Development 139, 4644-4655 (2012) doi:10.1242/dev.081729

Regulation of retinal interneuron subtype identity by the *Iroquois* homeobox gene *Irx6*

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SUMMARY

Interneuronal subtype diversity lies at the heart of the distinct molecular properties and synaptic connections that shape the formation of the neuronal circuits that are necessary for the complex spatial and temporal processing of sensory information. Here, we investigate the role of *Irx6*, a member of the *Iroquois* homeodomain transcription factor family, in regulating the development of retinal bipolar interneurons. Using a knock-in reporter approach, we show that, in the mouse retina, *Irx6* is expressed in type 2 and 3a OFF bipolar interneurons and is required for the expression of cell type-specific markers in these cells, likely through direct transcriptional regulation. In *Irx6* mutant mice, presumptive type 3a bipolar cells exhibit an expansion of their axonal projection domain to the entire OFF region of the inner plexiform layer, and adopt molecular features of both type 2 and 3a bipolar cells, highlighted by the ectopic upregulation of neurokinin 3 receptor (Nk3r) and Vsx1. These findings reveal *Irx6* as a key regulator of type 3a bipolar cell identity that prevents these cells from adopting characteristic features of type 2 bipolar cells. Analysis of the *Irx6;Vsx1* double null retina suggests that the terminal differentiation of type 2 bipolar cells is dependent on the combined expression of the transcription factors Irx6 and Vsx1, but also points to the existence of *Irx6;Vsx1*-independent mechanisms in regulating OFF bipolar subtype-specific gene expression. This work provides insight into the generation of neuronal subtypes by revealing a mechanism in which opposing, yet interdependent, transcription factors regulate subtype identity.

KEY WORDS: Retina, Transcription factor, Bipolar interneuron, Cell type diversity, Mouse

INTRODUCTION

The simple organization of the retina into five major neuronal classes makes it an excellent model for studying the developmental mechanisms that underlie cell type diversity (Ohsawa and Kageyama, 2008). The simple neuronal cell class organization of the retina, however, is accompanied by high degree of cell type heterogeneity. In mammals, the five major retinal neuronal classes give rise to 55 morphological cell types (Masland, 2001), which comprise functionally diverse circuits within the retina (Gollisch and Meister, 2010). The current literature defines 11 bipolar cell types based on morphology, gene expression and axonal tiling properties (Wässle et al., 2009) (see Fig. 1).

Combinatorial transcription factor coding based on the differential expression of homeodomain and basic helix-loop-helix transcription factors has been identified as a common mechanism underlying neuronal cell type diversity (Fode et al., 2000; Guillemot, 2007; Ma, 2006; Ohsawa and Kageyama, 2008; Shirasaki and Pfaff, 2002). Several homeodomain and basic helix-loop-helix transcription factors are important for the development and homeostasis of distinct retinal bipolar cell subtypes. The development of type 2 and 3 OFF bipolar cells is regulated by the overlapping requirements of several transcription factors (Cheng et al., 2005; Chow et al., 2004; Feng et al., 2006; Kerschensteiner et al., 2008; Ohtoshi et al., 2004). In mice lacking *Vsx1* or *Irx5* homeobox gene function, type 2 and 3 bipolar cells are specified but have defects in their terminal differentiation marked by reduced expression of type 2 and 3 cell-specific markers (Chow et al., 2004; Ohtoshi et al., 2004; Shi et al., 2012; Cheng et al., 2005). In *Vsx1*-mutant mice, expression of the type 2 cell markers recoverin (Rcvrn), neurokinin 3 receptor (Nk3r; Tacr3 – Mouse Genome Informatics) and Neto1 is reduced (Chow et al., 2004; Ohtoshi et al., 2004), type 3 cells have reduced Cabp5 immunolabeling in the axon terminal region (Chow et al., 2004), and there is a reduction in the total number of Hcn4-positive type 3a bipolar cells (Shi et al., 2012). In *Irx5* mutants, type 2 bipolar cells have reduced levels of recoverin immunolabeling, but normal levels of Nk3r immunolabeling, and type 3 cells exhibit reduced levels of Cabp5 within their axon terminals (Cheng et al., 2005). Mice lacking the basic helix-loop-helix transcription factor Bhlhb5 (Bhlhb22 – Mouse Genome Informatics) have a reduction of recoverin and Nk3r immunolabeled type 2 cells, and also have a reduction in the total number of Vsx1-labeled bipolar cells (Feng et al., 2006). Although *Vsx1* is not necessary for Irx5 bipolar cell expression (Cheng et al., 2005), it negatively regulates its own expression (Chow et al., 2004) and is a positive regulator of Bhlhb5 (M.Z. and R.L.C., unpublished). Conversely, *Bhlhb5* functions as a positive regulator of Vsx1 in putative type 2 cells (Feng et al., 2006).

In the present study, we investigate the role of the Iroquois family member *Irx6* in retinal development. The Iroquois (Irx) gene family encodes transcription factors that harbor a 63 amino acid TALE family homeodomain and a 9 amino acid conserved motif outside of the homeodomain known as the Iro box (Bilioni et al., 2005). Mammals have six *Irx* genes that exist in two clusters: the IrxA cluster (*Irx1*, *Irx2* and *Irx4*) and IrxB cluster (*Irx3*, *Irx5* and *Irx6*) (Gómez-Skarmeta and Modolell, 2002). Studies in both

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Accepted 12 September 2012

Drosophila and vertebrate models have implicated Iroquois genes in axon targeting events (Grillenzoni et al., 1998; Jin et al., 2003; Sato et al., 2006).

We show that *Irx6* is expressed in type 2 and 3a bipolar cells where it is required for the expression of cell type-specific markers, including Bhlhb5. Presumptive type 3a cells in the *Irx6* mutant stratify correctly to the OFF sublamina of the inner plexiform layer, but instead of the normal restricted segregation of their axon termini to sublamina 2, they can stratify within both OFF sublamina 1 and 2. Furthermore, these cells adopt molecular features of both type 2 and 3a cells, highlighted by the ectopic expression of Vsx1 and Nk3r. These defects in type 3a cell development in the *Irx6* mutant appear to be independent of *Vsx1* function. Our findings reveal *Irx6* as a core regulator of type 3a bipolar cell identity that prevents them from adopting features characteristic of type 2 bipolar cells, and suggest that terminal differentiation of type 2 bipolar cells is dependent on the combined activity of *Vsx1* and *Irx6*.

MATERIALS AND METHODS

Generation of *Irx6^{lacZ}* knock-in mice

Gene targeting was performed in R1 (Svj129) embryonic stem (ES) cells. The targeting construct was generated by screening an M13 mouse R1 genomic library (Stratagene) with probes for *Irx6* (supplementary material Fig. S1). Chimeric mice were generated by blastocyst injection of homologous recombinant ES cells (Scripps Research Institute Transgenic Core Facility), and the resulting mice were bred with the 129S1/SvImJ mouse strain (Jackson Laboratory). All experiments on mice were conducted with the approval of the University of Victoria Animal Care Committee.

Immunohistochemistry and microscopy

Unless otherwise noted, all retinas were taken from 2- to 3-month-old mice, and central sections of the retina were used for imaging. Eyes were fixed in 4% paraformaldehyde in PBS for 1 hour (on ice) or for 20 minutes (room temperature), cryoprotected in 30% sucrose, embedded in Tissue-Tek OCT (Sakura Finetek), and cryosectioned at 14 μm. Primary antibody information is given in supplementary material Table S1. Secondary antibodies were conjugated to Alexa dyes (Invitrogen) or Cy3 (anti-guinea pig; Jackson ImmunoResearch). Draq5 (Alexis Biochemicals) was used for nuclear labeling. For detection of β-galactosidase activity, sections were incubated in 1 mg/ml bromo-chloro-indolyl-galactopyranoside (X-gal) overnight at 37°C, as previously described (Mombaerts et al., 1996). Images were taken on a confocal microscope (Nikon Eclipse TE2000-U C1) and processed using Adobe Photoshop CS3. Cell counting and measurement of the fluorescence intensity within a 100 × 10 μm region of interest was carried out using EZ-C1 Version 3.60 software (Nikon Instruments). Data are presented as mean±s.e.m. Statistical analysis was performed using Student's *t*-test.

PCR genotyping

The genomic DNA was prepared from mice ear clips. The following primers were used for *Irx6* genotyping: IRX6-PF (GGCGGCCTGCTCCT-GCAGCCC), IRX6-IR (GGATGTGCTGCCATACGGGTGT) and LacZR (AGATGAAACGCCGAGTTAACGC). The *Vsx1* mutant mouse (AltB5 or Δ1-4 line) has been previously described (Chow et al., 2004).

Luciferase assay

HEK cells were transfected and assayed 24 hours later for luciferase activity with Dual-Glo luciferase assay system (Promega) according to the manufacturer's protocol. Luciferase activity was measured by a Wallac 1420 Multilabel Counter (PerkinElmer). The vectors used in the assay are described in supplementary material Table S2.

Electroretinography

Retinal function of mice from four experimental groups (*Irx6$^{+/lacZ}$;Vsx1$^{+/AltB5}$*, *Vsx1$^{AltB5/AltB5}$*, *Irx6$^{+/lacZ}$* and *Irx6$^{lacZ/lacZ}$*) was studied using the full field electroretinogram (ERG) as previously described (Alvarez et al., 2007). Light stimulation (10 μsecond duration flashes), signal amplification (0.3-300 Hz bandpass) and data acquisition were provided by the Espion E^2 system (Diagnosys). Scotopic intensity responses consisted of single flash presentations at 19 increasing strengths from −5.22 to 2.86 log cds/m^2. Ten minutes after transition from dark to light (30 cd/m^2 white light background) adaptation, photopic intensity responses (30 cd/m^2 background light) were recorded at 11 increasing flash strengths ranging from −1.6 to 2.9 log cds/m^2, followed by OFF responses obtained with a square wave stimulus of 562.5 cd/m^2, lasting 800 mseconds.

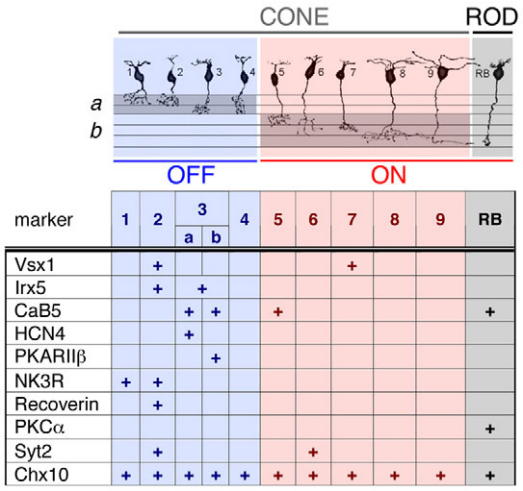

Fig. 1. Bipolar cell type-specific markers used in this study. Morphological cell types [adapted with permission from Ghosh et al. (Ghosh et al., 2004)]. Markers used are: Vsx1 (Chow et al., 2001; Chow et al., 2004; Shi et al., 2011); Irx5 (Cheng et al., 2005); CaB5 (Haverkamp et al., 2003; Ghosh et al., 2004); Hcn4 and PKARII-β (Matargua et al 2007); Nk3r (Haverkamp et al., 2003; Ghosh et al., 2004); recoverin (Haverkamp et al., 2003); PKCα (Negishi et al., 1988; Haverkamp and Wassle, 2000); Syt2 (Wassle, 2009); Chx10 (Burmeister et al., 1996).

