

# Gene-regulation logic in retinal ganglion cell development: *Isl1* defines a critical branch distinct from but overlapping with *Pou4f2*

Xiuqian Mu<sup>\*†</sup>, Xueyao Fu<sup>\*</sup>, Phillip D. Beremand<sup>‡</sup>, Terry L. Thomas<sup>‡</sup>, and William H. Klein<sup>\*†</sup>

<sup>\*</sup>Department of Biochemistry and Molecular Biology, M. D. Anderson Cancer Center and Graduate Training Program in Genes and Development, Graduate School of Biomedical Sciences, University of Texas, Houston, TX 77030; and <sup>‡</sup>Department of Biology and Laboratory for Functional Genomics, Texas A&M University, College Station, TX 77843

Communicated by Eric H. Davidson, California Institute of Technology, Pasadena, CA, March 18, 2008 (received for review September 9, 2007)

Understanding gene regulatory networks (GRNs) that control neuronal differentiation will provide systems-level perspectives on neurogenesis. We have previously constructed a model for a GRN in retinal ganglion cell (RGC) differentiation in which four hierarchical tiers of transcription factors ultimately control the expression of downstream terminal genes. *Math5* occupies a central node in the hierarchy because it is essential for the formation of RGCs and the expression of the immediate downstream factor *Pou4f2*. Based on its expression, we also proposed that *Isl1*, a LIM-homeodomain factor, functions in parallel with *Pou4f2* and downstream of *Math5* in the RGC GRN. To determine whether this was the case, a conditional *Isl1* allele was generated and deleted specifically in the developing retina. Although RGCs formed in *Isl1*-deleted retinas, most underwent apoptosis, and few remained at later stages. By microarray analysis, we identified a distinct set of genes whose expression depended on *Isl1*. These genes are all downstream of *Math5*, and some of them, but not all, also depend on *Pou4f2*. Additionally, *Isl1* was required for the sustained expression of *Pou4f2*, suggesting that *Isl1* positively regulates *Pou4f2* after *Math5* levels are diminished. The results demonstrate an essential role for *Isl1* in RGC development and reveal two distinct but intersecting branches of the RGC GRN downstream of *Math5*, one directed by *Pou4f2* and the other by *Isl1*. They also reveal that identical RGC expression patterns are achieved by different combinations of divergent inputs from upstream transcription factors.

cell differentiation | gene regulatory network | transcription factors | retinogenesis

Retinal development is especially attractive for gene regulatory network (GRN) analysis because it represents a relatively simple, highly amenable sensory tissue with a small number of neuronal cell types connected to visual centers in the brain by the optic nerve (1, 2). A canonical GRN has been proposed for retinal determination genes in insects and mammals that is composed of seven to eight highly conserved transcription factors (3). In the mouse, one of the retinal determination factors, the pair-rule homeobox factor *Pax6*, is essential for the formation of all retinal cell types with the exception of amacrine cells (4). *Pax6* is required in retinal progenitor cells (RPCs) for the expression of several critical transcription factors that then control the development of individual retinal cell types. One of these factors is *Math5*, a proneural basic helix–loop–helix factor that is essential for specifying retinal ganglion cells (RGCs) (5, 6), the first cells to differentiate in the developing retina. *Pax6-Math5*-expressing RPCs define a competence field that permits the subsequent steps of RGC formation to proceed (7, 8). Based on these facts, we have constructed a GRN model for RGC development that features a downward cascade of transcription factors occupying distinct hierarchical tiers (8).

In the RGC GRN, *Math5* activates genes that encode immediate downstream transcription factors. One of these, POU domain

factor *Pou4f2*, is essential for RGC differentiation; retinas from *Pou4f2*<sup>-/-</sup> mice have severe defects in RGC differentiation but not in their initial specification (9, 10). In gene expression-profiling experiments in which we compared genes whose expression was altered in *Math5*<sup>-/-</sup> and *Pou4f2*<sup>-/-</sup> retinas, we identified transcription-factor genes whose expression was substantially reduced in *Math5*<sup>-/-</sup> but not *Pou4f2*<sup>-/-</sup> retinas. One of these genes encodes *Isl1* (also known as *Islet-1*), a LIM homeobox protein expressed in many tissues during development, including the developing retina (11–14). *Isl1* is activated immediately after the birth of RGCs, and its expression is identical with that of *Pou4f2* before E14.5 (ref. 11 and data not shown).

