNL-GFP (N1-CKO) into *Notch1*$^{fl/fl}$ retinas labeled GFP+ photoreceptors and some amacrine cells, but not bipolar cells or Müller glial cells (B). *Notch1*$^{fl/fl}$ retinas were electroporated at P0 *in vivo* with CAG:GFP to mark WT cells, or CAG:Cre and CALNL-GFP, to mark N1-CKO cells. After 3 days *in vivo*, retinas were dissociated and single GFP+ cells were harvested for profiling. Each single cell was subjected to reverse transcription and PCR, with the resulting probes hybridized to Affymetrix arrays. The average signal levels for selected Notch target and pro-neurogenic genes are shown (C). *Notch1*$^{fl/fl}$ retinas were electroporated at P0 *in vitro* with CAG:GFP to mark WT cells, or CAG:Cre and CALNL-GFP, to mark N1-CKO cells. After 3 days in culture, GFP+ cells were collected by flow cytometry and used to prepare cDNA. Samples were subjected to semi-quantitative real time PCR in order to detect differences in expression of Notch target and pro-neurogenic genes between N1-CKO and WT cells (D). All expression values were normalized to *actin* expression levels in each sample. n=3 retinas per condition. p-value < 0.05. r-rod photoreceptor, bp-bipolar cell, Mg-Müller glial cell, ac-amacrine cell. Cellular laminae are denoted: ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.

Figure 2. Classification of single profiled cells

Profiled cells were classified as RPCs, rod photoreceptors, bipolar cells, amacrine cells, ganglion cells, or Müller glia based on the expression levels of genes associated with known markers of that particular cell type. Genes associated with one or several cell type-specific markers were determined by a Fisher's exact test and the relative expression level for each associated gene was calculated by dividing the cell's signal level by the maximum signal level found in a large collection of single cells (Trimarchi et al., 2008). These values were summed and normalized to generate a cell type score for each cell, with 10 being the maximum score for each cell type. Genes associated with *FGF15, Sfrp2,* and *μ-crystallin* were used to generate a RPC score, genes associated with *Blimp1* for rod photoreceptors, genes associated with *Og9x* for bipolar cells, genes associated with *Tcfap-2β, Gad1,* and *Glyt1* for amacrine cells, genes associated with *NF68* and *Ebf3* for ganglion cells, and genes associated with *Apoe* for Müller glia. For comparison, previously profiled cells, which were classified as amacrine (cells P0 A4, P0 B1, P0 D1, P0 G3)(Cherry et al., 2009) or rod precursors (cells P0 E1, P5 C4, P5 D2, P5 C2) (Trimarchi and Cepko, unpublished), are shown. The highest score for each cell is boxed in red.

Figure 3. Gene expression of selected Notch target, cell cycle, progenitor, and proneurogenic genes in WT and N1-CKO single cells

The microarrays performed using cDNA from single cells from WT and N1-CKO cells (described in Figure 1) were analyzed for the expression of selected genes. The signals for different types of genes are shown as a heatmap that was generated using Treeview software. The expression levels of Notch target, cell cycle, progenitor, and pro-neurogenic genes are shown. See also Tables S1 and S2. The signal intensity from Affymetrix microarray chips has been scaled and is represented by a gradation in color, from bright red to black. Signals below 3,000 are black and signals from 3,000 to 10,000 are a scaled shade of red. For Affymetrix identifier, Unigene number, and full gene name, see Table S3.

Figure 4. Analysis of *Blimp1* associated genes

The microarrays performed using cDNA from single cells from WT and N1-CKO cells (described in Figure 1) were analyzed to identify genes whose expression patterns significantly correlated with *Blimp1* (p-value listed next to gene row). A heatmap was generated using Treeview software to visualize expression levels of *Blimp1* and its associated genes in N1-CKO and WT cells (A). The signal intensity from Affymetrix microarray chips has been scaled and is represented by a gradation in color, from bright red to black. Signals below 3,000 are black and signals from 3,000 to 10,000 are a corresponding shade of red. For Affymetrix identifier, Unigene number, and full gene name, see Table S3. *In situ* hybridization for a novel gene, *Epha8*, correlated with *Blimp1* expression was performed at P3 (B, E), P9 (C, F), and adult (D, G) stages. Probes used were *Blimp1* (B-D) and *Epha8* (E-G). Cellular laminae are denoted: ONBL = outer neuroblastic layer, INBL = inner neuroblastic layer, ONL = outer nuclear layer, INL = inner nuclear layer. Scale bar = 50 µm.