RESULTS

Irx6 reporter expression in the developing and mature mouse retina

To investigate the role of *Irx6* in mouse retinal development, we generated a knock-in *lacZ* reporter allele (*Irx6^{lacZ}*) in which *Irx6* exon 1 and part of exon 2 was replaced by the *lacZ* gene (supplementary material Fig. S1). Whole-mount X-gal detection of the *lacZ* gene product, β-galactosidase in *Irx6$^{+/lacZ}$* mice at embryonic day 12.5 (E12.5) revealed scattered expression of the reporter throughout the embryo (Fig. 2B). X-gal staining was first detected in the central ganglion cell region of the retina in *Irx6$^{+/lacZ}$* mice beginning at embryonic day 13.5 (E13.5) (Fig. 2D). Owing to the lack of a suitable antibody, we were unable to confirm reporter expression using Irx6 immunohistology. However, the onset and localization of the reporter expression is consistent with the previously described expression of *Irx6* mRNA in the retina (Cohen et al., 2000; Erkman et al., 2000) (supplementary material Fig. S1), suggesting that the knock-in *lacZ* reporter faithfully recapitulates the normal *Irx6* expression pattern. By E16.5, there was strong expression of the *Irx6^{lacZ}* reporter in the developing retinal ganglion cell layer and optic nerve region (Fig. 2E). At postnatal day zero (P0), X-gal staining persisted in the ganglion cell layer, and was also observed in the apical margin region of the retina (Fig. 2G). By P7, reporter expression was detected in a

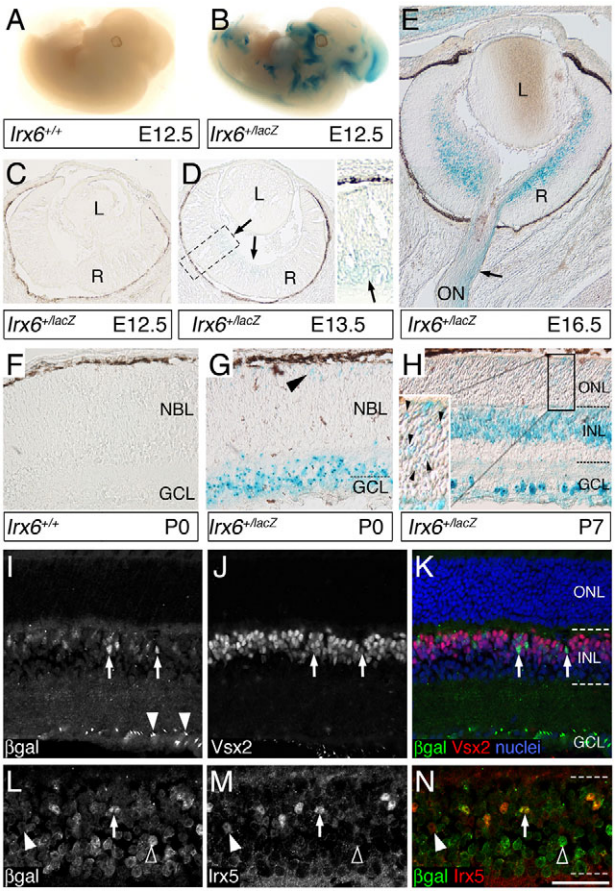

Fig. 2. Expression of the *Irx6*:βgal reporter during development of the mouse. (A-H) X-gal staining results. (**A**,**B**) E12.5 wild-type (A) and *Irx6*$^{+/lacZ}$ (B) mouse, showing expression of *Irx6*:βgal in the midbrain. (**C**,**D**) Retinal section from an *Irx6*$^{+/lacZ}$ mouse at E12.5 (C) and E13.5 (D); arrows in D indicate faint X-gal precipitate. (**E**) E16.5 retinal section from an *Irx6*$^{+/lacZ}$ mouse showing staining down the optic nerve. (**F-H**) Wild type (F) and *Irx6*$^{+/lacZ}$ (G,H) retina at P0 (F,G) and P7 (H), with arrowheads (G and H, inset) indicating staining in the newly formed ONL. (**I**,**J**) Immunohistochemistry of an *Irx6*$^{+/lacZ}$ retina showing *Irx6*:βgal expression (I, arrows) and Vsx2/Chx10 expression (J, arrows). Arrowheads in I indicate *Irx6*:βgal expression in the GCL. (**K**) Colocalization of *Irx6*:βgal and Vsx2/Chx10 (arrows). The nuclear stain is Draq5. (**L**,**M**) *Irx6*:βgal (L, arrow) and Irx5 (M, closed arrowhead) expression in the *Irx6*$^{+/lacZ}$ retina. (**N**) An Irx5-positive, *Irx6*:βgal-negative cell (closed arrowhead); an Irx5-positive, *Irx6*:βgal-positive cell (arrow); and an Irx5-negative, *Irx6*:βgal-positive cell (open arrowhead). L, lens; R, retina; ON, optic nerve; NBL, neuroblast layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 3 mm in A,B; 60 μm in C,D; 100 μm in E; 50 μm in F-K; 25 μm in L-N.

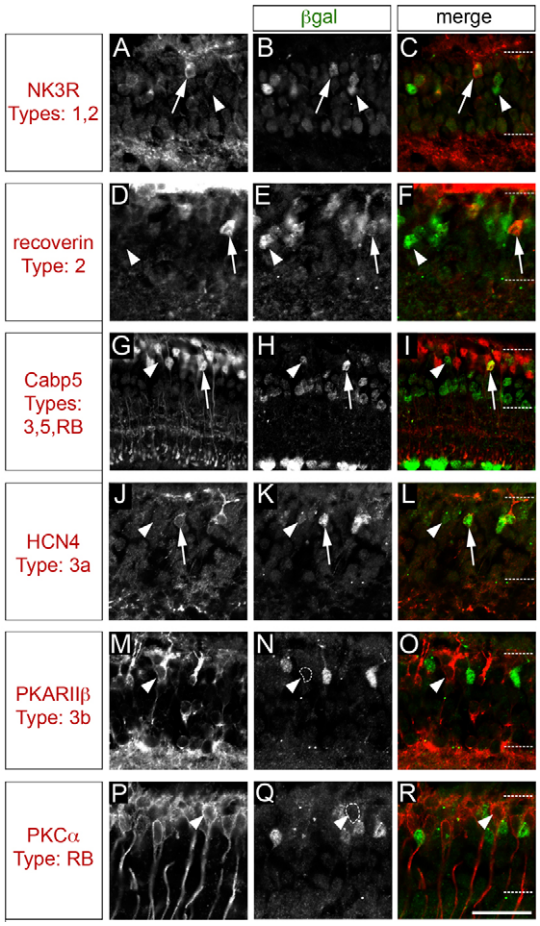

Fig. 3. The *Irx6*:βgal reporter is expressed in a subset of retinal bipolar cells. (**A-F**) In the mature *Irx6*$^{+/lacZ}$ retina, the *Irx6*:βgal reporter is expressed in a subset of Nk3r- (A-C, arrow) and recoverin- (D-F, arrow) positive cells. (**G-I**) *Irx6*:βgal is also expressed in a subset of Cabp5 cells (arrows). (**J-O**) *Irx6*:βgal colocalizes with the type 3a marker Hcn4 (J-L, arrows), but not with the type 3b marker PKARIIβ (M-O). Arrowheads in A-O indicate βgal-labeled cells that are not co-labeled with the respective bipolar marker. (**P-R**) There is no colocalization between PKCα expression and *Irx6*:βgal expression. Arrowhead indicates a PKCα-positive cell that does not colabel with *Irx6*:βgal. Scale bar: 25 μm.

number of cells within the outer half of the inner nuclear layer and in the ganglion cell layer (Fig. 2H).

In the mature *Irx6*$^{+/lacZ}$ retina, immunolabeling of the β-galactosidase reporter (*Irx6*:βgal) together with Vsx2/Chx10, a pan-bipolar transcription factor (Burmeister et al., 1996), indicated *Irx6* expression in a subset of bipolar cells (Fig. 2I-K). The *Irx6* homologue *Irx5*, was co-expressed in a subset of *Irx6*:βgal-immunolabeled bipolar cells (Fig. 2L-N). Not all Irx5-positive cells in the inner nuclear layer co-labeled with *Irx6*:βgal, and not all *Irx6*:βgal positive cells co-labeled with Irx5 (Fig. 2L-N). Additionally, *Irx6*:βgal co-immunolabeled with a subset of cells expressing the ganglion cell marker Brn3b (Pou4f2 – Mouse, Genome Informatics) (supplementary material Fig. S2A). These results indicate that *Irx6* is expressed in a subset of retinal ganglion cells and bipolar cells. *Irx6* also appeared to be transiently expressed in newly born photoreceptor cells (Fig. 2H, inset), but not in adult photoreceptor cells (Fig. 2I).

Irx6^{lacZ} is expressed in a subset of OFF bipolar cells

To determine the identity of the bipolar cell types that express the *Irx6*^{lacZ} reporter, retinal sections of Irx6$^{+/lacZ}$ mice were co-immunolabeled for *Irx6*:βgal and a series of bipolar cell type specific markers (Figs 1, 3). *Irx6*:βgal co-immunolabeled in a subset of cells expressing two type 2 cell markers, Nk3r (Fig. 3A-C) and recoverin (Fig. 3D-F) (Ghosh et al., 2004; Haverkamp and Wässle, 2000; Milam et al., 1993). *Irx6*:βgal labeling was also detected in a subset of cells immunolabeled for the type 3 and 5, and the rod bipolar cell marker Cabp5 (Fig. 3G-I) (Ghosh et al., 2004; Haverkamp et al., 2003). Type 3 cells are defined by their

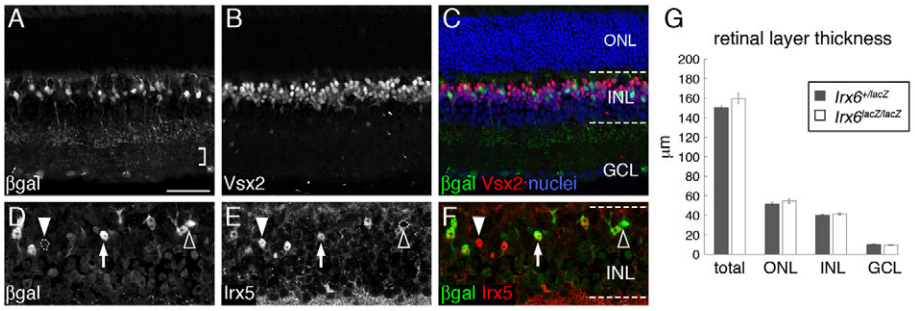

Fig. 4. *Irx6*$^{lacZ/lacZ}$ mice have normal retinal morphology. (**A**) Immunolabeling for βgal in the *Irx6*$^{lacZ/lacZ}$ mouse. Bracket indicates immunolabeling in the ON sublamina of the retina. (**B**) Immunolabeling for Vsx2. (**C**) Merged image of A and B, including Draq5 labeling of nuclei in the *Irx6*$^{lacZ/lacZ}$ mouse. (**D-F**) *Irx6*:βgal (D) and Irx5 (E) expression in the *Irx6*$^{lacZ/lacZ}$ mouse, showing *Irx6*:βgal expression in a subset of Irx5-positive bipolar cells (F); an *Irx6*:βgal-negative, Irx5-positive (closed arrowhead) bipolar cell; *Irx6*:βgal-positive, Irx5-positive (arrow) bipolar cell; and *Irx6*:βgal-positive, Irx5-negative (open arrowhead) bipolar cell. INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bar: 50 μm in A-C; 25 μm in D-F. (**G**) Retinal thickness in *Irx6*$^{+/lacZ}$ and *Irx6*$^{lacZ/lacZ}$ mice (*n*=6 retinas, each genotype). Data are mean±s.e.m.

morphology (Ghosh et al., 2004), but can be further categorized into two distinct cell types (type 3a and type 3b) based on their differential immunolabeling for Hcn4 and PKARIIβ (Prkar2b – Mouse Genome Informatics), respectively (Mataruga et al., 2007). *Irx6*:βgal was detected in type 3a cells co-immunolabeled with Hcn4 (Fig. 3J-L), but not in PKARIIβ-positive type 3b cells (Fig. 3M-O). Furthermore, *Irx6*:βgal expression was not detected in type 4 cells immunolabeled for calsenilin (Haverkamp et al., 2008) (supplementary material Fig. S2B), or in rod bipolar cells identified by PKCα immunolabeling (Haverkamp and Wässle, 2000; Negishi et al., 1988) (Fig. 3P-R). In summary, within the bipolar cell population of the mature retina, *Irx6* knock-in reporter gene expression is observed in type 2 and 3a bipolar cells.

Loss of Irx6 does not disrupt retinal morphology or number of retinal ganglion cells

To examine the role of *Irx6* during retinal development, we next examined the phenotype of *Irx6*$^{lacZ/lacZ}$ mice. Loss of *Irx6* did not disrupt the gross morphology and layering of the retina (Fig. 4C,G). *Irx6*:βgal immunolabeling was evident in a subset of Vsx2-positive bipolar cells and ganglion cells (Fig. 4A-C) and was visibly more robust than in the *Irx6*$^{+/lacZ}$ retina (Fig. 2I). In addition to the presence of *Irx6*:βgal in the OFF sublamina of the inner plexiform layer, immunolabeling was also detected in the ON sublamina of the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 4A; see below). Similar to the *Irx6*$^{+/lacZ}$ retina, Irx5 and *Irx6*:βgal immunolabeling exhibited overlapping and non-overlapping expression patterns in the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 4D-F).

Since reporter expression indicated that *Irx6* is expressed in retinal ganglion cells (Fig. 2E,G,H; supplementary material Fig. S2A), we investigated whether ganglion cell number was affected in the *Irx6*$^{lacZ/lacZ}$ retina. We did not observe a difference in the number of Brn3b positive ganglion cells between the *Irx6*$^{+/lacZ}$ (10.3 cells/100 μm ±0.6) and *Irx6*$^{lacZ/lacZ}$ (10.6 cells/100 μm ±0.4) retina (*n*=3 mice).

Irx6 is required for the terminal differentiation of OFF bipolar cells

Given that the *Irx6*:βgal reporter is expressed in a subset of OFF bipolar cells, we next examined whether the formation of these cell types was affected in *Irx6*$^{lacZ/lacZ}$ mice. Loss of *Irx6* led to the complete loss of recoverin in type 2 cells (Fig. 5A,B). Synaptotagmin II (Syt2) expression, which labels type 2 and 6 bipolar cells (Fox and Sanes, 2007; Wässle et al., 2009), was greatly reduced in the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 5C,D). Reduced Syt2 immunolabeling of axon termini in the inner plexiform layer was observed for both putative type 2 and 6 bipolar cells (Fig. 5C,D). Axon terminals of Syt2-labeled putative type 6 cells in *Irx6*$^{lacZ/lacZ}$ mice co-immunolabeled with *Irx6*:βgal (supplementary material Fig. S3), suggesting that the defects in this cell type are cell-autonomous. In contrast to the reduction in recoverin and Syt2 expression, Nk3r immunolabeling of type 1 and 2 cells was still present in the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 5E,F).