We have hypothesized that other factors in addition to *Pou4f2* must function at the same level in the RGC GRN (8, 15). This is because only a subset of RGC genes whose expression depends on the presence of *Math5* also depends on *Pou4f2*. It is therefore likely that *Pou4f2* represents one branch of the RGC GRN and that genes whose expression is independent of *Pou4f2* are regulated by other transcription factors positioned at the same level in the hierarchy as *Pou4f2*. These hypothesized factors would represent other branches of the RGC GRN. Moreover, some RGC genes are likely to be under the control of more than one transcription factor, thereby resulting in intersecting branches. Identifying additional transcription factors lying immediately downstream of *Math5* would substantially broaden our understanding of the regulatory events that direct RGC differentiation. Based on its expression pattern and its regulatory relationship with *Math5* and *Pou4f2*, we proposed that *Isl1* is one of these factors.

*Isl1*<sup>-/-</sup> embryos from *Isl1* germ-line knockout mice demonstrate abnormalities in the development of multiple organs/tissues including motor neuron and heart (16, 17) and consequently die at approximately embryonic day 11 (E11), a time when the differentiation of retinal cell types has yet to begin. Despite the fact that *Isl1* has been used as a RGC marker for many years (13), its role in RGC formation has not been established. In addition, *Isl1* is also expressed in developing and mature on-bipolar cells and cholinergic amacrine cells (12, 18), and it is possible that *Isl1* has functions in the development and maintenance of these retinal cell types.

To study the function of *Isl1* in retinal development, we generated a floxed *Isl1* allele and deleted it specifically in the developing retina using a retina-specific *Six3-Cre* transgene (19, 20). We find

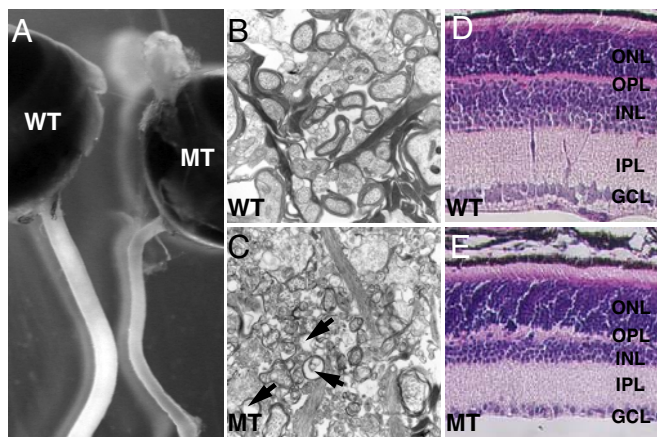
Author contributions: X.M. designed research; X.M. and X.F. performed research; P.D.B. and T.L.T. contributed new reagents/analytic tools; X.M. and X.F. analyzed data; and X.M. and W.H.K. wrote the paper.

The authors declare no conflict of interest.

<sup>†</sup>To whom correspondence may be addressed at: Department of Biochemistry and Molecular Biology, Unit 1000, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: xiumu@mdanderson.org or whklein@mdanderson.org.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0802627105/DCSupplemental](http://www.pnas.org/cgi/content/full/0802627105/DCSupplemental).