Figure 5. Expression of selected rod and amacrine marker genes in N1-CKO and WT cells

The microarrays performed using cDNA from single cells from WT and N1-CKO cells (described in Figure 1) were analyzed for the expression of genes that are expressed in rod photoreceptors and amacrine cells. A heatmap was generated using Treeview software to visualize the expression of these genes. For comparison, expression levels of selected genes in previously profiled cells, which were classified as amacrine (Cherry et al., 2009) or rod precursors (Trimarchi and Cepko, unpublished), are shown. The signal intensity from Affymetrix microarray chips has been scaled and is represented by a gradation in color, from bright red to black. Signals below 3,000 are black and signals from 3,000 to 10,000 are the appropriate shade of red. For Affymetrix identifier, Unigene number, and full gene name, see Table S3.

Figure 6. Simultaneous detection of RNA for two different cell type-specific marker genes by two color fluorescent *in situ* hybridization

WT P3 retinas were probed for expression of different cell type-specific markers by double fluorescent *in situ* hybridization on dissociated cells. Cells from dissociated retinas were probed for the expression of *Tcfap-2β* (amacrine marker) and *Crx* (photoreceptor marker) (A), or for *Crx* (photoreceptor marker) and *NF68* (ganglion cell marker) expression (B). Arrow indicates a double marker+ cell. Quantification of single and double marker+ cells (C). n=2047 total cells examined for *Tcfap-2β* and *Crx* expression. n=6278 total cells examined for *NF68* and *Crx* expression.

Table S1. Downregulated genes in selected N1-CKO cells versus selected WT cells

An unbiased search for significantly downregulated genes was conducted by comparing gene expression levels in cells classified as RPCs (WT cells 2-6, 8, 12) to those in cells classified as rod precursors (N1-CKO cells 1-10). T-test analysis (p-value<0.05) was performed to find significantly downregulated genes.

Table S2. Upregulated genes in selected N1-CKO cells versus selected WT cells

An unbiased search for significantly upregulated genes was conducted by comparing gene expression levels in cells classified as RPCs (WT cells 2-6, 8, 12) to those in cells classified as rod precursors (N1-CKO cells 1-10). T-test analysis (p-value<0.05) was performed to find significantly upregulated genes.

Table S3. Gene identities

Affymetrix identifier, Unigene number, gene symbol, and gene title are listed for all genes depicted in heatmaps in Figures 3, 4, 5, S1 and S2.

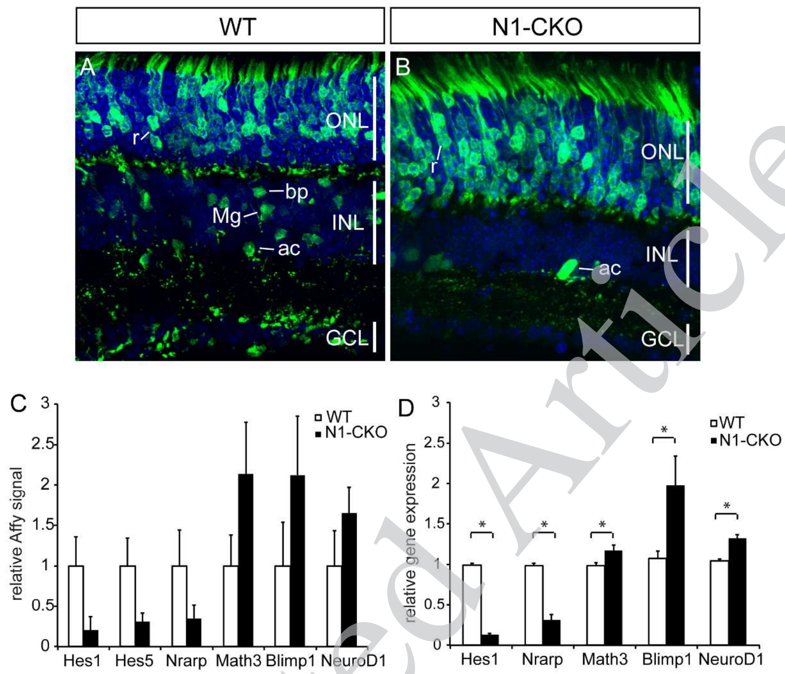

Figure 1

215x279mm (300 x 300 DPI)