As *Irx6*:βgal is also expressed in type 3a bipolar cells, we next examined whether this cell type was affected by the loss of *Irx6*. In *Irx6*$^{lacZ/lacZ}$ mice, we observed a specific reduction in the level of Cabp5 immunolabeling within the OFF region corresponding to the position where type 3 axon terminals are located (Fig. 5G,H). Cabp5 immunolabeling of putative type 5 and rod bipolar cell axon terminals was unaffected (Fig. 5G,H). The identification of a Cabp5 immunolabeling defect in type 3 cells was correlated with a slight, but statistically significant, decrease in the number of cells expressing the type 3a cell marker Hcn4 (Fig. 6B,H; Fig. 8I). Furthermore, within the cell bodies of the Hcn4-expressing type 3a cells in the *Irx6*$^{lacZ/lacZ}$ retina, a loss of Cabp5 immunolabeling was observed (Fig. 6C,D,I,J). The reduction of Cabp5 immunolabeling was specific to type 3a cells and was not observed in PKARIIβ-positive type 3b cells (Fig. 6E,F,K,L; data not shown). Together, these results identify a specific defect in type 3a bipolar cells of the *Irx6*$^{lacZ/lacZ}$ retina marked by a loss of Cabp5 immunolabeling and a reduction in the total number of Hcn4-expressing bipolar cells.

Hcn4-expressing OFF bipolar cells exhibit stratification defects in *Irx6* mutant mice

In addition to a reduction in the number of Hcn4-immunolabeled putative type 3a bipolar cells in the *Irx6*$^{lacZ/lacZ}$ retina, we observed defects in the stratification of the remaining Hcn4-immunolabeled axon terminals (Fig. 6H; Fig. 7). These defects were characterized by the expansion of Hcn4-immunolabeled axon terminals into the entire OFF region of the inner plexiform layer (Fig. 7B,C). To determine more precisely the nature of this defect, we performed co-immunolabeling experiments with calretinin, a marker used to distinguish specific inner plexiform layer sublaminae (Ghosh et al., 2004; Haverkamp and Wässle, 2000). The five distinct sublaminae defined by calretinin immunolabeling were unaltered in the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 7H,I). The axon terminals of type 3 bipolar

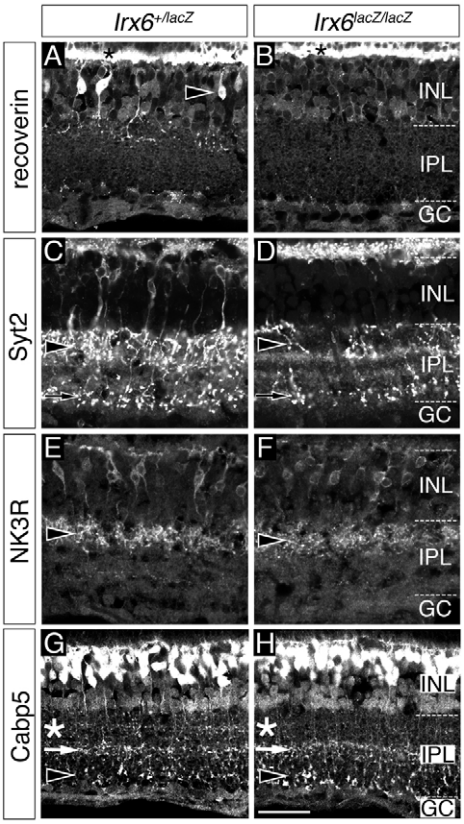

Fig. 5. OFF cone bipolar cell gene expression defects in *Irx6*:βgal mice. (**A-D**) Recoverin (A,B) and synaptotagmin 2 (C,D) immunolabeling of type 2 cells (arrowheads in A,C,D) is reduced in *Irx6*$^{lacZ/lacZ}$ mice. The asterisks in A,B indicate recoverin immunolabeling of photoreceptor cells. Synaptotagmin 2 immunolabeling of the axon terminals of type 6 cells is also reduced in *Irx6*$^{lacZ/lacZ}$ mice (arrows in C,D). (**E**,**F**) Nk3r immunolabeling is not reduced in *Irx6*$^{lacZ/lacZ}$ mice, as judged by labeling of soma and axon terminals (arrowheads). (**G**,**H**) Cabp5 expression is reduced in the region of the inner plexiform layer normally occupied by the axon terminals of type 3 cells (compare asterisks in G and H), but is unaffected in the axon terminal of type 5 (arrows in G and H) and rod bipolar cells (arrowheads in G and H) in the *Irx6*$^{lacZ/lacZ}$ mouse. INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar: 25 μm.

cells are normally positioned in sublamina 2 (between the two outermost calretinin-labeled bands), whereas the axon terminals of type 1 and 2 bipolar cells are positioned within sublamina 1, located above the outermost calretinin-labeled band (Ghosh et al., 2004). In contrast to the invariable positioning of Hcn4-immunolabeled axon terminals in sublamina 2 in the wild-type (data not shown) and *Irx6*$^{+/lacZ}$ retina (Fig. 7D-F), we observed Hcn4 immunolabeling spanning both sublamina 1 and 2 in *Irx6*$^{lacZ/lacZ}$ mice (Fig. 7G-I). This expansion of Hcn4-labeled termini in *Irx6*$^{lacZ/lacZ}$ mice was observed across the entire retina. Hcn4-labeled axon terminals were not observed in sublaminae 3-5, where ON bipolar cell axon terminals reside (Fig. 7G-I). In support of the observation of expanded Hcn4-immunolabeled axon termini, we measured a slight but statistically significant increase in the ratio of fluorescence intensity between a region of interest located directly below the inner nuclear layer (sublamina 1) and region of equal size located immediately below and corresponding to sublamina 2 (0.84±0.02 for *Irx6*$^{+/lacZ}$ retina; 0.90±0.02 for *Irx6*$^{lacZ/lacZ}$ retina; n=5, P<0.03), suggesting that there is a shift in the axon stratification of putative type 3a bipolar cells in the *Irx6*$^{lacZ/lacZ}$ mouse. These results suggest that *Irx6* is required for the correct axon stratification of Hcn4-expressing cells within the OFF sublamina of the inner plexiform layer.

Dysregulation of Vsx1 and Bhlhb5 in *Irx6* mutant mice

The OFF bipolar cell axon stratification defects observed in putative type 3a bipolar cells of *Irx6*$^{lacZ/lacZ}$ mice raises the possibility that OFF bipolar cell subtype identity is affected in the *Irx6* mutant. We therefore asked whether the expression of Vsx1 and Bhlhb5, two transcription factors required for OFF bipolar cell differentiation, was affected in *Irx6*$^{lacZ/lacZ}$ mice. Vsx1 expression is undetectable in Hcn4-positive type 3a cells in the wild-type retina (Shi et al., 2012). In the *Irx6*$^{+/lacZ}$ retina, Vsx1 was normally not detected in Hcn4-expressing type 3a cells (Fig. 8C), but in rare instances, Hcn4-positive bipolar cells exhibited faint levels of Vsx1 immunolabeling (data not shown). By contrast, in the *Irx6*$^{lacZ/lacZ}$ retina, ectopic Vsx1 immunolabeling was prominent in Hcn4-expressing bipolar cells (Fig. 8B,D,H). Despite the presence of ectopic Vsx1 immunolabeling, there was no significant change in the number of Vsx1-labeled cells in *Irx6*$^{lacZ/lacZ}$ mice (Fig. 8I). Unlike Vsx1, a significant reduction in the level of Bhlhb5 immunolabeling of type 2 cells was observed in the bipolar cell region of the inner nuclear layer in *Irx6*$^{lacZ/lacZ}$ mice (Fig. 8E,F,I). A previously uncharacterized subset of Hcn4-positive type 3a cells in wild-type and *Irx6*$^{+/lacZ}$ mice that weakly co-immunolabeled for Bhlhb5 was also reduced in size in the *Irx6*$^{lacZ/lacZ}$ retina (Fig. 8E; data not shown). The population of Bhlhb5-positive amacrine cells, identified by both position and co-immunolabeling with the amacrine cell marker syntaxin, remained unchanged in the absence of *Irx6* (Fig. 8E,F). These data demonstrate a differential requirement for *Irx6* in the regulation of Bhlhb5 and Vsx1 such that *Irx6* is required for the repression of Vsx1 in type 3a cells and for the activation of Bhlhb5 in type 2 and 3a bipolar cells.

Mixed subtype identity in Hcn4-expressing OFF bipolar cells in the Irx6-mutant retina

As *Vsx1* is necessary for the expression of several type 2 bipolar cell-specific markers (Chow et al., 2004; Ohtoshi et al., 2004), we next examined the possibility that the presence of ectopic Vsx1 in Hcn4-expressing cells in the *Irx6*-mutant was correlated with the ectopic expression of type 2 markers regulated by *Vsx1*. We focused our attention on the type 1 and 2 bipolar cell marker Nk3r, because, unlike recoverin, its expression is not lost in the *Irx6* mutant (Fig. 5E,F). In the wild type, *Irx6*$^{+/lacZ}$ and *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$ double heterozygous retina, Nk3r and Hcn4 are expressed in a mutually exclusive manner in presumptive type 1, 2 and 3a cells, respectively (Fig. 9A-C′,J; data not shown). In both the *Irx6*$^{lacZ/lacZ}$ and *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{+/AltB5}$ retina, ectopic Nk3r immunolabeling was observed in Hcn4-immunolabled cell somas (Fig. 9D-F,J; data not shown) and in the axon terminals of putative type 3a cells (Fig. 9D′-I′). A population of Nk3r-positive putative type 1 or 2 cells was not labeled with Hcn4 (Fig. 9D-F). In agreement with our observation of co-immunolabeling between Hcn4 and Nk3r in the *Irx6*$^{lacZ/lacZ}$ retina, we measured a significant increase in the number of Nk3r-positive bipolar cells in the *Irx6*$^{lacZ/lacZ}$ retina when compared with the *Irx6*$^{+/lacZ}$ retina (Fig. 8I). We also observed a decrease in the fluorescence intensity ratio between sublamina 1 and 2 for Nk3r immunolabeling in the *Irx6*$^{lacZ/lacZ}$ retina (1.2±0.07) when compared with the *Irx6*$^{+/lacZ}$

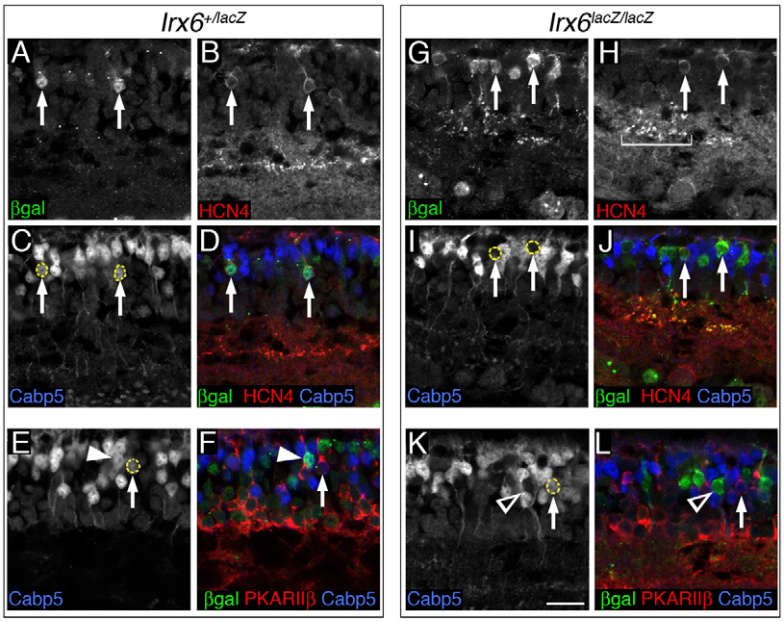

Fig. 6. *Irx6*$^{lacZ/lacZ}$ mice have a reduction in Cabp5 staining in type 3a bipolar cells. (**A-L**) *Irx6*$^{+/lacZ}$ (A-F) and *Irx6*$^{lacZ/lacZ}$ mice (G-L) were immunolabeled for *Irx6*:βgal (A,G), Hcn4 (B,H) and Cabp5 (C,I). Arrows indicate cells in the heterozygous retina (A-D) that are positive for *Irx6*:βgal, Hcn4 and Cabp5, and cells in the *Irx6*$^{lacZ/lacZ}$ retina (G-J) that are *Irx6*:βgal and Hcn4 positive, but have lost Cabp5 expression. Bracket in H indicates Hcn4 staining in the inner plexiform layer of the mutant mouse. Staining for *Irx6*:βgal (E,K) and PKARIIβ (F,L) shows that there is no change in the colocalization of these markers with Cabp5 in the heterozygous and *Irx6*$^{lacZ/lacZ}$ mouse. Arrows in E,F,K,L indicate cells that are positive for Cabp5 and PKARIIβ expression. Arrowheads in E,F,K,L indicate *Irx6*:βgal-expressing cells that are positive (E,F) or negative (K,L) for Cabp5. Scale bar: 12 μm.

retina (1.5±0.1; P<0.03, n=3 mice), providing further support for the finding that there is an increase in Nk3r immunolabeling in sublamina 2. Together, these findings suggest that NKR3 is ectopically expressed in putative type 3a bipolar cells in both the *Irx6*$^{lacZ/lacZ}$ and *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{+/AltB5}$ retina.