© 2008 by The National Academy of Sciences of the USA



**Fig. 1.** *Isl1*-deleted optic nerves and retinas have abnormal structures. (A) The optic nerve from *Isl1*-deleted (MT) eye is significantly thinner than the WT control. (B and C) Transmission electron microscopy on thin sections across the optic nerves. Compared with WT controls (B), the axons in the optic nerve from *Isl1*-deleted eyes (C) are undermyelinated and disorganized. Arrows point to empty or partially empty myelin enclosures. (D and E) Hematoxylin and eosin staining of retina sections reveal that there are fewer cells in the INL and GCL of *Isl1*-deleted retinas (E) than in WT controls (D).

that *Isl1* is required for the differentiation and survival of RGCs as well as for the formation of subpopulations of bipolar and amacrine cells. Alterations in gene expression show that *Isl1* indeed functions in parallel with *Pou4f2* and represents a distinct branch of the RGC GRN, which intersects with *Pou4f2* and is downstream of *Math5*. Our results predict the existence of additional RGC gene regulation branches. RGC-expressed genes are therefore subject to combinatorial regulation controlled by distinct transcription factors positioned upstream in the GRN.

## Results

**Structural Defects in Mature *Isl1*-Deleted Retinas.** We first generated a floxed *Isl1* allele (*Isl1<sup>lox</sup>*) by placing two loxP sites flanking exon 3, which encodes the second LIM domain [supporting information (SI) Fig. S1]. Crossing this allele with the retina-specific transgenic Cre line (*Six3-Cre*) efficiently deleted *Isl1* at E14.5 (Fig. S1). To determine whether retinal defects were associated with the deletion of *Isl1*, we first examined *Isl1<sup>lox/lox</sup>;Six3-Cre* eyes at postnatal day (P)16, when cell differentiation in the retina is completed. Eyes of *Isl1<sup>lox/lox</sup>;Six3-Cre* mice were slightly smaller than those of WT controls, and the optic nerves were significantly thinner (Fig. 1A). These results indicated that RGCs were defective in emitting axons. Transmission electron microscopy with cross-sections from P16 optic nerves showed major optic nerve defects in *Isl1*-deleted mice. In WT controls, axons within the optic nerve were well organized and myelinated (Fig. 1B). However, optic nerves from *Isl1<sup>lox/lox</sup>;Six3-Cre* mice were severely disrupted (Fig. 1C). The axons were significantly thinner, and most were not properly myelinated. For those that were, the myelin sheath was thinner with fewer, less tightly packed layers compared with controls. In addition, numerous empty myelin-sheath enclosures were observed that were devoid of axons (Fig. 1C). Because myelination requires the presence of axons (21), the absence of axonal structures in the empty myelin enclosures suggested that the axons initially formed but subsequently degenerated. We also observed partially filled enclosures in which axons were not surrounded tightly by myelin sheaths. These axons were likely in the process of being degraded.

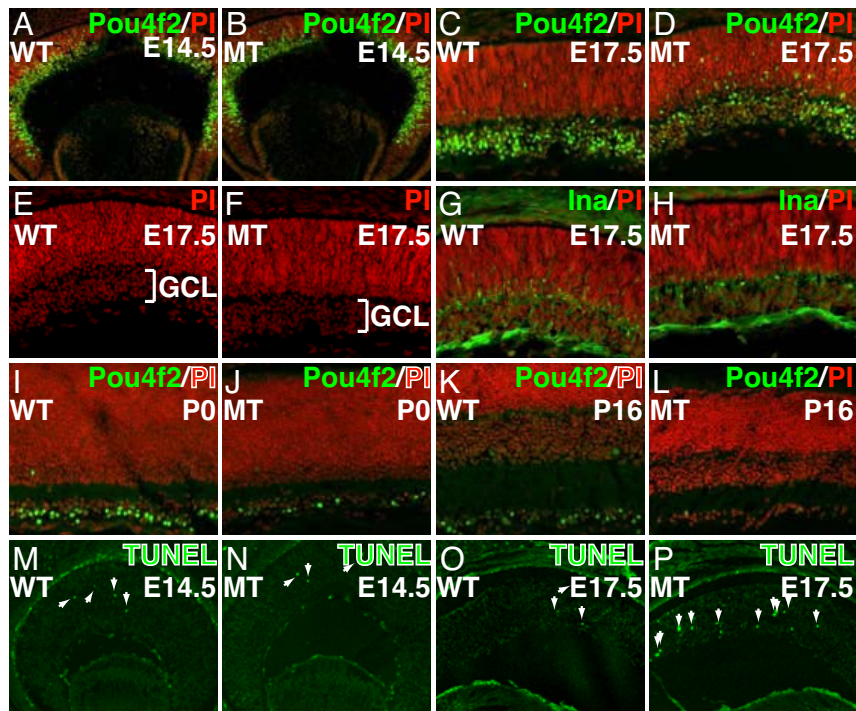
Histology of *Isl1<sup>lox/lox</sup>;Six3-Cre* retinas dissected from P16 mice showed that, although gross laminar organization was maintained, the *Isl1*-deleted retinas were nonetheless abnormal compared with the WT controls (Fig. 1D and E). In particular, the number of cells

