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

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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*$^{-/-}$ 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*$^{-/-}$ and *Pou4f2*$^{-/-}$ retinas, we identified transcription-factor genes whose expression was substantially reduced in *Math5*$^{-/-}$ but not *Pou4f2*$^{-/-}$ 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$^{-/-}$ 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

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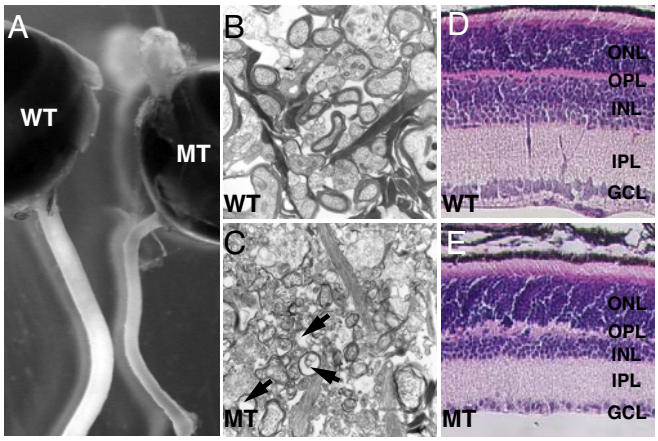

Fig. 1. *Isl1*-deleted optic nerves and retinas have abnormal structures. (*A*) The optic nerve from *Isl1*-deleted (MT) eye is significantly thinner than the WT control. (*B* and *C*) Transmission electron microscopy on thin sections across the optic nerves. Compared with WT controls (*B*), the axons 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 ($Isl1^{flox}$) 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 $Isl1^{flox/flox}$;*Six3-Cre* eyes at postnatal day (P)16, when cell differentiation in the retina is completed. Eyes of $Isl1^{flox/flox}$;*Six3-Cre* mice were slightly smaller than those of WT controls, and the optic nerves were significantly thinner (Fig. 1*A*). 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. 1*B*). However, optic nerves from $Isl1^{flox/flox}$;*Six3-Cre* mice were severely disrupted (Fig. 1*C*). 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. 1*C*). 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 $Isl1^{flox/flox}$;*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. 1 *D* 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 $Isl1^{flox/flox}$;*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, amarine 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. 2 *A* and *B*), and the expression levels of both Pou4f2 protein and mRNA were unchanged (Fig. 2 *A* and *B* and Fig. 3 *G* 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. 2 *A* 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 γ (Sncg, also known as persyn), and internexin α (Ina, also known as Nf66) (Fig. 3 *A*, *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. 2 *C*, *D*, *G*, and *H*). Additionally, the thickness of GCL did not change as demonstrated by nuclear staining (Fig. 2 *E* 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. 2 *C*, *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. 3 *A* 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. 2 *C* 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

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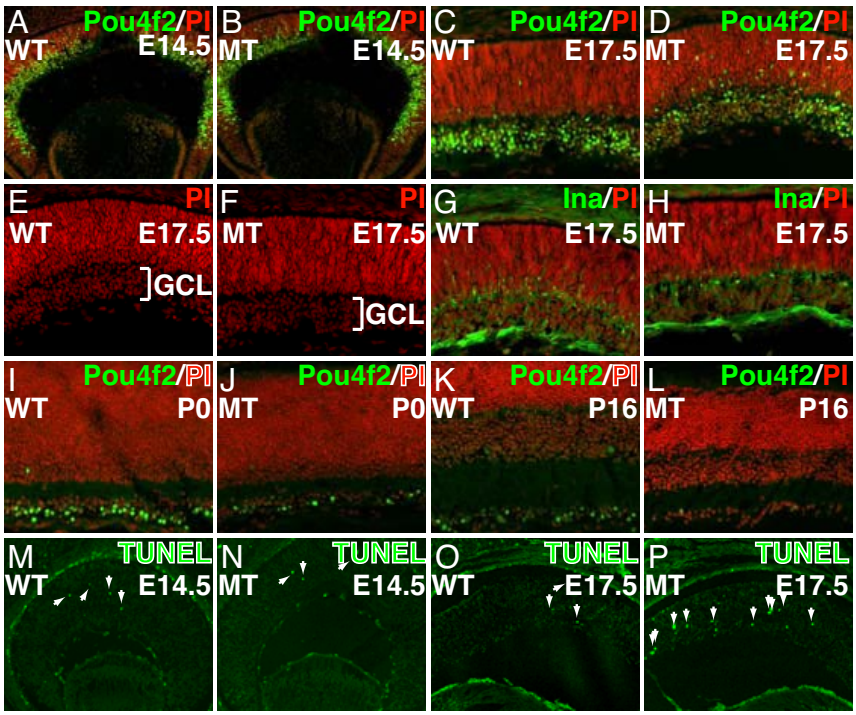