Given that both Nk3r and Hcn4 are downregulated in the *Vsx1*-null retina (Chow et al., 2004; Shi et al., 2012), we next asked whether their co-expression in putative type 3a bipolar cells of the *Irx6* mutant retina would be affected in mice deficient for both *Irx6* and *Vsx1*. Unexpectedly, despite the predicted reduction in overall immunolabeling of the two markers Hcn4 and Nk3r, co-immunolabeled bipolar cells were still observed in the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ double homozygous mutant retina (Fig. 9G-J). In the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ mutant retina, all of the Hcn4-expressing bipolar cells co-immunolabeled with Nk3r (Fig. 9J). These data indicate that the expression of Nk3r in Hcn4-expressing cells appears to be independent of *Vsx1*. We also observed a population of Nk3r-positive cells that did not label with Hcn4 in the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ double homozygous retina. In agreement with previous findings in the *Vsx1*-null retina (Chow et al., 2004), we observed a decrease in the number of Nk3r immunolabeled cells in the *Irx6*$^{+/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ retina when compared with the *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$ retina (Fig. 9J). In the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ double homozygous retina we observed a further decrease in the number of Nk3r-only immunolabeled cells (Fig. 9J), suggesting that *Irx6* and *Vsx1* can both induce Nk3r expression.

To determine whether *Vsx1* expression has an effect on the expression of the *Irx6*, we examined the expression of the *Irx6*:βgal reporter in the presence and absence of *Vsx1*. There was a significant increase in the number of *Irx6*:βgal positive cells in the *Irx6*$^{+/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ retina when compared with the *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$ retina (Fig. 9K). These results suggest that *Vsx1* has a partial inhibitory effect on the expression of *Irx6*.

Irx6 can activate or repress transcription through Iroquois-binding sites found proximal to recoverin, Vsx1 and Nk3r

Members of the Iroquois family are known to bind in vitro to a DNA sequence known as the Iroquois-binding site (IBS) and function as transcriptional repressors (Bilioni et al., 2005; Berger et al., 2008). To gain insight into the molecular mechanism by which the expression of the OFF bipolar cell markers recoverin, Vsx1 and Nk3r is disrupted in the *Irx6*$^{lacZ/lacZ}$ mouse, we searched for candidate sequences that match the IBSs in the regions

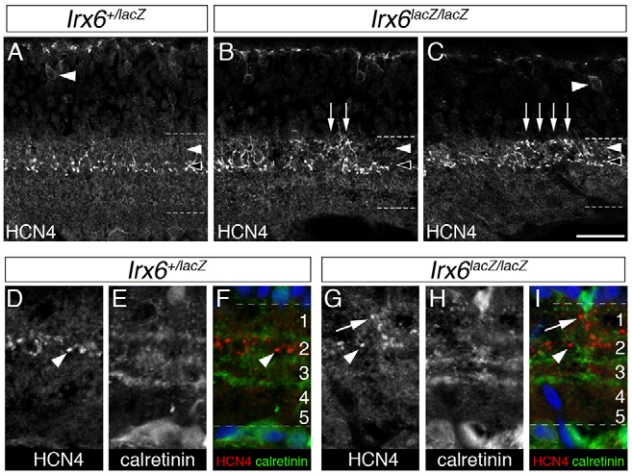

Fig. 7. Abnormal distribution of Hcn4 immunolabeling in the inner plexiform layer of the *Irx6*$^{lacZ/lacZ}$ retina. (**A-C**) Immunolabeling for Hcn4 in the *Irx6*$^{+/lacZ}$ (A) and *Irx6*$^{lacZ/lacZ}$ (B,C) retina. Arrowheads indicate Hcn4-positive cell bodies in the inner nuclear layer of the retina. Arrows indicate increased expression of Hcn4 in the upper zone of the inner plexiform region. Broken lines define the boundary of the inner plexiform layer (A-C,F,I). (**D-I**) Immunolabeling using calretinin to further define the projection zones of the inner plexiform region of the retina. In the *Irx6*$^{+/lacZ}$ retina, Hcn4 immunolabeled axon terminals of putative type 3a cells (D) are restricted to sublamina 2 in the inner plexiform layer, as defined by calretinin immunolabeling (E). Arrowheads (D,F) indicate Hcn4-positive axon termini in sublamina 2. By contrast, in the *Irx6*$^{lacZ/lacZ}$ retina (G-I), Hcn4 immunolabeling (G) is detected in both sublamina 1 and 2, as defined by calretinin immunolabeling (H). Hcn4-positive axon termini in sublamina 1 (G,I, arrows) and sublamina 2 (G,I, arrowheads). Scale bar: 25 μm in A-C; 18 μm in D-I.

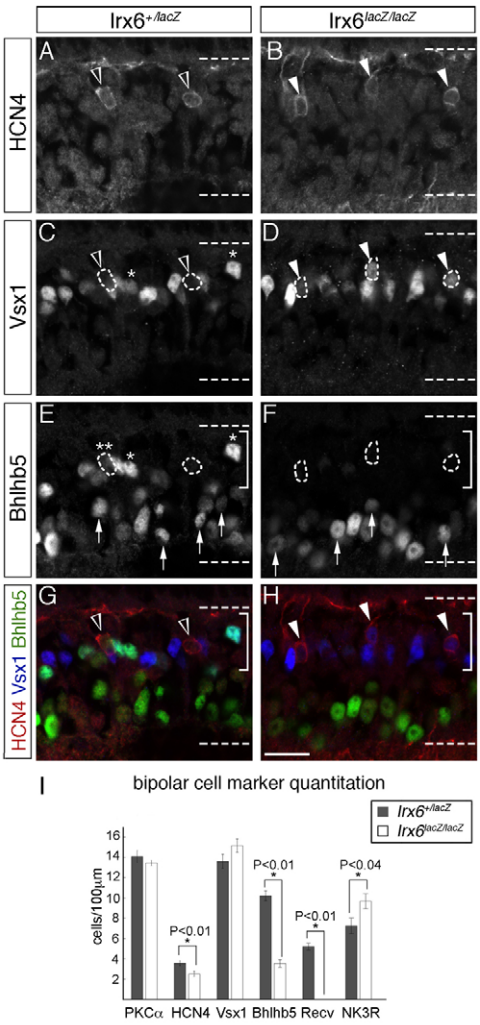

Fig. 8. Irx6 regulates the expression of transcription factors required for OFF bipolar cell development. (**A-H**) Vsx1 immunolabeling is ectopically upregulated in Hcn4-expressing cells (A,B, arrowheads) in the *Irx6*$^{lacZ/lacZ}$ retina (compare C,D, arrowheads). The single asterisks in C and E indicate a putative type 2 cell, based on its co-labeling of Vsx1 and Bhlhb5 and on the lack of Hcn4 immunolabeling. In the *Irx6*$^{lacZ/lacZ}$ retina, Bhlhb5 immunolabeling of the bipolar cell region of the inner nuclear layer (indicated by the bracketed regions in E-H) is greatly reduced compared with the *Irx6*$^{+/lacZ}$ retina (E,F). Amacrine cell labeling of Bhlhb5 (E,F, arrows) is retained in the *Irx6*$^{lacZ/lacZ}$ retina. An example of a putative type 3a cell expressing low levels of Bhlhb5 is indicated by the double asterisk in E. Scale bar in H: 15 µm. (**I**) Quantitation of bipolar cell marker expression in the retina of *Irx6*$^{+/lacZ}$ (n=5 retinas) and *Irx6*$^{lacZ/lacZ}$ (n=6 retinas) mice. Recv, recoverin. Data are mean±s.e.m.

proximal to these genes. Candidate IBSs in *Vsx1*, *Tacr3* (the gene encoding Nk3r) and *Rcvrn* were identified (Fig. 10A; supplementary material Table S2). We then performed a series of transcriptional reporter assays to determine whether Irx6 might directly regulate the expression of these factors. Regions of ~200 bp of *Vsx1* or *Tacr3* surrounding the predicted IBSs were cloned upstream of an SV40 promoter-driven luciferase reporter plasmid. In three of the four IBS sequences examined, we observed repression of luciferase expression in the presence of co-transfected Irx6 (Fig. 10B). Upstream of *Rcvrn* we identified a fragment containing a potential IBS (Fig. 10A), and positioned a 1.3 kb region containing this site in front of a mini-promoter-driven luciferase reporter construct (supplementary material Table S2). Unexpectedly, in contrast to the well-characterized repressor activity of Irx transcription factors, reporter assays showed that the addition of Irx6 had an activating effect on transcription of the *Rcvrn* reporter (Fig. 10C). Interestingly, addition of Irx5 had no effect on transcription and did not alter Irx6-mediated activation (Fig. 10C). Together, these findings suggest that Irx6 can function in a context-dependent manner as either a transcriptional repressor or activator, and may explain the increased expression of Vsx1/Nk3r and decreased expression of recoverin in the *Irx6*$^{lacZ/lacZ}$ mouse.

Visual signaling defects in Irx6 mutant mice

To determine whether the retinal defects in *Irx6* mutant mice were accompanied by defects in visual signaling, electroretinography (ERG) was performed on mice at 3-4 months of age. In contrast to wild-type and *Irx6*$^{+/lacZ}$ mice, *Irx6*$^{lacZ/lacZ}$ mice exhibited a reduction of dark-adapted and light-adapted a-wave amplitude (Fig. 11A; data not shown), which reflects photoreceptor activity. Consistent with the reduced a-wave amplitude, a decreased ERG b-wave amplitude, which is indicative of post photoreceptor retinal activity, was also observed (Fig. 11C). No changes were observed in a-wave or b-wave kinetics (Fig. 11B,D), suggesting that the biochemical integrity of the photoreceptors and inner retina was not lost. Despite the presence of OFF bipolar differentiation defects in *Irx6*$^{lacZ/lacZ}$ mice, light-adapted OFF responses were intact in *Irx6*$^{lacZ/lacZ}$ mice compared with controls (Fig. 11E,F; data not shown). In summary, *Irx6*$^{lacZ/lacZ}$ mice exhibit a reduced ERG a-wave and b-wave, but have intact light-adapted OFF visual signaling responses.

DISCUSSION

Here, we show that *Irx6* is required downstream of bipolar cell specification for the terminal differentiation of type 2, type 3a and possibly type 6 bipolar cells (Fig. 12A).

Our results suggest there is a complex regulatory network of transcription factors (i.e. Irx6, Vsx1 and Bhlhb5) that function to regulate the development of type 2 and 3a bipolar cells (Fig. 12B). In the *Irx6*$^{lacZ/lacZ}$ mutant mouse, type 2 cells have reduced recoverin and Bhlhb5 immunolabeling, but retain Vsx1 and some Nk3r immunolabeling. In the *Irx6;Vsx1* homozygous mutant mouse, we see a decrease in the number of Nk3r alone-positive cells compared with either the *Irx6*$^{+/lacZ}$*;Vsx1*$^{AltB5/AltB5}$ mouse or *Irx6*$^{lacZ/lacZ}$*;Vsx1*$^{+/AltB5}$ mouse. This genetic interaction reveals that both *Irx6* and *Vsx1* contribute to the expression of Nk3r in type 2 cells in the mouse retina. Based on our observations and published work (Chow et al., 2004; Feng et al., 2006; Kerschensteiner et al., 2008; Ohtoshi et al., 2004), we suggest that both *Vsx1* and *Bhlhb5* have a central role in directing the terminal differentiation and maturation of type 2 bipolar cells, with *Irx6* taking on a more minor role in regulating the formation of this cell type (Fig. 12B).