in the inner nuclear layer (INL) and ganglion cell layer (GCL) were significantly reduced. As a result, the thickness of the *Isl1*-deleted INL was only half that of the WT control. The margins of the outer plexiform layer (OPL) were ragged, and the inner plexiform layer (IPL) was slightly thinner. However, the number and organization of the photoreceptor cells in the outer nuclear layer (ONL) appeared unaltered in *Isl1*-deleted retinas. These results suggested that cells residing in the INL and GCL in *Isl1<sup>lox/lox</sup>;Six3-Cre* retinas had defects in their development. *Isl1*-deleted retinas from mice 2 months of age showed no further degeneration (data not shown). Because *Isl1* is expressed in RGCs and in subsets of bipolar and amacrine cells, the defects observed in the GCL and INL were likely to be directly caused by the absence of *Isl1*.

**RGCs Form Normally in *Isl1*-Deleted Retinas but Are Lost at Later Stages.** Next, we performed cell type-specific marker analysis by immunofluorescence staining. We detected defects in the development of bipolar cells, amacrine cells, and ganglion cells but found no change in the numbers of cone and rod photoreceptors, horizontal cells, and Müller cells (Fig. 2 and Figs. S2–S4). We observed a significant reduction in on-bipolar cells and cholinergic amacrine cells (SI Text and Fig. S3). Similar results have been reported recently regarding the defects of these two cell types in *Isl1*-deleted retinas (22). We therefore focus our analysis on the role of *Isl1* on RGC development.

The morphological changes in the optic nerve and GCL caused by the loss of *Isl1* were likely to reflect defects in RGC development. *Pou4f2* is an early marker of RGCs, and its expression mirrors the status of RGC differentiation. At E14.5, the number of RGCs expressing *Pou4f2* was the same in *Isl1*-deleted as in WT control retinas (Fig. 2A and B), and the expression levels of both *Pou4f2* protein and mRNA were unchanged (Fig. 2A and B and Fig. 3G and H). In *Isl1*-deleted retinas, *Pou4f2*-expressing RGCs were mostly located toward the inner side of the retina where the GCL was forming, suggesting that RGCs were being born and migrating normally (Fig. 2A and B). At E14.5, *Isl1*-deleted retinas were the same size as retinas of WT controls. The normal birth of RGCs in *Isl1*-deleted retinas was also demonstrated by the unaltered expression of neurofilament middle chain (*Nefm*, also known as NF160), synuclein  $\gamma$  (*Sncg*, also known as *persyn*), and internexin  $\alpha$  (*Ina*, also known as *Nf66*) (Fig. 3A, B, I, J, M, and N). The normal appearance of these markers at E14.5 demonstrates that *Isl1* is not required for the initial specification of RGC fate.