	RPC	rod	bipolar	amacrine	ganglion	Muller
WT cell 1	1.9	2.4	0.5	2.0	1.3	0.3
WT cell 2	3.2	1.7	0.9	0.8	0.7	0.6
WT cell 3	3.8	3.1	0.5	0.9	0.7	0.6
WT cell 4	4.2	1.1	0.7	1.2	0.7	0.6
WT cell 5	3.2	2.4	0.5	0.8	0.5	0.5
WT cell 6	2.9	2.6	0.6	1.5	0.7	0.5
WT cell 7	0.9	1.8	0.5	2.6	1.9	0.2
WT cell 8	3.8	1.4	0.6	1.4	0.5	0.6
WT cell 9	5.6	9.9	0.9	2.1	1.5	0.3
WT cell 10	2.8	4.7	1.0	1.7	1.9	0.5
WT cell 11	1.8	8.4	0.9	0.9	1.1	0.5
WT cell 12	5.1	2.6	0.7	1.7	0.5	0.6
WT cell 13	1.8	6.6	0.6	1.1	0.7	0.4
N1-CKO cell 1	1.6	6.8	0.6	1.0	0.7	0.3
N1-CKO cell 2	1.9	3.3	0.6	2.7	1.3	0.3
N1-CKO cell 3	3.2	4.3	1.0	1.1	0.6	0.5
N1-CKO cell 4	1.9	5.8	0.7	1.2	0.7	0.5
N1-CKO cell 5	0.8	9.6	0.7	1.1	1.1	0.4
N1-CKO cell 6	1.4	10.0	1.4	1.3	0.3	0.3
N1-CKO cell 7	1.0	6.8	0.8	1.5	0.9	0.3
N1-CKO cell 8	1.7	8.6	1.7	1.4	1.0	0.3
N1-CKO cell 9	2.2	6.3	0.9	1.7	0.3	0.6
N1-CKO cell 10	2.2	8.9	0.9	1.9	0.7	0.5
N1-CKO cell 11	1.7	2.1	0.7	2.8	1.9	0.4
N1-CKO cell 12	1.0	2.7	1.2	4.2	1.7	0.2
N1-CKO cell 13	1.1	2.2	1.1	3.6	2.2	0.4
P0 cell E1	1.1	7.1	1.4	1.0	0.9	0.4
P5 cell C4	1.1	7.8	0.9	1.5	0.7	0.2
P5 cell D2	1.9	5.0	1.2	1.3	1.2	0.3
P5 cell C2	1.8	4.1	1.0	1.1	1.0	0.3
P0 cell A4	1.7	1.9	1.3	6.8	1.8	0.3
P0 cell B1	1.9	1.4	1.1	3.8	2.9	0.6
P0 cell D1	1.6	0.9	1.7	8.9	2.6	0.4
P0 cell G3	1.1	1.4	0.8	5.6	3.2	0.4

Figure 2

215x279mm (300 x 300 DPI)

Developmental Dynamics

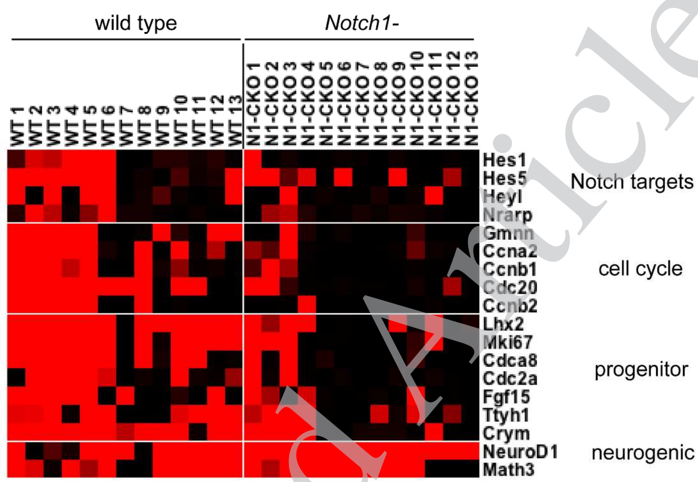

Figure 3

215x279mm (300 x 300 DPI)