In type 3a bipolar cells, our findings reveal that *Irx6* has a key role in defining cell type specific gene expression. Results from the transcriptional reporter assays suggest that Irx6 could directly repress the expression of both Vsx1 and Nk3r, and therefore is likely to be responsible in part for repressing the expression of these genes in type 3a cells. Members of the Irx family have been shown to act as transcriptional repressors (Bilioni et al., 2005; Costantini et al., 2005). Although the measured increase in Vsx1 immunolabeling in the *Irx6*$^{lacZ/lacZ}$ retina was not statistically significant, we think this is due to Vsx1 also being expressed in

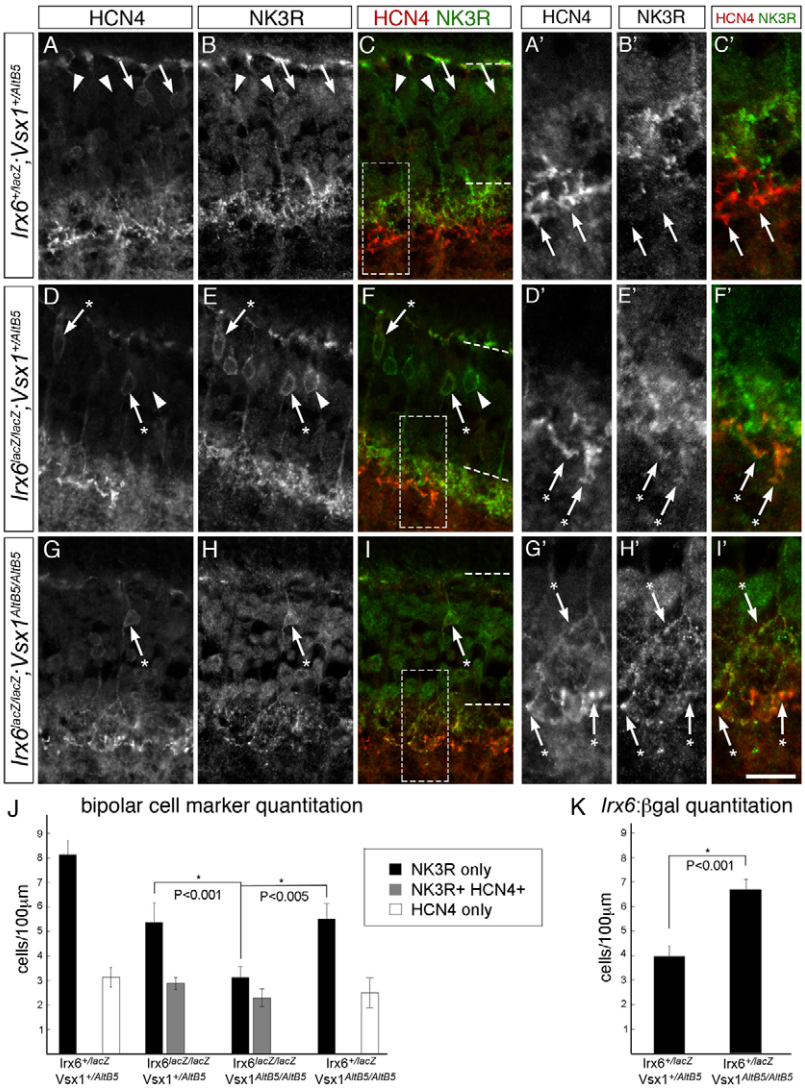

Fig. 9. Co-labeling of Nk3r in putative type 3a bipolar cells in the *Irx6*$^{lacZ/lacZ}$ retina. (**A-C′**) In the *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$ retina (A-C), Nk3r and Hcn4 immunolabeling of putative types 1 or 2 (arrowheads) and type 3a (arrows) cells, respectively, does not overlap in cell somas or axon terminals (A′-C′). (**D-F′**) By contrast, in the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{+/AltB5}$ retina, Hcn4 immunolabeling in putative type 3a cells that exhibit type 3a axon stratification are co-immunolabeled with Nk3r (indicated by cells with asterisked arrows). Putative type 1 or 2 cells expressing only Nk3r and not Hcn4 are labeled with an arrowhead. (**G-I′**) In the *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ retina, co-labeling of Hcn4 and Nk3r is observed in the cell soma (indicated by asterisked arrows) and in the inner plexiform layer. Scale bar: 15 µm in A-I; 10 µm in A′-I′. (**J**) Quantitation of bipolar cells immunolabeled with Nk3r alone (black), Nk3r and Hcn4 (gray), and Hcn4 alone (white) in the retina of *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$, *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{+/AltB5}$, *Irx6*$^{lacZ/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ and *Irx6*$^{+/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ mice (*n*=3, 3, 4, 2 mice, respectively). (**K**) Quantitation of *Irx6*:βgal expression in the retina of *Irx6*$^{+/lacZ}$;*Vsx1*$^{+/AltB5}$ and *Irx6*$^{+/lacZ}$;*Vsx1*$^{AltB5/AltB5}$ mice (*n*=3 mice for both genotypes). Data are mean±s.e.m.

type 7 bipolar cells, and that any increase in Vsx1-positive cell number in the *Irx6*$^{lacZ/lacZ}$ retina is masked by the additional expression of Vsx1 in the ON bipolar cells.

Irx6 also promotes the expression of Bhlhb5 and Cabp5, and partially promotes the expression of Hcn4 in type 3a cells. In the *Irx6*$^{lacZ/lacZ}$ mouse retina, we observe a 'hybrid' cell that shows characteristics of both type 2 and 3a bipolar cells, and extends axon terminal projections throughout the OFF sublamina, suggesting that *Irx6* expression is necessary to generate a type 3a bipolar cell with a separate identity from a type 2 cell (Fig. 12B). We propose that, in the *Irx6*$^{lacZ/lacZ}$ mouse, the type 3a bipolar cells adopt molecular features of type 2 cells, as we observe a large increase in the total number of Nk3r immunolabeled cells in the *Irx6*$^{lacZ/lacZ}$ retina. This increase in Nk3r immunolabeled cells is consistent with the ectopic expression of Nk3r in Hcn4-positive type 3a cells, but would not be expected if Nk3r immunolabeled type 1 or 2 cells ectopically expressed Hcn4. Additionally, in both the *Irx6*$^{lacZ/lacZ}$ and *Irx6;Vsx1* double homozygous mutant retinas, we observe *Irx6*:βgal cells that are either positive for Hcn4 (and Nk3r) or negative for Hcn4, suggesting that there are two types of *Irx6*:βgal reporter cells in the *Irx6*$^{lacZ/lacZ}$ mouse (supplementary material Fig. S4). The putative type 2/3a hybrid cell is still present in the retina of the *Irx6;Vsx1* homozygous mutant mouse, even in the absence of robust Bhlhb5 bipolar cell expression (supplementary material Fig. S4), suggesting that *Irx6* is a primary regulator in controlling the formation of type 3a cells in a mechanism that is independent of *Vsx1* function (Fig. 12B), but that other factors must also be functioning to regulate OFF bipolar cell specific gene expression. Together, these observations support a model in which the identities of type 2 and 3a bipolar cells are defined, in part by opposing, yet interdependent, transcription factor gene expression established in these cell types.

In *Irx6* mutant retinas, recoverin immunolabeling in type 2 bipolar cells was lost. Results from the transcriptional reporter assay suggest that the putative IBS upstream of *Rcvrn* can induce Irx6-dependent transcriptional activation, and this is consistent with our genetic data showing loss of recoverin in the *Irx6*$^{lacZ/lacZ}$ mouse. In *Irx5* mutant retinas, recoverin immunolabeling in type 2 cells is also downregulated, demonstrating that *Irx5* and *Irx6* are not functionally redundant. As we did not observe any effect in our transcriptional assay with the addition of Irx5, the *Rcvrn* transcriptional activation site for Irx5 remains unclear, and our data suggest that the function of putative Iroquois-binding sites is highly dependent on the surrounding sequence.

Although no ganglion cell defects were observed in *Irx6*$^{lacZ/lacZ}$ mice (supplementary material Fig. S5), nor in the individual *Irx2*,

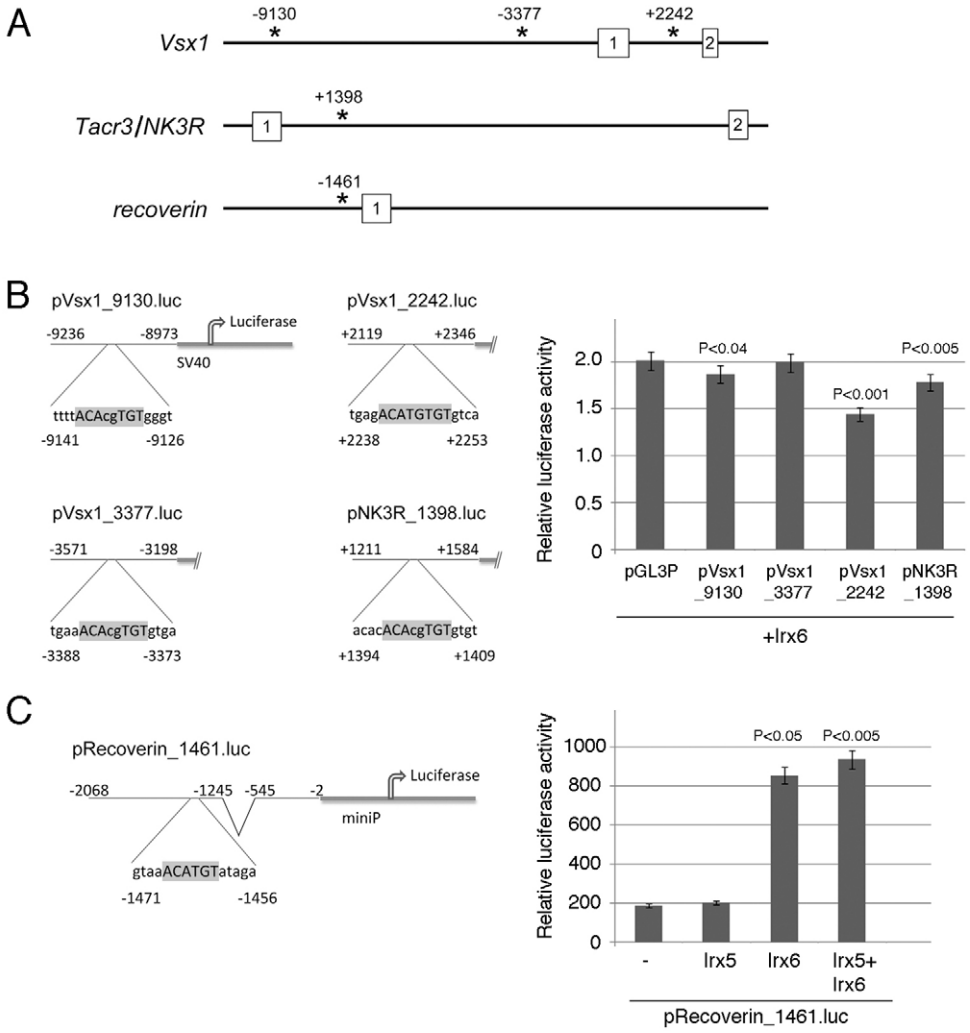

Fig. 10. Irx6 can act as a transcriptional repressor or activator on sites found proximal to OFF bipolar cell markers. (**A**) Potential IBS found proximal to *Vsx1*, *Tacr3* (*Nk3r*) and *Rcvrn*. Numbered boxes indicate exons. (**B**,**C**) Irx6 represses both Vsx1-luciferase (B) and Nk3r-luciferase and activates Rcvrn-luciferase (C) expression in HEK cells. Data are mean±s.e.m.

Irx4 or *Irx5* gene knockout mice (Bruneau et al., 2001; Cheng et al., 2005; Lebel et al., 2003) previous studies in chick suggest a role for Iroquois genes in these cells (Jin et al., 2003). Although in situ hybridization studies have shown that ganglion cells express both IrxA and IrxB cluster genes, only the Iroquois cluster B genes are expressed in the bipolar cell region of the inner nuclear layer (Cohen et al., 2000) (R.L.C., unpublished). This difference in expression could explain why defects are observed in bipolar cells and not in ganglion cells of both *Irx6* and *Irx5* mutants, and suggests that the absence of a ganglion cell phenotype in individual *Irx* loss-of-function mutants is due to functional redundancy of the Irx proteins.

Iroquois genes appear to have a conserved function in subdividing neuronal projection domains. In the *Drosophila* notum, the *Iro* complex appears to control axonal projections of medial versus lateral mechanosensory bristles (Grillenzoni et al., 1998). In addition, the *Iro* complex prevents dorsal photoreceptor axons from misprojecting to the ventral lamina by inhibiting *Drosophila* Wnt4 (Sato et al., 2006). Similar to the fly, our results reveal that *Irx6* functions in subdividing neuronal projection domains in the mammalian retinal inner plexiform layer. In $Irx6^{lacZ/lacZ}$ mice, axonal projections of presumptive type 3a bipolar cells, which are normally restricted to sublamina 2, are also observed in sublamina 1. Interestingly, the presumptive type 3a cells did not misproject to sublamina 3-5, where the axon terminals of ON bipolar cells reside. This observed axonal termini misprojection phenotype is reminiscent of the $Sema5a^{-/-};Sema5b^{-/-}$ mouse retinal phenotype, where Cabp5 immunolabeled type 3 bipolar cells also had ectopic projections to sublamina 1 (Matsuoka et al., 2011). These findings suggest that at least two mechanisms are required for bipolar cell type axonal development: one that defines the division of ON versus OFF boundaries; and one (involving *Irx6*) that is required for proper positioning within the OFF region. Parallels between the axon guidance defects in the mouse retina and in *Drosophila* suggest a conserved role for Irx genes in regulating axon projection domain specificity.