At E17.5, the number of RGCs remained unchanged in *Isl1*-deleted retinas as revealed by the cells expressing the RGC markers *Pou4f2* and *Ina* (Fig. 2C, D, G, and H). Additionally, the thickness of GCL did not change as demonstrated by nuclear staining (Fig. 2E and F). However, within most RGCs, the expression of *Pou4f2* was substantially reduced, and only a few newly formed RGCs located in and near the neuroblast layer and at the distal retina expressed *Pou4f2* at levels comparable with those of controls, whereas the expression level of *Ina* did not change (Fig. 2C, D, G, and H). This suggested that the reduced level of *Pou4f2* at E17.5 was not due to the reduced fitness of RGCs. Because the absence of *Isl1* did not affect the expression of *Pou4f2* at E14.5, the decreased expression of *Pou4f2* at E17.5 suggests that *Isl1* is required for the maintenance of *Pou4f2* expression rather than for its initial activation. Supporting this notion, we found that the expression of *Nefm*, whose expression depends on *Pou4f2*, did not change at E14.5 in *Isl1*-deleted retinas (Fig. 3A and B) but was down-regulated at E17.5 (data not shown). At E17.5, we observed many more *Pou4f2*-expressing RGCs located in the neuroblast layer in *Isl1*-deleted retinas than were observed in WT controls (Fig. 2C and D). Because newly born RGCs arise from progenitors within the neuroblast layer, these results imply that the production of RGCs is prolonged in the absence of *Isl1*. RGCs regulate their own



**Fig. 2.** RGCs are born normally but lost at later stages in *Isl1*-deleted retinas. Development of RGCs was examined by anti-Pou4f2 or anti-Ina antibody staining (green) with WT (WT) and mutant (MT) retinas at different developmental stages. Red is nuclear staining by propidium iodide (PI). (A–H) There are equivalent numbers of RGCs at E14.5 (A and B) and E17.5 (C–H) in *Isl1*-deleted and control retinas, and they migrate to the GCL normally. PI staining shows that the GCLs (E and F, indicated by half brackets) in the WT and mutant retina are the same thickness. At E17.5, most RGCs express Pou4f2 at a lower level (D) than in the control (C), but they express Ina at an equivalent level (G and H). (I–L) At P5 and P16, the number of RGCs was dramatically reduced (J and L) compared with the control (I and K). (M and N) At E14.5, an equivalent number of apoptotic cells (green, indicated by white arrowheads) is seen in WT and *Isl1*-mutant retinas. (O and P) At 17.5, significantly more apoptotic cells are seen in *Isl1*-deleted retinas compared with that of WT controls; most of the apoptotic cells are located in or near the GCL (P).

production through feedback mechanisms mediated by secreted factors (23, 24). The increased number of RGCs in the neuroblast layer in *Isl1*-deleted retinas may reflect a role for *Isl1* in this feedback process.

Although there were no changes in the number of RGCs in *Isl1*-deleted retinas during the early stages of retinogenesis, there was a significant reduction at later stages. At P5, compared with WT controls, only  $28 \pm 4\%$  ( $n = 3$ ,  $P < 0.001$ ) of Pou4f2-expressing RGCs remained in the *Isl1*-deleted retinas (Fig. 2 I and J). The reduction in RGCs was even more apparent at later stages, and by P16,  $5 \pm 2\%$  ( $n = 3$ ,  $P < 0.001$ ) of Pou4f2-expressing RGCs remained (Fig. 2 K and L). Normal specification of RGCs and their gradual loss at later stages was further confirmed by using an anti-Nefm antibody (Fig. 3 A and B and Fig. S2 A and F). The loss of RGCs at later stages was consistent with the reduced thickness of optic nerves at P16 in *Isl1*-deleted mice. Because the majority of RGCs are born between E12 and E17, the reduction in RGC number could not have been caused by decreased RGC production after E17.5. More likely is that normal numbers of RGCs were produced initially, but most of them died subsequently. TUNEL analysis to determine the extent of apoptosis showed that although there were similar numbers of apoptotic cells in *Isl1*-deleted and WT retinas at E14.5 ( $n = 3$ ,  $P = 0.5$ ), significantly more apoptotic cells were found in *Isl1*-deleted retinas at E17.5 ( $n = 3$ ,  $P < 0.001$ ) (Fig. 2 M–P and Fig. S5). Most of the dying cells were located in or near the GCL (Fig. 2P). Thus, *Isl1* is not required for the specification of RGCs but for their differentiation and survival.