Fig. 2. RGCs are born normally but lost at later stages in *Isl1*-deleted retinas. Development of RGCs was examined by anti-Pou4f2 or anti-Ina antibody staining (green) with WT (WT) and mutant (MT) retinas at different developmental stages. Red is nuclear staining by propidium iodide (PI). (*A–H*) There 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 ± 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 ± 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. 2*P*). 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,

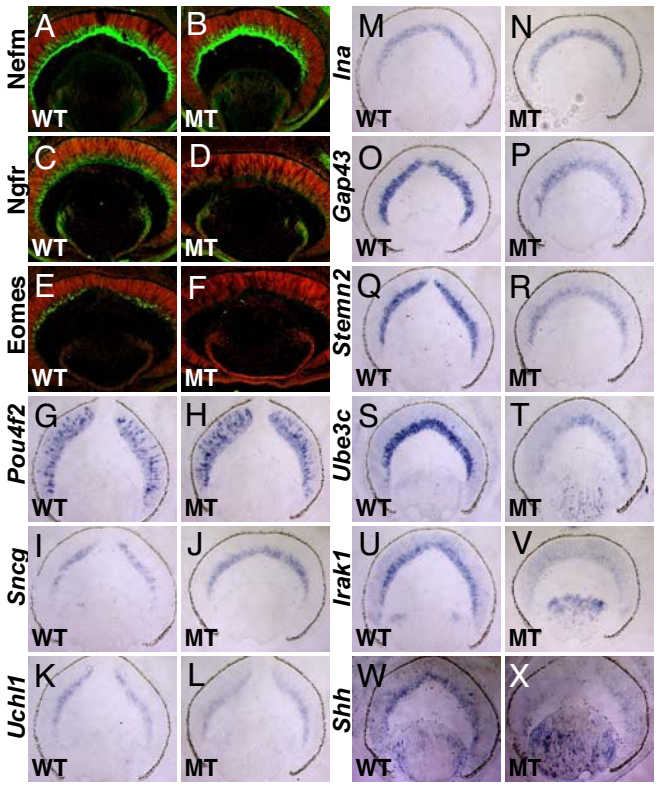

Fig. 3. Expression of a set of RGC genes revealed by immunofluorescence and *in situ* hybridization. Isl1 regulates a subset of RGC genes distinct from but overlapping with those that depend on Pou4f2. The expression of RGC genes was examined by immunostaining or *in situ* hybridization on E14.5 WT and mutant retinal sections. Some genes (*C–F* and *O–X*) are down-regulated in *Isl1*-deleted retinas, and others do not change (*A*, *B*, and *G–N*); some of these genes (*A*, *B*, *E*, *F*, *I*, *J*, *O*, *P*, *S*, *T*, *W*, and *X*) depend on Pou4f2 for their expression, whereas others (*C*, *D*, *K–N*, *Q*, *R*, *U*, and *V*) do not.

Gli1 and *Cyclin D1*, two genes that are targets of Shh signaling in RPCs (20), were also down-regulated in *Isl1*-deleted retinas. These findings indicate that Isl1 also participates in this non-cell-autonomous function and are consistent with the prolonged production of RGCs at E17.5 in *Isl1*-null retinas (Fig. 2*D*). Perhaps most notable, Isl1 regulates the expression of other transcription-factor genes, indicating that, like Pou4f2, Isl1 confers its function in part by activating transcription-factor genes at lower tiers in the RGC GRN.