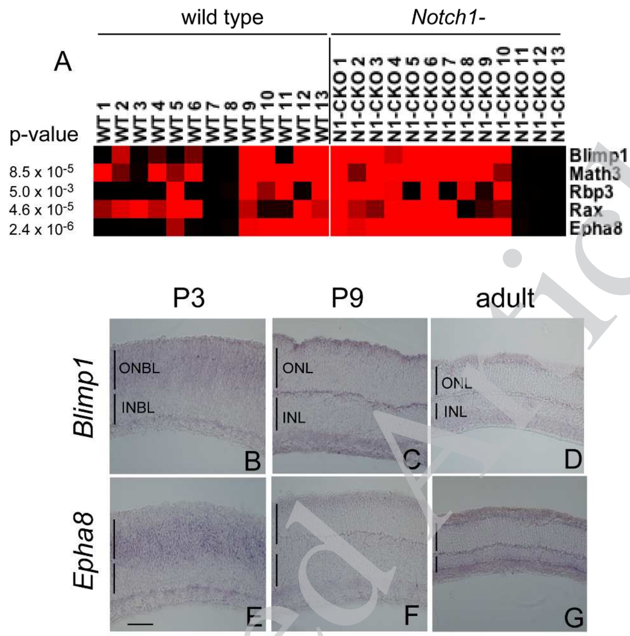

Figure 4

215x279mm (300 x 300 DPI)

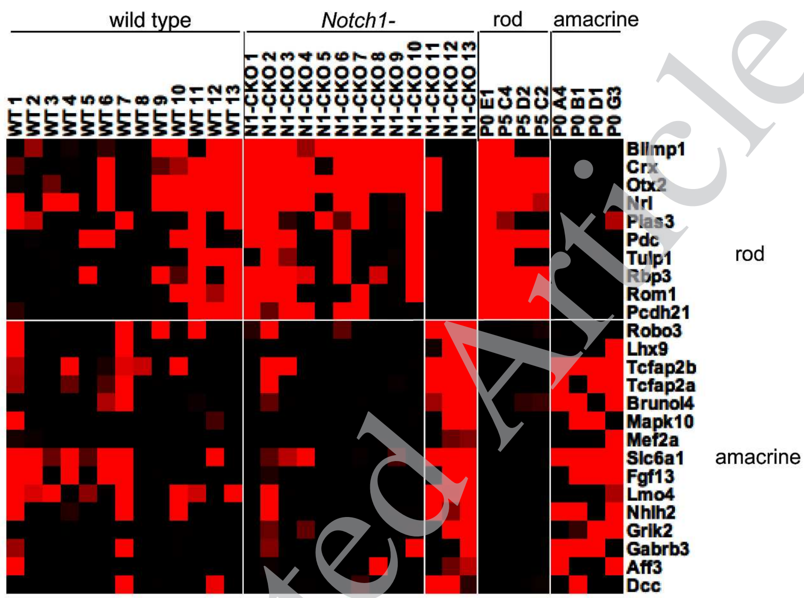

Figure 5

5
215x279mm (300 x 300 DPI)

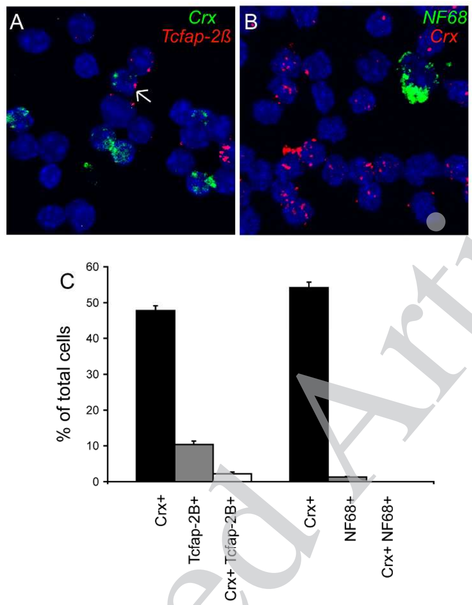

Figure 6

215x279mm (300 x 300 DPI)