**Identification of *Isl1*-Dependent Genes in RGC Development.** Because *Isl1* is a well described transcription factor, the loss of RGCs in

*Isl1*-deleted retinas should correlate with corresponding alterations in RGC gene expression. To determine the genes that *Isl1* regulates, we performed a microarray analysis to compare global gene expression in WT and *Isl1*-null retinas at E14.5 using retinal cDNA microarrays that we have described (8, 15, 25). In addition, we performed *in situ* hybridization and immunofluorescence staining to confirm the microarray results (Fig. 3) and examined additional RGC-expressed genes not represented in the cDNA microarrays (Fig. 3 and Table S1). We chose E14.5 because there was no obvious RGC loss in *Isl1*-null retinas at this stage; alterations in gene expression in the mutant retinas were thus likely to be the direct result of the absence of *Isl1*. We identified 63 genes whose expression changed at least by 1.45-fold in *Isl1*-deleted retinas, including 45 down-regulated genes and 19 up-regulated genes (Table S1). The *Isl1*-dependent genes encode proteins of all functional classes, analogous to what we had observed for Pou4f2-dependent genes. These classes included neuronal integrity and function (*Gap43*, *Stemn2*, *Nefl*, *Mapt*, *Syt4*, *Syt13*), signaling (*Shh*, *Fgf15*, *Gdf8*), transcription factors (*Ebf*, *Zfp704*, *Nr0b1*, *Isl2*, *Eomes*), and genes required for cell cycle progression (*cyclin D1*) (Fig. 3 and Table S1).

Similar to Pou4f2-dependent genes, the largest functional class of *Isl1*-dependent genes was represented by those encoding proteins for neuronal integrity and function. This includes neuronal cytoskeletal proteins and other proteins with known neuronal functions. It is therefore likely that the down-regulated expression of these genes was the cause of defective RGC differentiation and subsequent cell death. *Gdf8* and *Shh* are regulated by Pou4f2 (15) and are involved in the non-cell-autonomous function of RGCs, in which RGCs regulate their own production and RPC proliferation (20, 24). As expected,





intermediate-level transcription factor in the RGC GRN. *Eomes*-deleted retinas exhibit defects in RGC differentiation that are less severe than those seen in *Pou4f2*<sup>-/-</sup> retinas (31), implying that the higher tier branches of the RGC GRN are further divided into multiple subbranches.

## Materials and Methods

**Gene Targeting and Mouse Breeding.** A gene-targeting construct was generated by recombining by following the procedure of Liu *et al.* (32). By gap repair, a 7.9-kb fragment of the *Isl1* gene was cloned from a BAC clone (RP24-127E11) into a targeting vector containing a TK cassette. A loxP site and a Neo cassette flanked by two FRT sites and one loxP site were inserted sequentially into the second and third introns, respectively, by homologous recombination (see Fig. S1A for details). The targeting construct was linearized with Pacl and electroporated into the G4 129xC57BL/6 F1 hybrid ES line (33), and G418 and FIAU double-resistant clones were genotyped by Southern blot hybridization (Fig. S1B). Two positive clones were expanded and injected into albino C57BL/6 blastocysts to generate chimeric mice, which were further mated for germ-line transmission. Positive offspring were mated with the FLPer mice (34) to delete the Neo cassette (Fig. S1A).

All mice were bred onto a 129 × C57BL/6 background. In all experiments using mice, the U. S. Public Health Service Policy on Humane Care and Use of Laboratory Animals was followed.