We also identified 19 genes whose expression was up-regulated in *Isl1*-null retinas (Table S1). Among them was *Dlx1* (Fig. 4), a transcription factor gene that is repressed by Pou4f2 (15) and required for the development of a subset of RGCs (26). This suggests that both Isl1 and Pou4f2 function to repress *Dlx1* in RGC development. We also found that some genes were up-regulated in *Isl1*-null retinas but not in *Pou4f2*-null retinas. These include *Nhlh2*, *Sema3a*, and microRNA genes *Mirn124a-1* and *Mirn216a* (see Fig. 4 and Table S1). The finding that microRNA genes are subject to Isl1 regulation suggests that they are also an integral part of the RGC GRN.

Isl1 and Pou4f2 Define Distinct but Overlapping Gene Regulation Branches in the RGC GRN. Because *Isl1* is expressed in a spatio-temporal pattern highly similar to that of *Pou4f2* (11), and its expression depends on Math5 but is independent of Pou4f2 (8), it is possible that Isl1 regulates a set of Math5-dependent genes distinct from but overlapping with the gene set regulated by Pouf42. To determine the extent of overlap among these gene sets, we focused on the down-regulated genes, because most of these genes are expressed in RGCs and are likely to be directly downstream of Pou4f2 and Isl1 in the RGC GRN.

We have, to date, identified 156 and 50 down-regulated genes in *Math5*$^{-/-}$ and *Pou4f2*$^{-/-}$ retinas, respectively. We compared these genes with those down-regulated in the absence of Isl1 (Fig. 4). Virtually all RGC-expressed genes dependent on Isl1 also depended on Math5 (Fig. 4). However, 28 RGC-expressed genes, were identified that depended on both Isl1 and Pou4f2 (e.g., *Gap43*, *Eomes*, *Ube3c*, and *Shh*), implying that these genes are coregulated by both factors. In contrast, 17 genes depended only on Isl1 (e.g., *Irak1*, *Ngfr*, and *Nr0b1*), and 23 genes depended only on Pou4f2 (e.g., *Scng*, *Nefm*). These results demonstrate that Pou4f2 and Isl1 represent two distinct but interacting gene regulation branches in the RGC GRN. Notably, 88 (56%) of Math5-dependent RGC genes do not have altered expression in either *Isl1*- or *Pou4f2*-null retinas (e.g., *Ina*, *Ngfr*). We predict the expression of these genes depends on other transcription factors. As depicted in Fig. 5, RGC-expressed genes fall into four regulatory modes; some genes are regulated by both Pou4f2 and Isl1, some by Pou4f2 and not Isl1, some by Isl1 and not Pou4f2, and some by neither Pou4f2 nor Isl1.

Discussion

Our current study revealed an essential role for Isl1 in RGC differentiation and survival but not in the initial specification of RGC fate. The results also establish a requirement for Isl1 in the differentiation of on-bipolar and cholinergic amacrine cells. The role of Isl1 in RGC development is very similar to that of Pou4f2. In *Pou4f2*$^{-/-}$ retinas, RGCs are specified but most die at later stages (9, 10). However, there are two distinctions in the phenotypes of *Pou4f2*$^{-/-}$ and *Isl1*-deleted retinas. In *Isl1*-deleted retinas, the number of RGCs at E17.5 is comparable with WT retinas. In contrast, in *Pou4f2*$^{-/-}$ retinas, a significant reduction in RGC number is already apparent by E17.5 (27). Nevertheless, far fewer RGCs survive in *Isl1*-deleted retinas than in *Pou4f2*$^{-/-}$ retinas; in retinas of adult mice, 30% of RGCs survive in the absence of Pou4f2, whereas only 5% remain in the absence of Isl1. It is possible that loss of Pou4f2 contributes to RGC death in both mutants. In *Pou4f2*$^{-/-}$ retinas, Pou4f2 is never expressed. In contrast, in *Isl1*-deleted retinas, Pou4f2 expression is normal at the time when RGCs are born and diminishes only at later stages between E14.5 and E17.5, which coincides with the delayed onset of RGC loss. However, because both Pou4f2 and Isl1 are lost at later stages in *Isl1*-deleted retinas, the expression of more RGC genes is altered than in *Pou4f2*$^{-/-}$ retinas, and virtually all RGCs are lost by the adult stage. Compensation by the Pou4f2-related factors Pou4f1 and Pou4f3 may also contribute to the higher survival rate observed in *Pou4f2*$^{-/-}$ retinas compared to *Isl1*-deleted retinas (28). (S. Wang and W.H.K., unpublished results).