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1455820_x_at	Mm.282242	Scarb1	6.3037E-05
1437378_x_at	Mm.282242	Scarb1	8.001E-05
1450644_at	Mm.235132	Zfp36l1	9.9196E-05
1424508_at	Mm.276362	Ttc5	0.00010865
1423682_a_at	Mm.313185	Cdca4	0.00011868
1416050_a_at	Mm.282242	Scarb1	0.00015139
1449345_at	Mm.181767	Ccdc34	0.00017166
1415810_at	Mm.42196	Uhrf1	0.00019712
1439407_x_at	Mm.271711	Tagln2	0.00024061
1437197_at	Mm.479578	Sorbs2	0.00030126
1426642_at	Mm.193099	Fn1	0.00033615
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1454804_at	Mm.309520	Nploc4	0.00059536
1416581_at	Mm.28265	Wdr5	0.00060242
1416962_at	Mm.255045	Rcc1	0.0007834
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1447919_x_at	Mm.3014	Ndufab1	0.04573358
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1448698_at	Mm.273049	Ccnd1	0.0463349
1460626_at	Mm.428571	11-Sep	0.04644243
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1444368_at	Mm.63569	Chrna3	0.0466412
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1430638_at	Mm.479615	Ccdc30	0.04703568
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1454739_at	Mm.89845	Cdc27	0.04853475
1454960_at	Mm.7320	Smad3	0.04861309
1456729_x_at	Mm.11333	Rtel1	0.04867628
1452295_at	Mm.478899	Pmepa1	0.04870265
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1417848_at	Mm.212908	Zfp704	0.04916075
1456351_at	Mm.45602	Brd8	0.04924745
1416542_at	Mm.479548	Phf1	0.04928638
1415692_s_at	Mm.248827	Canx	0.0493322
1448791_at	Mm.273379	LOC100048642 /,	0.04935144
1436200_at	Mm.327654	Lonrf3	0.04939446
1440492_at	---	---	0.04952174
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Probe Set ID	UniGene ID	Gene Symbol	P-value
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1420505_a_at	Mm.278865	Stxbp1	0.00169602
1431177_a_at	Mm.336955	Rpl10a /// Rpl10a-ps1	0.00224578
1433658_x_at	Mm.286394	Pcbp4	0.00229353
1423892_at	Mm.38469	Apbb1	0.00256851
1419341_at	Mm.1390	Epha8	0.00286174
1426412_at	Mm.4636	Neurod1	0.00334169
1448233_at	Mm.648	Prnp	0.00342182
1451597_at	Mm.240139	Tmprss11d	0.00423101
1417308_at	Mm.326167	Pkm2	0.00427551
1435732_x_at	Mm.30155	Atp6v0c	0.00451569
1436568_at	Mm.41758	Jam2	0.00452929
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1418449_at	Mm.36726	Lad1	0.00566364
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1422866_at	Mm.300931	Col13a1	0.00868479
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1417484_at	Mm.4987	Ibsp	0.00935364
1416731_at	Mm.130362	Top2b	0.00964003
1416473_a_at	Mm.209041	Igdcc4	0.01023362
1448430_a_at	Mm.3746	Naca	0.01025747
1423883_at	Mm.210323	Acsl1	0.01067544
1449196_a_at	Mm.180003	Gm9354 /// LOC1000	0.01076538
1416134_at	Mm.2381	Aplp1	0.01079886
1450031_at	Mm.395281	Aff4	0.01082612
1450658_at	Mm.112933	Adamts5 /// LOC1000	0.01099518
1441799_at	---	6030422H21Rik	0.01128589
1429091_at	Mm.302754	1600002K03Rik	0.01136624
1416474_at	Mm.209041	Igdcc4	0.01179838
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1420760_s_at	Mm.30837	Ndrg1	0.01607493
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1449602_at	---	---	0.01749353
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1422418_s_at	Mm.7821	Gm3258 /// Supt4h1	0.01837947
1416407_at	Mm.544	Pea15a	0.01840541
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1442251_at	Mm.274493	Vcpip1	0.018576
1432898_at	---	4930544L18Rik	0.01877585
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1431559_at	Mm.75260	6430710C18Rik	0.01936235
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1425298_a_at	Mm.6898	Naip1	0.02037353
1443757_x_at	Mm.458319	Ankzf1 /// Glb1l	0.02050764
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1444203_at	---	---	0.02288392
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1448715_x_at	Mm.86541	BC094435 /// Ccrn4l /	0.02384733
1432157_at	---	4930515G13Rik	0.02391841
1442147_at	Mm.441031	---	0.02397445
1424092_at	Mm.30038	Epb4.1	0.02402342
1450072_at	Mm.130752	Ash1l	0.02408985
1456467_s_at	Mm.9001	Nlk	0.02445064
1418054_at	Mm.10695	Neurod4 (Math3)	0.02469134