How the loss of *Irx6* affects visual signaling is unclear at this point. In *Irx6* mutants, the ERG b-wave amplitude, an indicator of bipolar cell activity was intact, but reduced. However, as the amplitude of the a-wave (a measure of photoreceptor activity) was also reduced, it was not possible to determine whether the reduced b-wave was due to defects in bipolar cell function. The reduced a-wave amplitude in $Irx6^{lacZ/lacZ}$ mice was unexpected. Although a

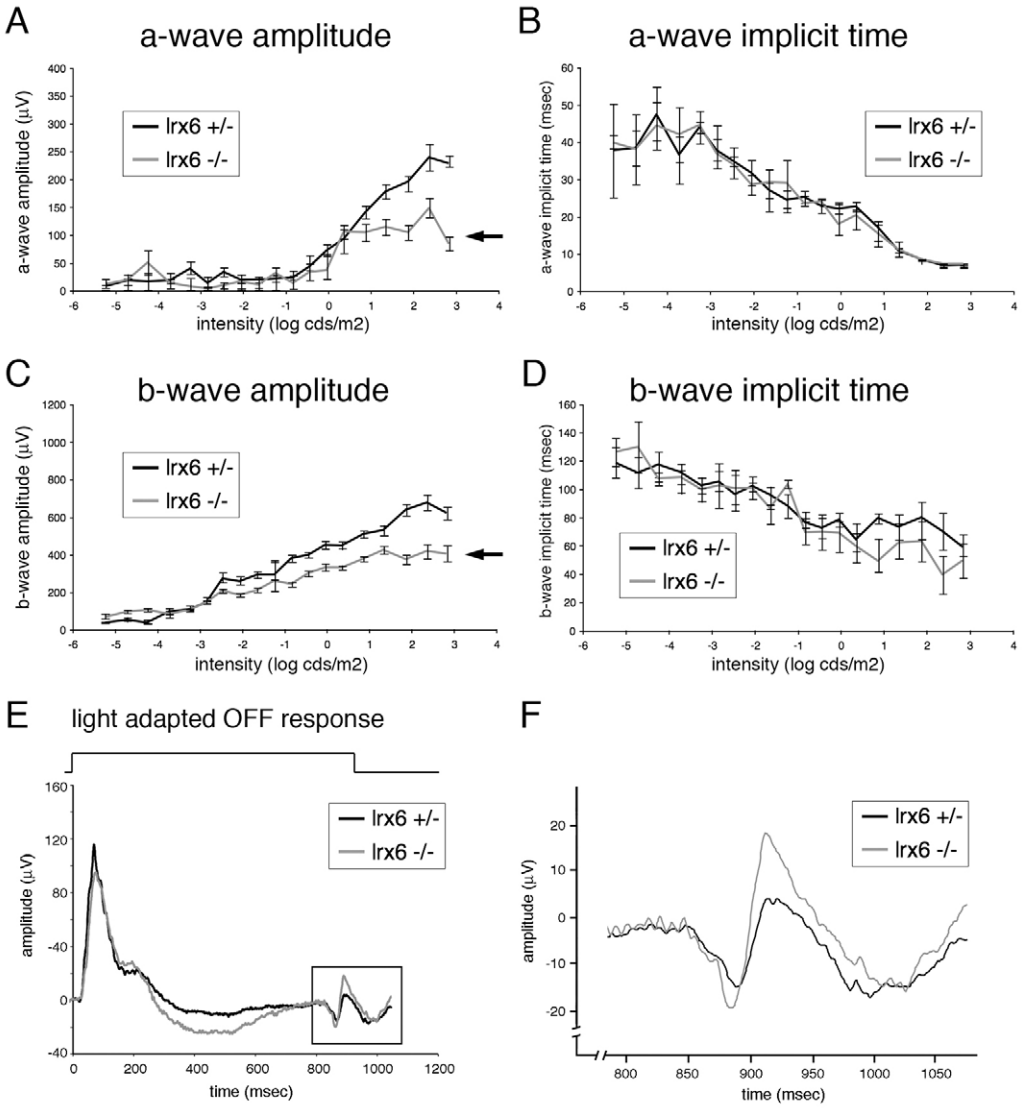

Fig. 11. Visual signaling defects in *Irx6*$^{lacZ/lacZ}$ mice. (**A-D**) Dark-adapted ERG a-wave (A) and b-wave (C) amplitudes were reduced in *Irx6*$^{lacZ/lacZ}$ (n=5) compared with *Irx6*$^{+/lacZ}$ mice (n=4). Both the a-wave (B) and b-wave (D) implicit time values were unaffected in *Irx6*$^{lacZ/lacZ}$ mice. Data are mean±s.e.m. (**E**) An example of the light-adapted OFF response in *Irx6*$^{lacZ/lacZ}$ and *Irx6*$^{+/lacZ}$. (**F**) The inset in E revealing an intact d-wave that is generated in response to light OFF-set.

reduced a-wave amplitude was previously observed in *Irx5* mutant mice, this was attributed to the overall reduced size of *Irx5* mice compared with controls (Cheng et al., 2005). *Irx6*$^{lacZ/lacZ}$ mice, however, do not exhibit any significant reduction in overall size (data not shown) or photoreceptor layer thickness, raising the possibility that the reduced a-wave amplitude is due to a defect in photoreceptor function. How this defect could be manifest is presently unclear given that the a-wave implicit time kinetics were unaffected in the *Irx6* mutants. Interestingly, transient expression of the *Irx6*:βgal reporter was observed in photoreceptor cells, suggesting that there may be a requirement for *Irx6* during photoreceptor development. The light adapted d-wave, a specific indicator of OFF bipolar cell function, was intact in *Irx6* mutants; this result contrasts the OFF visual signaling defects previously observed in *Vsx1* mutant mice (Chow et al., 2004; Kerschensteiner et al., 2008) and with our observation that *Vsx1*-null mice have a reduced d-wave (data not shown). This difference in visual signaling phenotype is surprising given that the *Vsx1* and *Irx6* mutants have overlapping gene expression defects in OFF bipolar cells. However, the *Vsx1* and *Irx6* mutant phenotypes are not identical. In particular, Vsx1 and Nk3r expression are upregulated in type 3a bipolar cells in the *Irx6* mutant. Future work examining the signaling properties of *Irx6* and *Vsx1* mutant OFF bipolar cells in isolation and slice preparations will be important to begin to understand how these genes regulate functional differences in OFF bipolar subtypes.

One question that remains unanswered is to what extent the specification of diverse retinal bipolar cell subtypes is driven by intrinsic (i.e. transcription factor coding) and extrinsic mechanisms. Although dissociation studies have suggested that intrinsic factors play a dominant role in regulating E16-17 retinal progenitor cell cycle exit and cell fate determination, these studies have focused on retinal cell classes and not on subtype determination (Cayouette et al., 2003). In the developing neural tube, specification of ventral interneurons is determined in part by a dorsal-ventral Hedgehog signaling gradient that regulates the expression of homeodomain

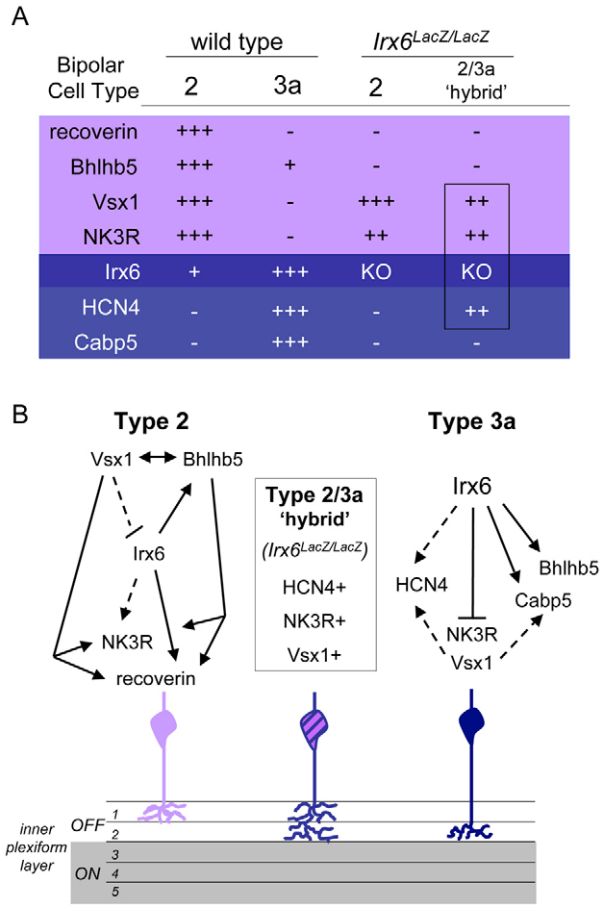

Fig. 12. Regulatory network model of Irx6, Vsx1 and Bhlhb5 in directing terminal gene expression in OFF bipolar cells. (**A**) Expression of type 2 and 3a cell markers in the *Irx6*$^{+/lacZ}$ (wild-type retina), and the resulting changes in marker expression in the *Irx6*$^{lacZ/lacZ}$ retina. (**B**) We propose that *Vsx1* and *Bhlhb5* are key players in regulating the terminal differentiation and maturation of type 2 cells, while *Irx6* has a leading role in regulating the formation of type 3a cells. *Irx6* is necessary for preventing type 3a cells from adopting type 2 cell characteristics in a mechanism that is independent of *Vsx1* activity. Dashed lines indicate partial repression or activation.

and basic helix-loop-helix transcription factors (Briscoe et al., 2000). As is the case in the retina, subtypes exist within different ventral interneuronal classes of the neural tube that possess distinct axonal targeting properties and neuronal activity (Shirasaki and Pfaff, 2002). Retinal type 7 bipolar cell dendrite stratification is not affected by the absence of photoreceptors (Keeley and Reese, 2010), supporting the idea that intrinsic mechanisms specify some aspects of bipolar cell type development that is independent of extrinsic cues. Future experiments aimed at understanding how *Irx6* and its downstream targets are regulated by intrinsic and extrinsic cues will provide insight into the control of retinal bipolar cell type diversity.

Acknowledgements
We thank F. Haeseleer, R. R. McInnes, F. Müller, A. Hirano and C. C. Hui for reagents and members of the R.L.C. lab for comments and suggestions.

Funding
This work was supported by operating grants from the Canadian Institutes for Health Research, The Foundation Fighting Blindness – Canada (R.L.C.) and National Institutes of Health/National Heart, Lung, and Blood Institute (NIH/NHLBI) [R01 HL93414 ARRA to B.G.B.]. Y.S. is an Alberta Innovates-Health Solutions (AIHS) Senior Scholar [200800242]. E.N.S. is supported by a Michael Smith Foundation for Health Research Postdoctoral Fellowship. R.L.C. is supported by a Tier 2 Canada Research Chair. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.081729/-/DC1

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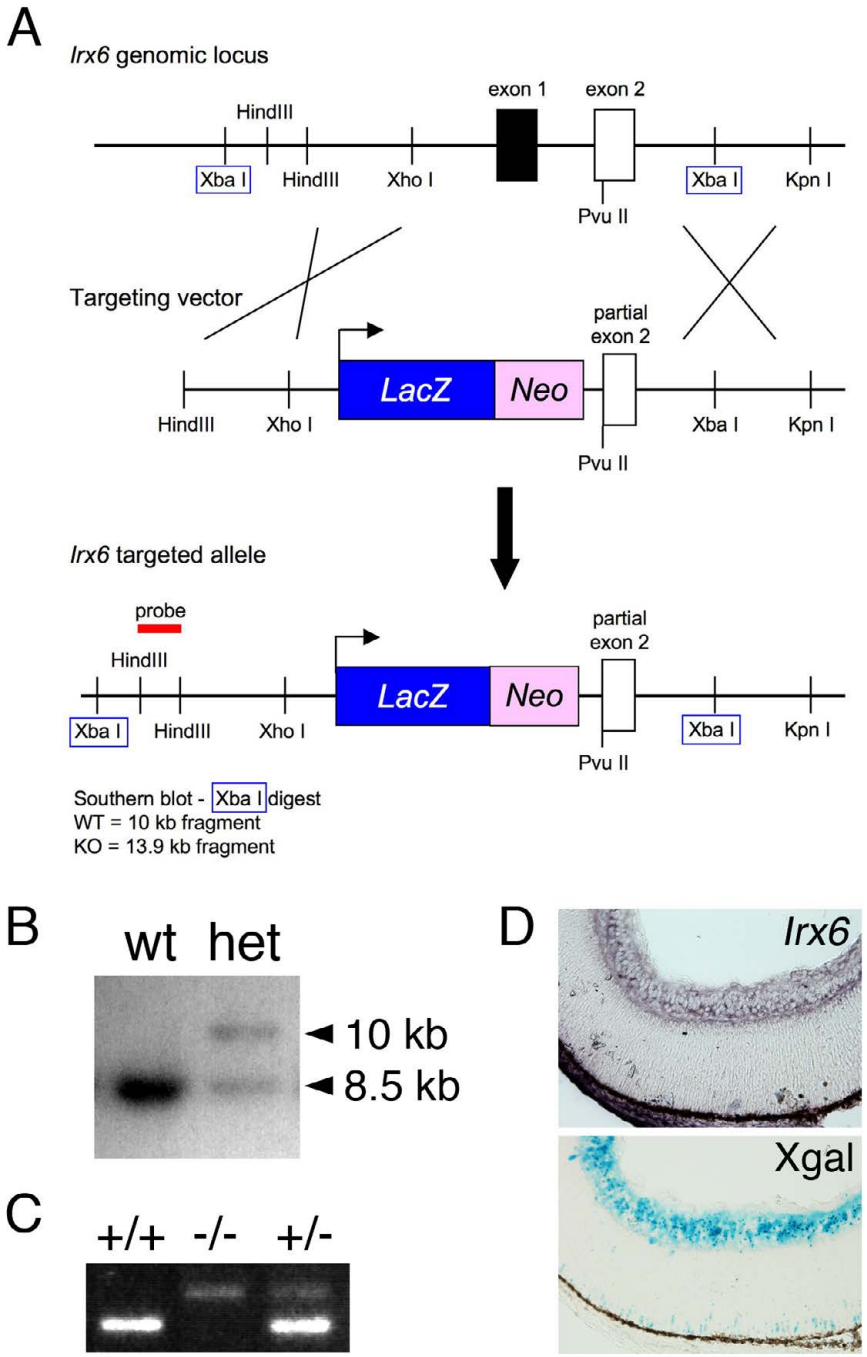

Fig. S1. Targeted disruption of *Irx6*. A 5.5 kb *Nco*I/*Hin*dIII *Irx6* fragment was cloned as the 5′ arm of the targeting construct upstream of the *lacZ* gene. The targeting construct contained a neomycin resistance cassette and a thymidine kinase cassette (*Pgk-Neo-SV40 polyA*) that was cloned in reverse orientation with respect to the targeting vector. A 2 kb *Pvu*II/*Not*I *Irx6* fragment was cloned as the 3′ arm of the targeting construct. Following homologous recombination, a mutated *Irx6* allele was generated that had lost all of exon 1 and part of exon 2. (**A**) Construct used. (**B**) Southern blot of the targeted ES cell DNA, clone B3. Southern blot analysis for homologous recombinants was carried out following an *Xba*I digest using a 2 kb *Hin*dIII fragment probe 5′ to the left arm of the targeting construct. The wild-type band size is 8.5 kb and the targeted band size is 10 kb. Two positive clones were used to give rise to the knock-in founder mice. (**C**) PCR showing wild-type, homozygous ($Irx6^{lacZ/lacZ}$) and heterozygous ($Irx6^{+/lacZ}$) mice. $Irx6^{+/lacZ}$ heterozygous crosses produced offspring (*+/+, +/lacZ, lacZ/lacZ*) with the expected Mendelian ratio and $Irx6^{lacZ/lacZ}$ mice were able to reproduce successfully. Both developing and adult $Irx6^{lacZ/lacZ}$ mice were indistinguishable from their wild-type littermates in terms of size and general behavior. (**D**) In situ hybridization (upper) for Irx6 expression in the P0 $Irx6^{+/lacZ}$ mouse retina and X-gal staining (lower) in an adjacent section showing the overlapping expression pattern between endogenous *Irx6* expression and expression of the *Irx6*:βgal reporter. The riboprobe corresponds to the full-length cDNA for Irx6. The protocol for in situ hybridization has been previously described (Chow et al., 2001), except the hybridization temperature was 56°C.