Our previous studies on gene regulation by Math5 and Pou4f2 suggested a hierarchical and complex regulatory network in RGC development (8, 15). Based on the differences in gene expression changes in *Math5*-null and *Pou4f2*-null retinas, we predicated the existence of independent regulatory branches parallel to the branch defined by Pou4f2 and proposed that Isl1 mediates one such branch. Our current study demonstrates that one principal function of Isl1 is to regulate the expression of a subset of RGC genes downstream of Math5. Isl1 is therefore a key transcription factor in the RGC GRN, functioning downstream of Math5 and parallel to Pou4f2 (8). These results not only confirmed the existence of this branch but also expanded our understanding of the network by revealing the extensive interaction of the Isl1 and Pou4f2 branches.

Thus, similar to Pou4f2, Isl1 is required for the expression of a subset of Math5-dependent genes and defines a distinct gene regulation branch in the RGC GRN. Nevertheless, the functions

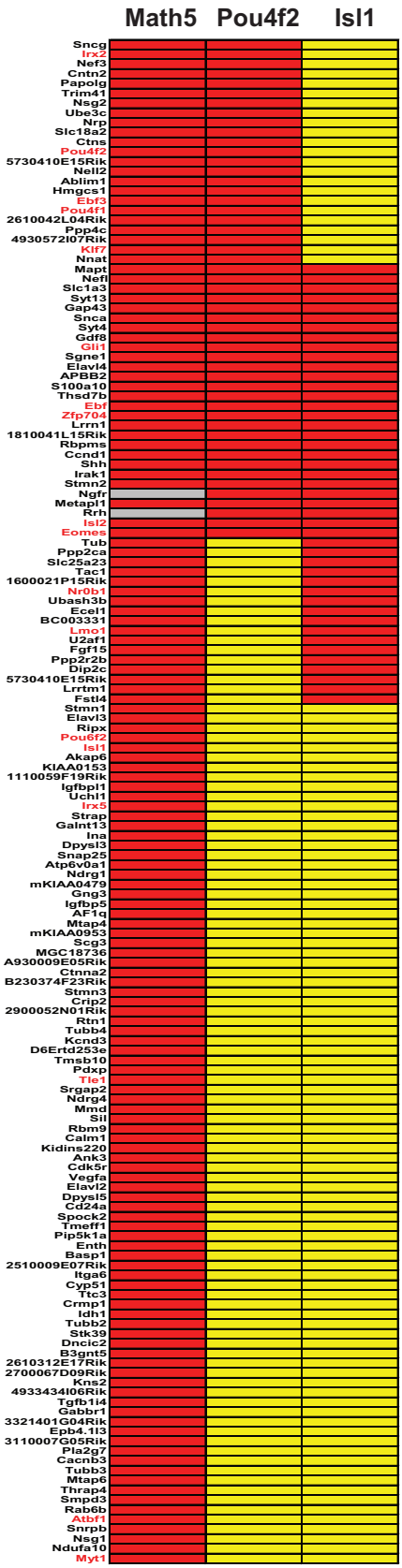

Fig. 4. Comparison of the three gene sets whose expression depends on Math5, Pou4f2, and Isl1. Gene symbols are listed on the left, and the 17 transcription-factor genes are highlighted in red. The three bars in each row represent the changes of each gene in the retinas of the three mutant genotypes (*Math5*-, *Pou4f2*-, and *Isl1*-null), respectively. Significant changes are indicated with red bars, yellow bars mean no significant changes, and gray bars indicate data not available. Note that Isl1 showed no change in *Isl1*-null retinas, probably because deletion of exon 3 did not affect its mRNA level.