1438925_x_at	Mm.30155	Atp6v0c	0.02473745
1417380_at	Mm.207619	Iqgap1	0.02478975
1444938_at	Mm.361172	---	0.02480743
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1431436_a_at	Mm.35803	Katnal2	0.02525751
1422215_at	---	4930542C12Rik	0.02537668
1422575_at	Mm.259260	Mxd4	0.02558057
1435062_at	Mm.146779	Srrm4	0.02596614
1453389_a_at	Mm.425294	Sh2b2	0.02613074
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1420752_at	Mm.271724	Dtx3 /// LOC1000450	0.02644921
1447512_at	---	---	0.02650334
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1419412_at	Mm.190	Xcl1	0.02696518
1416828_at	Mm.45953	Snap25	0.02766148
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1420425_at	Mm.4800	Prdm1 (Blimp1)	0.02825884
1416785_at	Mm.252514	Kcnip1	0.02839476
1450989_at	Mm.5090	Tdgf1	0.02843628
1435256_at	Mm.159258	Clip3	0.02917019
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1456393_at	Mm.375091	Ncrna00081	0.02964904
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1434051_s_at	Mm.39739	Hspa12a	0.0297259
1455097_at	Mm.321312	Wdr70	0.02984169
1434548_at	Mm.218473	Serinc3	0.02987151
1439256_x_at	---	Gpr137b-ps	0.02992631
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1452173_at	Mm.200497	Hadha	0.03027367
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1426491_at	Mm.20929	Herc2	0.03089658
1460533_at	---	4933440K10Rik	0.03090498
1439255_s_at	Mm.379269	Gpr137b /// Gpr137b-	0.03115066
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1433852_at	Mm.250641	Kidins220	0.03132041
1420138_at	Mm.265060	Slc19a1	0.0313692
1427191_at	Mm.103477	Npr2	0.03159904
1436843_at	Mm.430709	C430048L16Rik	0.03179969
1427338_at	Mm.80685	Crocc	0.03192782
1418373_at	Mm.219627	Pgam2	0.03193551
1424880_at	Mm.40298	Trib1	0.03194705
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1457051_at	Mm.314056	Trim27	0.03277408
1418375_at	Mm.372826	Mbd6	0.03291178
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1430238_at	Mm.272794	Got1l1	0.03376931
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1416298_at	Mm.4406	Mmp9	0.03392681
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1443565_at	---	AI849538	0.03417771
1437724_x_at	Mm.1860	Pitpnm1	0.03429358
1456838_at	Mm.40213	Lingo3	0.0345211
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1459665_s_at	Mm.479246	Mrvi1	0.038908