**Genotyping *Isl1* Alleles.** Southern blot hybridization on BamHI-digested genomic DNA was performed to identify targeted positive clones with an external probe (Fig. S1A). The probe detected a 10-kb band in the WT allele and a 6-kb band in the targeted allele (Fig. S1B). PCR was used to detect the deletion of the Neo cassette and to genotype the established *Isl1* alleles. The PCR conditions and primers used are listed in *SI Text*.

**Histology.** Embryos or adult retinas were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 7 μm. The sections were dewaxed and stained with hematoxylin and eosin as described (20).

**Immunofluorescence, TUNEL Assay, and Cell Counting.** The immunofluorescence procedure and most antibodies used in this study have been described in our

previous studies (19, 20). Antibodies that were first used by our group are listed in *SI Text*. TUNEL assay was performed with the ApoTag plus fluorescein *in situ* apoptosis detection kit (Chemicon) by following the manufacturer's protocol. Positive cells were counted on arbitrary length unit on a computer screen, and three sections were counted for each genotype. Student *t* test with equal variance was used to determine the significance of the difference observed.

**In Situ Hybridization.** *In situ* hybridization with digoxin-labeled riboprobes was carried out on 7 μM paraffin sections as described (15, 19).

**Transmission Electron Microscopy.** As described (20), P16 optic nerves were dissected from euthanized P16 mice, fixed, and embedded in EPON medium. Ultrathin sections across the optic nerve were cut 3 mm from the optic disk, stained with uranyl acetate and lead citrate, and examined in a JEM 1010 transmission electron microscope (JEOL).

**Microarray Hybridization, Data Acquisition, and Data Analysis.** RNA isolation and aRNA amplification from E14.5 WT and *Isl1*-null retinas was performed as described (8, 15), and four WT/mutant pairs of aRNA samples were obtained. cDNA microarray slides containing 18,816 mouse retinal cDNA clones (8, 15) were used for hybridization. Each pair of probes was used twice, and a total of eight hybridization reactions were performed.

Microarray data were acquired and analyzed as described (8, 15). One-way ANOVA was used to identify significant changes ( $P < 0.01$ , average fold change  $> 1.45$ ) in gene expression. The final gene list was generated by manual inspection to eliminate redundancy and annotate previously unknown genes.

**ACKNOWLEDGMENTS.** We acknowledge the excellent technical support from Hongxia Sun, Allison Chin, and Mark Russell. The *Isl1* conditional allele was generated with the help of the Genetically Engineered Mouse facility, and the TEM analysis was carried out at the High Resolution Electron Microscopy Facility at University of Texas M. D. Anderson Cancer Center. Both facilities are supported in part by National Cancer Institute Grant CA16672. This work was supported by a grant from the E. Matilda Ziegler Foundation for the Blind and an Institutional Research Support grant (to X.M.), by National Eye Institute Grants EY011930 and EY010608 (to W.H.K.), and by Robert A. Welch Foundation Grant G-0010.