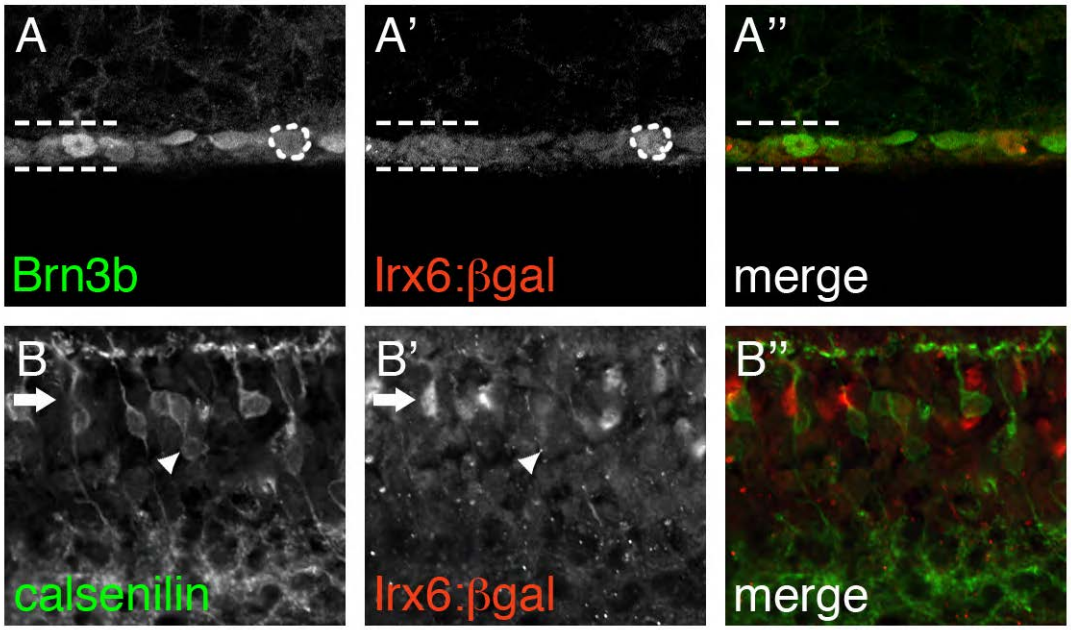

Fig. S2. The *Irx6*:βgal reporter is expressed in a subset of ganglion cells, but is not expressed in type 4 bipolar cells. (**A-A″**) The *Irx6*: βgal reporter co-immunolabels with the ganglion cell marker Brn3b in a subset of cells in the adult *Irx6*$^{+/lacZ}$ mouse retina. The dashed lines indicate the boundary of the ganglion cell layer. The outlined cell is both positive for Brn3b and *Irx6*: βgal. (**B-B″**) In the adult *Irx6*$^{+/lacZ}$ mouse retina, the type 4 bipolar cell marker calsenilin does not co-immunolabel with *Irx6*:βgal, indicating that Irx6 is not expressed in type 4 OFF bipolar cells. The arrow indicates a cell that is positive for *Irx6*:βgal, but not for calsenilin; the arrowhead indicates a calsenilin-positive cell that is negative for *Irx6*:βgal.

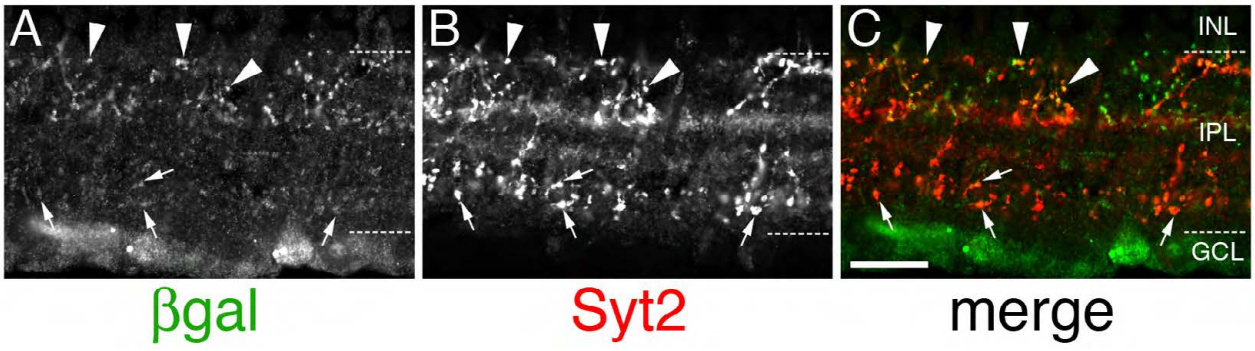

Fig. S3. *Irx6*:βgal is strongly expressed in the *Irx6*$^{lacZ/lacZ}$ mouse and can be visualized in the inner plexiform layer. Irx6:βgal (**A**) co-immunolabels with Synaptotagmin 2 (Syt2) (**B**,**C**) in both the upper and lower zones of the inner plexiform region, corresponding to the OFF and ON projecting regions. Other ON bipolar cells (type 5 or rod bipolar cells) do not show expression of the *Irx6*:βgal reporter as all of the Cabp5-expressing cells that co-immunolabeled with *Irx6*:βgal also expressed Hcn4. Scale bar 10 μm.

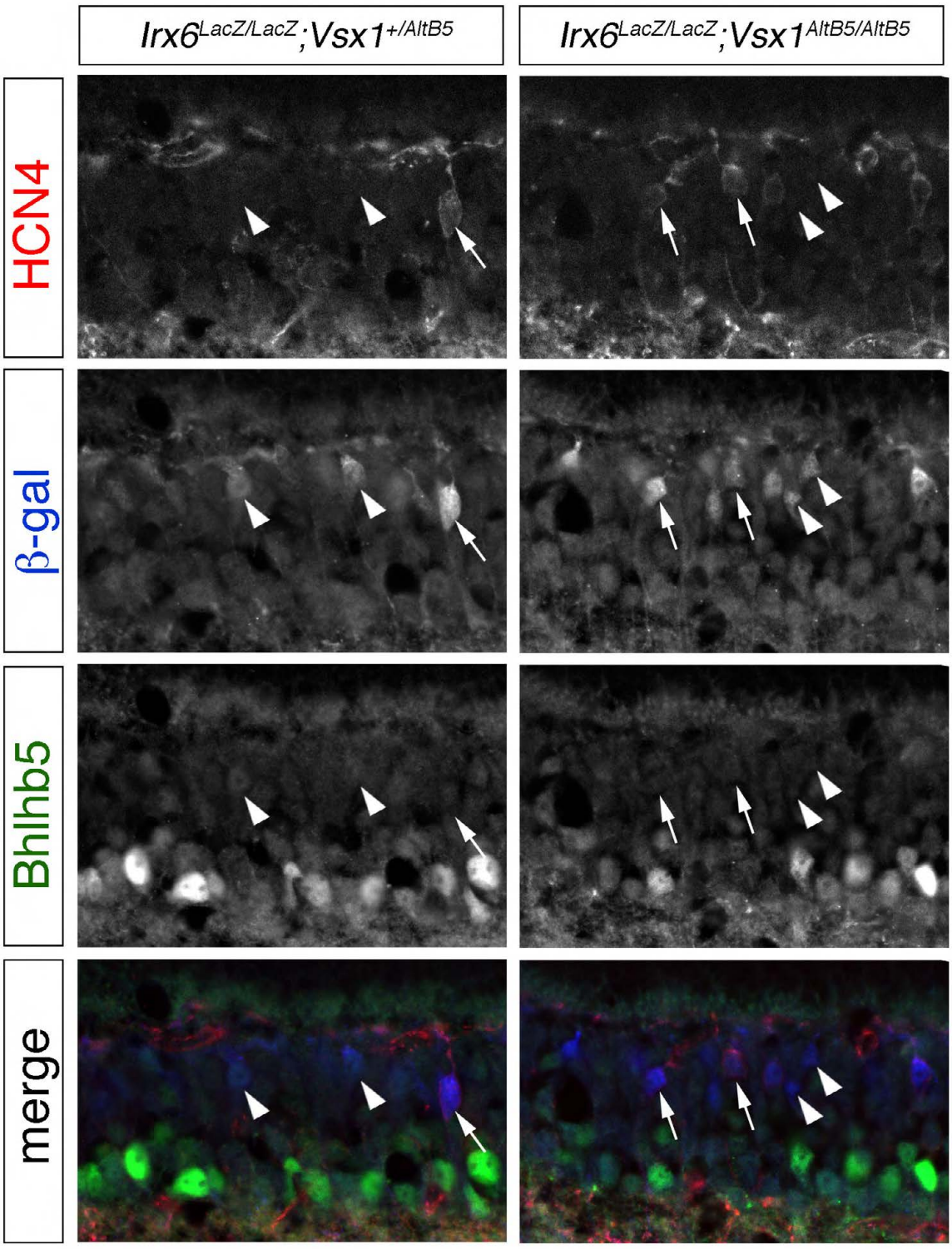

Fig. S4. In both the *Irx6*$^{lacZ/lacZ}$; *Vsx1*$^{+/AltB5}$ and *Irx6*$^{lacZ/lacZ}$; *Vsx1*$^{AltB5/AltB5}$ mouse, not all *Irx6*:βgal-positive cells express Hcn4. Arrowheads indicate *Irx6*:βgal-positive cells that do not express Hcn4 whereas arrows indicate *Irx6*:βgal-positive cells that express Hcn4. Bhlhb5 immunolabeling is present in some of the *Irx6*:βgal cells that do not co-immunolabel with Hcn4 in the *Irx6*$^{lacZ/lacZ}$; *Vsx1*$^{+/AltB5}$ retina.