1428458_at	Mm.248779	Pop1	0.03893919
1423816_at	Mm.328602	Cxx1a /// Cxx1b	0.03895164
1431251_at	Mm.133301	Lrrc3	0.03896938
1428347_at	Mm.154358	Cyfip2	0.03897795
1415929_at	Mm.28357	Map1lc3b	0.03903051
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1450574_at	Mm.377079	Cyp11b2	0.0423647
1452746_at	Mm.205625	Atp13a2	0.04264654
1454076_at	Mm.439963	Lrrc17	0.0426788
1436926_at	Mm.235550	Esrrb	0.0427797
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1421647_at	Mm.389465	Cd1d2	0.04284403
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1432752_at	---	4930403O18Rik	0.04310425
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1451757_at	Mm.463028	BC003883	0.04343596
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1446750_at	Mm.478864	Impact	0.04363916
1449055_x_at	Mm.286394	Pcbp4	0.04363929
1423153_x_at	Mm.8655	Cfh /// LOC10004801	0.04378076
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1425123_at	Mm.86446	Klhl36	0.04469519
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1415885_at	Mm.255241	Chgb	0.0448256
1426160_a_at	Mm.17461	Stk16	0.04500233
1435720_at	Mm.44530	Kcnd3	0.04507472
1444182_at	Mm.98583	2210010C17Rik	0.04523875
1448193_at	Mm.157648	5730403B10Rik	0.04537022
1420971_at	Mm.389330	Ubr1	0.04570947
1433817_at	Mm.141230	Agpat3	0.04603104
1425934_a_at	Mm.182377	B4galt4	0.04604431
1439165_at	Mm.446631	---	0.0460548
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1429993_s_at	Mm.375471	Gm10471 /// Speer4b	0.0464577
1429707_at	Mm.478340	Plaa	0.04662597
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1449311_at	Mm.26147	Bach1	0.04694977
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1441168_at	---	---	0.04761732
1416094_at	Mm.28908	Adam9	0.04767814
1455733_at	Mm.248296	Taok3	0.0477142
1438621_x_at	Mm.4128	Axl	0.04775626
1459664_at	Mm.155573	Wdr31	0.04796261
1441676_at	Mm.309526	Zfat	0.04798642
1428707_at	Mm.142187	Ptms	0.04828729
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1450596_at	Mm.445781	Olfr66	0.0486383
1417391_a_at	Mm.10137	Il16	0.04869307
1416521_at	Mm.42829	Sepw1	0.04870956
1450518_at	Mm.330897	Hnf4g	0.0487374
1451314_a_at	Mm.76649	Vcam1	0.04881765
1436715_s_at	Mm.28219	Cdipt	0.04886268
1417364_at	Mm.379129	Eef1g /// LOC100047	0.04886371
1422829_at	Mm.41075	Drd4	0.04892374
1446623_at	---	---	0.04896308
1443196_at	Mm.373919	---	0.04934486
1437722_x_at	Mm.272803	Pcbp3	0.04951643

1434988_x_at	Mm.284446	Aldh2	0.04969138
1436496_at	---	1700012O15Rik	0.04985789
1435435_at	Mm.478873	Cttnbp2	0.04996828

Probe Set ID	UniGene ID	Gene Symbol	Gene Title
1416776_at	Mm.9114	Crym	μ-crystallin
1417312_at	Mm.55143	Dkk3	dickkopf homolog 3 (Xenopus laevis)
1417506_at	Mm.12239	Gmnn	geminin
1417911_at	Mm.4189	Ccna2	cyclin A2
1417985_at	Mm.46539	Nrarp	Notch-regulated ankyrin repeat protein
1417999_at	Mm.4266	Itm2b	integral membrane protein 2B
1418054_at	Mm.10695	Neurod4 (Math3)	neurogenic differentiation 4
1418102_at	Mm.390859	Hes1	hairy and enhancer of split 1 (Drosophila)
1418304_at	Mm.156506	Cdhr1	cadherin-related family member 1
1418310_a_at	Mm.41653	Rlbp1	retinaldehyde binding protein 1
1418317_at	Mm.142856	Lhx2	LIM homeobox protein 2
1418376_at	Mm.3904	Fgf15	fibroblast growth factor 15
1418497_at	Mm.7995	Fgf13	fibroblast growth factor 13
1418558_at	Mm.478898	Rax	retina and anterior neural fold homeobox
1418705_at	Mm.441911	Crx	cone-rod homeobox containing gene
1419302_at	Mm.103615	Heyl	hairy/enhancer-of-split related with YRPW motif-like
1419324_at	Mm.250732	Lhx9	LIM homeobox protein 9
1419341_at	Mm.1390	Epha8	Eph receptor A8
1419628_at	Mm.4405	Vsx2	visual system homeobox 2
1419944_at	Mm.380027	Ccnb1	Cyclin B1
1420425_at	Mm.4800	Prdm1 (Blimp1)	PR domain containing 1, with ZNF domain
1420981_a_at	Mm.479165	Lmo4	LIM domain only 4
1421996_at	Mm.85544	Tcfap2a	transcription factor AP-2, alpha
1422694_at	Mm.29729	Ttyh1	tweety homolog 1 (Drosophila)
1423146_at	Mm.137268	Hes5	hairy and enhancer of split 5 (Drosophila)
1424103_at	Mm.29087	Atg4b	autophagy-related 4B (yeast)
1424118_a_at	Mm.272969	Spc25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)
1424547_at	Mm.342160	Car10	carbonic anhydrase 10
1424944_at	Mm.440882	Pcp2	Purkinje cell protein 2 (L7)
1425041_at	Mm.386765	Lhx3	LIM homeobox protein 3
1425105_at	Mm.436622	Rbp3	retinol binding protein 3, interstitial
1425926_a_at	Mm.134516	Otx2	orthodenticle homolog 2 (Drosophila)
1426236_a_at	Mm.210745	Glul	glutamate-ammonia ligase (glutamine synthetase)
1426508_at	Mm.1239	Gfap	glial fibrillary acidic protein
1426681_at	Mm.297706	Unk	unkempt homolog (Drosophila)
1426817_at	Mm.4078	Mki67	antigen identified by monoclonal antibody Ki 67
1427185_at	Mm.132788	Mef2a	myocyte enhancer factor 2A
1427482_a_at	Mm.119320	Car8	carbonic anhydrase 8
1429994_s_at	Mm.271190	Cyp2c65	cytochrome P450, family 2, subfamily c, polypeptide 65
1432466_a_at	Mm.305152	Apoe	apolipoprotein E
1433939_at	Mm.336679	Aff3	AF4/FMR2 family, member 3
1435021_at	Mm.8004	Gabrb3	gamma-aminobutyric acid (GABA) A receptor, subunit beta 3
1435670_at	Mm.137021	Tcfap2b	transcription factor AP-2 beta
1436205_at	Mm.326702	Nfasc	neurofascin
1436634_at	Mm.212826	Robo3	roundabout homolog 3 (Drosophila)
1436847_s_at	Mm.28038	Cdca8	cell division cycle associated 8
1436888_at	Mm.137286	Nhlh2	nescient helix loop helix 2
1437195_x_at	Mm.39253	Mapk10	mitogen-activated protein kinase 10