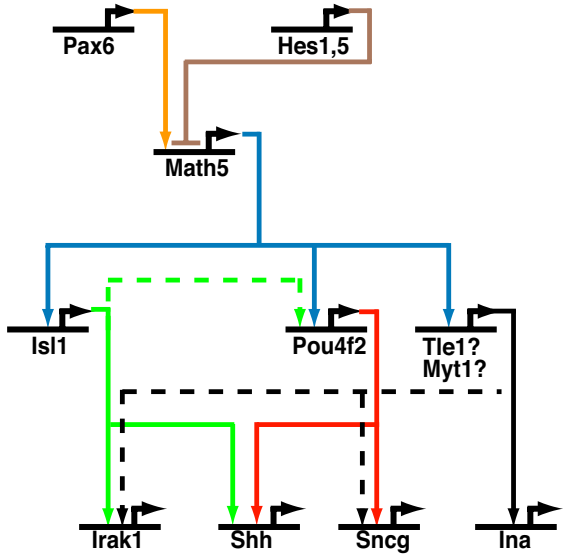

Fig. 5. A diagram depicting different gene regulation branches for RGC gene expression in the RGC GRN. Isl1 (green) and Pou4f2 (red) define two such parallel branches, which are both downstream of the retinal determination factors Pax6 and Notch (represented by its transcription factor effectors Hes1 and Hes5), and the RGC-specific regulator Math5. The Isl1 and Pou4f2 pathways interact by coregulating a subset of RGC-expressing genes. Isl1 may also regulate *Pou4f2* directly (green broken line). Other branch(es) (black line) mediated by other transcription factors such as Tle1 and Myt1 are postulated, and they may also interact with the Isl1 and Pou4f2 branches (represented by broken black lines). Individual RGC genes receive a distinct combination of upstream inputs from these branches to achieve RGC expression. One example each is provided for genes subject to the four possible combinations of upstream inputs.

of Isl1 and Pou4f2 are not completely independent of each other. Indeed, the expression of a significant number of Math5-dependent genes depends on both Pou4f2 and Isl1, suggesting that these downstream genes are coregulated by the two factors. On the other hand, many RGC-expressed genes depend on either Pou4f2 or Isl1 alone and still others are regulated by neither factor. Based on their relationships with Math5, Isl1, and Pou4f2, RGC-expressed genes fall into four regulatory modes (Fig. 5). In addition to regulating downstream genes, Isl1 appears to be required for the sustained expression of *Pou4f2* in differentiating RGCs after the down-regulation of *Math5* (Fig. 5), but further investigation is required to confirm this. We do not yet know whether Pou4f2 is required for the sustained expression of *Isl1*. Genes whose expression depends on Math5, but not on Isl1 or Pou4f2, are predicted to be regulated by other transcription factors. We propose that Myt1 and Tle1 are two such factors positioned at the same hierarchical tier as Pou4f2 and Isl1, based on their spatiotemporal expression (29, 30).

The regulatory modes depicted in Fig. 5 are defined by different combinations of upstream transcription factor inputs. It is likely that Isl1 and Pou4f2 act together within the regulatory regions of many RGC-expressing genes, along with other transcriptional regulators that are presently uncharacterized. The genes coregulated by Isl1 and Pou4f2 encode proteins from all functional classes, including genes encoding transcription factors. The T-box-containing transcription factor *Eomes* is activated later than *Pou4f2* in RGC differentiation and represents an

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*$^{-/-}$ 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 recombineering 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. S1*A* for details). The targeting construct was linearized with PacI 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. S1*B*). Two positive clones were expanded and injected into albino C57/BL6 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. S1*A*).

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. S1*A*). The probe detected a 10-kb band in the WT allele and a 6-kb band in the targeted allele (Fig. S1*B*). 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 ApopTag 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.

Mircoarray 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.

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