- Mu X, Klein WH (2004) A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. *Semin Cell Dev Biol* 15:115–123.
- Livesey FJ, Cepko CL (2001) Vertebrate neural cell-fate determination: Lessons from the retina. *Nat Rev Neurosci* 2:109–118.
- Silver SJ, Rebay I (2005) Signaling circuitries in development: Insights from the retinal determination gene network. *Development* 132:3–13.
- Marquardt T, *et al.* (2001) Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 105:43–55.
- Wang SW, *et al.* (2001) Requirement for math5 in the development of retinal ganglion cells. *Genes Dev* 15:24–29.
- Brown NL, Patel S, Brzezinski J, Glaser T (2001) Math5 is required for retinal ganglion cell and optic nerve formation. *Development* 128:2497–2508.
- Yang Z, *et al.* (2003) Math5 determines the competence state of retinal ganglion cell progenitors. *Dev Biol* 264:240–254.
- Mu X, *et al.* (2005) A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. *Dev Biol* 280:467–481.
- Gan L, Wang SW, Huang Z, Klein WH (1999) POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification. *Dev Biol* 210:469–480.
- Wang SW, Gan L, Martin SE, Klein WH (2000) Abnormal polarization and axon outgrowth in retinal ganglion cells lacking the POU-domain transcription factor Brn-3b. *Mol Cell Neurosci* 16:141–156.
- Rachel RA, *et al.* (2002) Spatiotemporal features of early neurogenesis differ in WT and albino mouse retina. *J Neurosci* 22:4249–4263.
- Elshatory Y, Deng M, Xie X, Gan L (2007) Expression of the LIM-homeodomain protein *Isl1* in the developing and mature mouse retina. *J Comp Neurol* 503:182–197.
- Austin CP, Feldman DE, Ida JA, Jr, Cepko CL (1995) Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* 121:3637–3650.
- Edqvist PH, Myers SM, Hallbook F (2006) Early identification of retinal subtypes in the developing, pre-laminated chick retina using the transcription factors Prox1, Lim1, Ap2alpha, Pax6, *Isl1*, *Isl2*, *Lim3* and *Chx10*. *Eur J Histochem* 50:147–154.
- Mu X, *et al.* (2004) Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. *Development* 131:1197–1210.
- Lin L, *et al.* (2006) *Isl1* is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. *Dev Biol* 295:756–763.
- Pfaff SL, *et al.* (1996) Requirement for LIM homeobox gene *Isl1* in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* 84:309–320.
- Galli-Resta L, Resto G, Tan SS, Reese BE (1997) Mosaics of islet-1-expressing amacrine cells assembled by short-range cellular interactions. *J Neurosci* 17:7831–7838.
- Fu X, Sun H, Klein WH, Mu X (2006) Beta-catenin is essential for lamination but not neurogenesis in mouse retinal development. *Dev Biol* 299:424–437.
- Mu X, *et al.* (2005) Ganglion cells are required for normal progenitor-cell proliferation but not cell-fate determination or patterning in the developing mouse retina. *Curr Biol* 15:525–530.
- Michailov GV, *et al.* (2004) Axonal neuregulin-1 regulates myelin sheath thickness. *Science* 304:700–703.
- Elshatory Y, *et al.* (2007) *Isl1* controls the differentiation of retinal bipolar and cholinergic amacrine cells. *J Neurosci* 27:12707–12720.
- Kim J, *et al.* (2005) GDF11 controls the timing of progenitor cell competence in developing retina. *Science* 308:1927–1930.
- Wang Y, *et al.* (2005) Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* 132:5103–5113.
- Mu X, *et al.* (2001) Gene expression in the developing mouse retina by EST sequencing and microarray analysis. *Nucleic Acids Res* 29:4983–4993.
- de Melo J, *et al.* (2005) *Dlx1* and *Dlx2* function is necessary for terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina. *Development* 132:311–322.
- Pan L, Yang Z, Feng L, Gan L (2005) Functional equivalence of Brn3 POU-domain transcription factors in mouse retinal neurogenesis. *Development* 132:703–712.
- Wang SW, *et al.* (2002) Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth. *Development* 129:467–477.
- Schneider ML, Turner DL, Vetter ML (2001) Notch signaling can inhibit *Xath5* function in the neural plate and developing retina. *Mol Cell Neurosci* 18:458–472.
- Visel A, Thaller C, Eichele G (2004) GenePaint.org: An atlas of gene expression patterns in the mouse embryo. *Nucleic Acids Res* 32:D552–D556.
- Mao CA, *et al.* (2008) *Eomesodermin*, a target gene of *Pou4f2*, is required for retinal ganglion cell and optic nerve development in the mouse. *Development* 135:271–280.
- Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* 13:476–484.
- George SH, *et al.* (2007) Developmental and adult phenotyping directly from mutant embryonic stem cells. *Proc Natl Acad Sci USA* 104:4455–4460.
- Farley FW, Soriano P, Steffen LS, Dymecki SM (2000) Widespread recombinase expression using FLP<sub>er</sub> (flipper) mice. *Genesis* 28:106–110.