1437458_x_at	Mm.200608	Clu /// LOC100046120	clusterin /// similar to clusterin
1437828_s_at	Mm.2437	Wdr46	WD repeat domain 46
1438118_x_at	Mm.268000	Vim	vimentin
1438782_at	Mm.321683	Cntn4	contactin 4
1439377_x_at	Mm.289747	Cdc20	cell division cycle 20 homolog (S. cerevisiae)
1440487_at	Mm.167882	Dcc	deleted in colorectal carcinoma
1447676_x_at	Mm.331185	S100a16	S100 calcium binding protein A16
1447745_at	Mm.250786	Aqp4	aquaporin 4
1448314_at	Mm.281367	Cdk1	cyclin-dependent kinase 1
1448752_at	Mm.1186	Car2	carbonic anhydrase 2
1448996_at	Mm.426094	Rom1	rod outer segment membrane protein 1
1449145_a_at	Mm.28278	Cav1	caveolin 1, caveolae protein
1449159_at	Mm.68889	Gnb3	guanine nucleotide binding protein (G protein), beta 3
1449179_at	Mm.440883	Pdc	phosducin
1450920_at	Mm.22592	Ccnb2	cyclin B2
1450945_at	Mm.222178	Prkca	protein kinase C, alpha
1450946_at	Mm.20422	Nrl	neural retina leucine zipper gene
1451115_at	Mm.1635	Pias3	protein inhibitor of activated STAT 3
1451534_at	Mm.255667	Scgn	secretagogin, EF-hand calcium binding protein
1451582_at	Mm.42102	Tulp1	tubby like protein 1
1451826_at	Mm.103669	Cabp5	calcium binding protein 5
1451835_at	Mm.478420	Sox21	SRY-box containing gene 21
1452142_at	Mm.5260	Slc6a1	solute carrier family 6 (neurotransmitter transporter, GABA), member 1
1452240_at	Mm.266435	Celf4	CUGBP, Elav-like family member 4
1453008_at	Mm.151594	Trnp1	TMF1-regulated nuclear protein 1
1455976_x_at	Mm.2785	Dbi	diazepam binding inhibitor
1457683_at	Mm.332838	Grik2	glutamate receptor, ionotropic, kainate 2 (beta 2)
1457946_at	Mm.134360	Sebox (Og9x)	SEBOX homeobox

Table S3. Gene identities