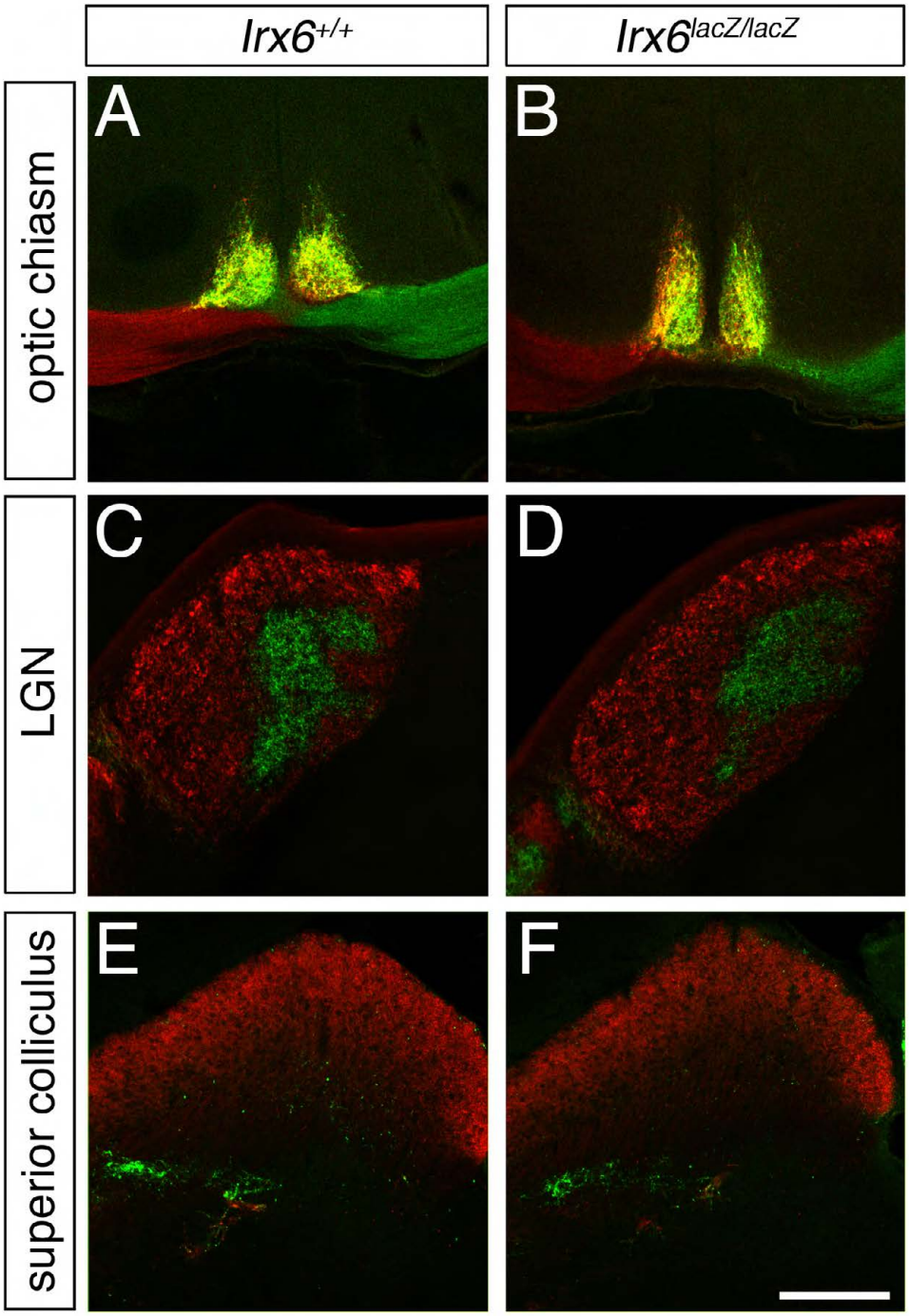

Fig. S5. Ganglion cell projections to the brain are grossly normal in *Irx6*$^{lacZ/lacZ}$ mice. As *Irx6* is expressed in a subset of retinal ganglion cells (supplementary material Fig. S2), we investigated whether *Irx6* is required for ganglion cell axon outgrowth, migration and targeting. Cholera toxin subunit B coupled to either Alexa Fluor 488 (left eye) or Alexa Fluor 555 (right eye) (Invitrogen), was injected into the intravitreal space of the eye (2 μl, 5 μg/μl per eye) using a 33G Hamilton needle at 2 months of age. After 24 hours, mice were euthanized and brain tissue fixed for 10 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS) by trans-cardial perfusion, followed by overnight fixation of tissue in 4% paraformaldehyde in PBS at 4°C. (**A**,**B**) The optic nerves carrying different dyes branched out contralaterally at the optical chiasm, with a subset of nerve fibers projecting ipsilaterally with no difference between *Irx6*$^{+/lacZ}$ (A) and *Irx6*$^{lacZ/lacZ}$ (B) mice. (**C**,**D**) In the lateral geniculate nucleus, nerves projecting contralaterally (red) and ipsilaterally (green) occupy distinct regions in a manner that was indistinguishable between control *Irx6*$^{+/lacZ}$ (C) and *Irx6*$^{lacZ/lacZ}$ (D) mice. (**E**,**F**) Similarly, in the superior colliculus, where most projections were formed contralaterally, no difference was observed between control *Irx6*$^{+/lacZ}$ (E) and *Irx6*$^{lacZ/lacZ}$ (F) mice. Scale bar: 100 μm in A,B,E,F; 200 μm in C,D.

Table S1. Antibody dilutions and sources

Antigen	Antiserum	Source	Working dilution[5]
Vsx1	Rabbit anti-Vsx1	R. L. Chow, University of Victoria, Victoria, BC	1:100
Recoverin	Rabbit anti-recoverin[1]	Millipore/Chemicon (AB5585)	1:500
NK3R	1. Rabbit anti-NK3R[2] 2. Rabbit anti-NK3R[3]	A. Hirano, Department of Neurobiology, Los Angeles, CA Calbiochem (480739)	1:500 1:5000
Cabp5	Rabbit anti-Cabp5	F. Haeseleer, Department of Ophthalmology, Seattle, WA	1:500
PKCα	Rabbit anti-PKCα	Sigma (P4334)	1:20,000
Calbindin	Mouse anti-calbindin	Sigma (C2724)	1:500
Chx10	1. Rabbit anti-Chx10 2. Sheep anti-Chx10	R. R. McInnes, Hospital for Sick Children, Toronto Exalpha (X1180P)	1:500 1:1000
β-Gal	1. Chicken anti-β-Gal[4] 2. Rabbit anti-β-Gal 3. Mouse anti-β-Gal	Abcam (ab9361) Cappel (55976), MP Biomedicals Sigma (G8021)	1:300/1:12,500 1:5000 1:500
Bhlhb5	Goat anti-β3 (E17)	Santa Cruz (sc-6045)	1:1000
HCN4	1. Guinea pig anti-HCN4γ 2. Rat anti-HCN4γ PG2-1A4	F. Müller, Forschungszentrum, Jülich, Germany	1:500 1:1
Irx5	Rabbit anti-Irx5	C. C. Hui, Hospital for Sick Children, Toronto	1:50
PKA RIIβ	Mouse anti-PKA RIIβ	BD science (612550)	1:3000
Calretinin	Goat anti-calretinin	Chemicon (AB1559)	1:2500
Brn3b	Goat anti-Brn3b	Santa Cruz (sc-31989)	1:100
Syntaxin	Mouse anti-syntaxin	Sigma (S0664)	1:500
Calsenilin	Mouse anti-calsenilin	W. Wasco, Harvard Medical School, Charlestown, MA clone 40A5	1:2000
Synaptotagmin	Mouse anti-Syt2/ZNP-1	Zebrafish International Resource Center	1:250

[1]Labeling for recoverin was carried out using 0.1% Triton X-100 in place of Tween 20.
[2]Immunolabeling for NK3R was done in the absence of horse serum.
[3]Mice were perfused with 4% PFA prior to enucleation and the retina was then left in 4% PFA for 20 minutes at room temperature.
[4]Chicken anti-β-gal shows some non-specific labeling of amacrine cells in this system
[5]Antibodies were diluted in PBS containing 1% horse serum and 0.1% Tween 20, except as noted above.

Table S2. Vectors used

Vector	Host	Reference to sequence number below‡ or vector source	Putative Irx6-binding site (IBS)*
pRecoverin_1461.luc	pGL4.26 *Kpn*I and *Hin*dIII sites	1	ACATGT
pVsx1_9130.luc	pGL3P *Sac*I and *Bgl*II sites	2	ACACGTGT
pVsx1_3377.luc	pGL3P *Sac*I and *Bgl*II sites	3	ACACGTGT
pVsx1_2232.luc	pGL3P *Sac*I and *Bgl*II sites	4	ACATGTGT
pNK3R_1398.luc	pGL3P *Sac*I and *Bgl*II sites	5	ACAGGTGT
pIrx6	pBSK-EF1_ *Sfl*I and *Xba*I sites	C. C. Hui, Hospital for Sick Children, Toronto	
pIrx5	pBSK-EF1_ *Sfl*I and *Xba*I sites	C. C. Hui, Hospital for Sick Children, Toronto	
Renilla	pRL-TK	Promega	
Luciferase with mini-promoter	pGL4.26	Promega	
Luciferase with SV40 promoter	pGL3P	Promega	

*Putative IBS identified using FIMO (http://meme.nbcr.net/meme/intro.html).
‡Appropriate restriction enzyme sites have been integrated into the primers and are found in the sequence below (primers are italicized and putative IBS is in bold).

1.*TGGTGAACAGTGCTGTGGATGTGCAA*GCCAATCAAACCATTTTCCTCCTCAAGTTGC
TTTTGCTCACGGTGCTCCACGGCAGCAATAGAAACCCTAACTAACACAAGCAGAGAACT
AAGGCAGCCCATTCCTAGGCAGAAACGCAGGGGAAGATGGGAATACGTATGTAGTTCCT
GGGGATGCCTTGAAAAAGAGAAATCCCAGTGTGTAAGGTCAGTATTGTATGGAGTACCA
GGTAATAGGGTGGCATCAGAAGGAAGGGGTGGGGTTTGCTTTCTAAAACTTACTATACA
CCCCAACAGTCCTCTAGTCTCTAAGGAAATGGCAGGGGCAGCTATGAATAACACATAAA
CACAACACCAAAATGTATCTAAAATTACAACCCCTGAGACTACACTCAAATGACACATT
CACCATTGCCAATGTGACAGGCCTCTCTGTCTCTTGCCACCTGCCTGGTCTGCCTGCTC
ACTCCTCCTAGGCAGCTTTATTCTACCTTTGTTACTTAGGAACAATAACACCAGGGCTT
AACTTATAATAGAGACTCACAACGGACCCAGGTCCCCATGACCCAGTCTTGCCTTGAAC

ACACCATAAGTAA**ACATGT**ATAGAGCGCATCCAACACACTGAACTTTGTAACTCAGGCT
TACCCACCTCTGTCCGCTCAGAACACTCACGTTGGACAAAATCCCGGAGAAATAATCTC
CTGTGGTTGTATAAGGCATGTTGTGGTACTGTGGTAAAGTCAAAACATTTAAACTGTGG
TAGATCAAAACATTTAGGTGAGCCTATAGTGAGTCTGGAGCCGTGTTTGTGTGTGTGTG
TGTGTGTGTTCAGCACCTTTTCCAGCCAGTTTGAGCTATTTTGTACTGTTCTGGCAGTG
GAGATAACAACCCACAGAACACAATCTTTCCCTATCTGGCACTTCCATTCTGGCAGACA
GTTTGGGGTTTCCCAGCAGCGGTGAGGAAGGAGCCATCAGAGAGTGATGGATAAAAGGT
CTTGCTGAGAAGGTGAAGTGTCTAGGCCCAAATGTGACTGGGGGAGGATTAGAAAAACT
CCCATGCAGTGTAGGTTAAGTGGTAGGGCTGCCTTTAGACCCTAAGCTCCCAAACTCCT
GCACTATAAGGTCCCATGGGTGCCCCTCAGATGGGGAGACAAACAGCAAGCAGTGTGGC
TCTTCGGGGGCTTAAATGCCGAGGATGCTTGGTTCAAGGGTCCCTCCTTGTTCCTTTCT
TCTCAACAAGCACAGGAAAGGTAAGTGGCTGCTCTTGGCTCTTTATTTTAATCTCAGAA
GGGTGCCCCTCCTCCCTCCAAGGACTGGGCAGACCTGGCCGCAAATCCC*CCCTAATCCA*
TCAGCACCTGGGTC

***a region containing more than 44% GT repeat and 20% TA repeat has been deleted from the above sequence.

2.*TACGAGCTCTTCCATAATCTGTCCATTGG*TGAGAGTGGGGTGTTGAAATCTCCCACT
ATTATTATGTAATGTTCAATGTGTGTTTTGAGCTTTAGTAATGTTTCTTTT**ACACGTGT**
GGGTGCCCTTGCCTATGGGGCATACATGTTTAGAATTGAGAATTCAATTTGGATTTTTC
GTATGGTGGGTATGAATAAGATTTCCCATCTCTTTTGATAACTTTTGGTTGAAAGTCTA
TTTTACTGGATATTAGAAT*GGCTACTTCAGCTTGTTTCTAGATCTGTT*

3.*TACGAGCTCAGTACTTGACGATGAGGTCC*CTGGACACAAAGCTGGAGTCAGTGTCTG
AGTATAGATTCATGGGAGGGTCGGATCTCAGAACAGAGGGATAGCAGAGAAGCAGGAGA
AGGAGCCACGCAAAGTTGCCACTGCCTTATCCACGAATGGGGAGGAACTGGAAACAGAC
ACTGCAATTGTCCTCCTTGAA**ACACGTGT**GTGACCTCTTAGTAACTCAGTGCTGAGACT
CTGTTTTGTGGGTTTTCTGTCTACGGAAGAACAATTGCCCCCATCGAGGCAGGGTAAAA
AGCCAGTCCATATTTATCTAGTACAAGGGTCCAAGAACGGAGTCTGAGTGGGACACCAA
CAACCTGGGAA*GGAGAAAGCAATGTGGAACGAGATCTGCGATCTG*

4.*TACGAGCTCCGAATCTGATAATAGTTTGGC*TTTTAGCTTTTCTTACCCTGAGATTTT
TTGATATGTTGAATATGGGTAGATAACCTCACAGTTGAATGGTATTGTTTTCAGGGGAA
TTTATACATTACTGAG**ACATGTGT**GTCATGGGTGCACAAGCTTCCTGGGCTATGTCTCT
AGGATTCGTGCATCAGGGCAGGGTGAGTTTTGACAAAGATTT*GATGTCACAAATCCTGC*
CTTAGATCTGTT

5.*TACGAGCTCCAACAGGCAGTAATGATCAC*ATATTTATTTGATAAAAAAAAATGTGTC
TTTTTGAAATGCATTCTGCCTAATAGCATTGATTTCTTTTTCCACTTTGTAAATAACTC
CACCAAAATCGTGGATGTGACATC**ACAGGTGT**TAGTTTTCTACTGACATGTGGTACTTA
CTACATTATTAGCAATTCCTGAAAGCCTGTTTTGTTGCCTGAAAGACCACTTCTCTATC
GTCATCGCCACCTTGATACCCAGATCCCAAGTGAAAGGTATAATTACTTCTTCTA*CCGA*
TCTGAATATCGTTATCTAGATCTGTT