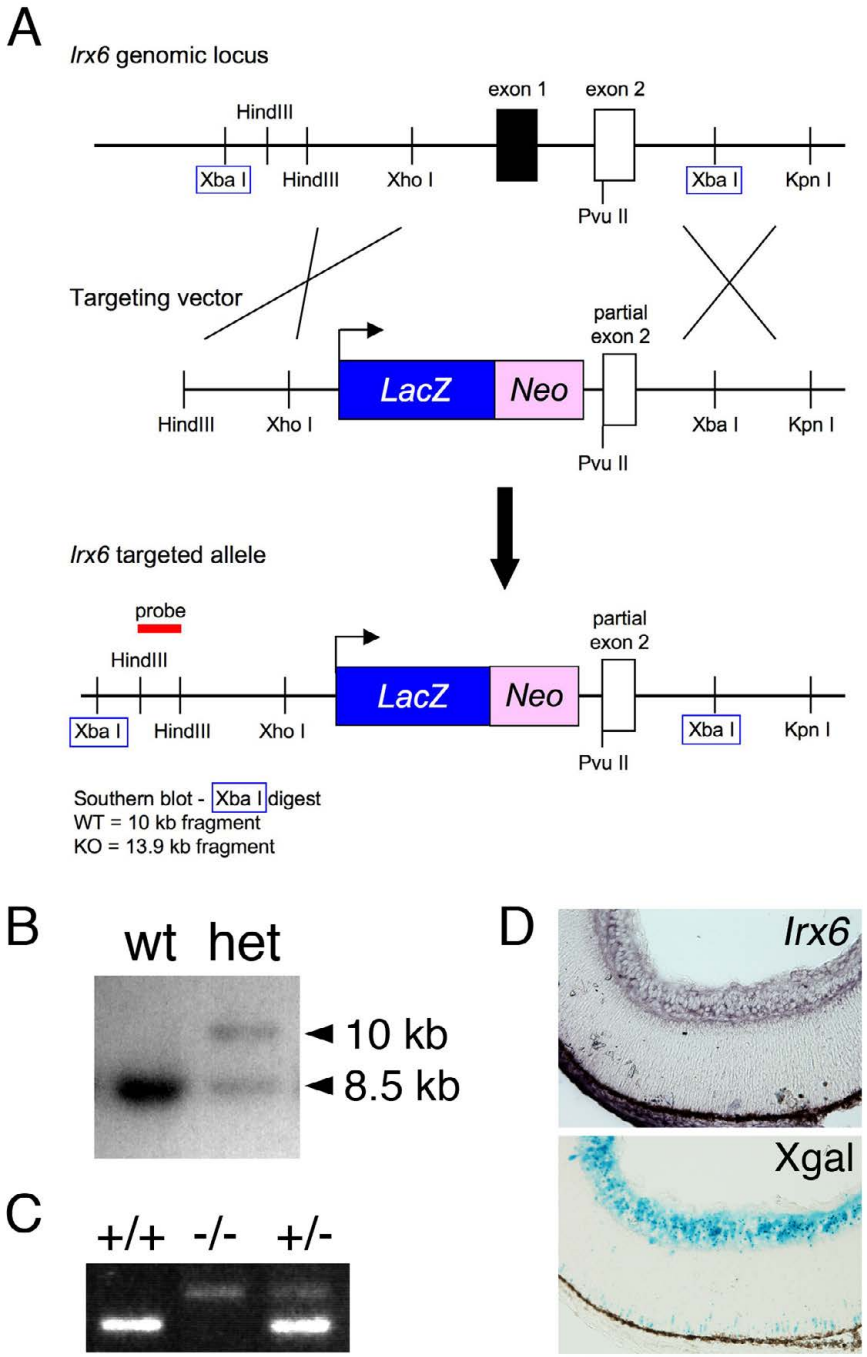

Fig. S1. Targeted disruption of *Irx6*. A 5.5 kb *Nco*I/*Hin*dIII *Irx6* fragment was cloned as the 5′ arm of the targeting construct upstream of the *lacZ* gene. The targeting construct contained a neomycin resistance cassette and a thymidine kinase cassette (*Pgk-Neo-SV40 polyA*) that was cloned in 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.

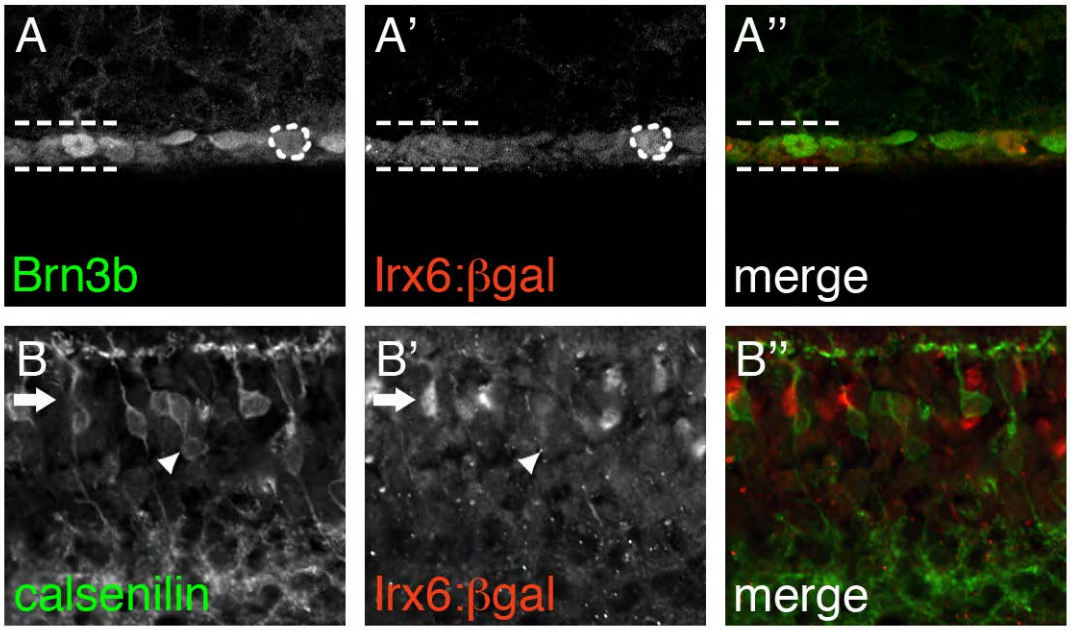

Fig. S2. The *Irx6*:βgal reporter is expressed in a subset of ganglion cells, but is not expressed in type 4 bipolar cells. (**A-A″**) The *Irx6*: βgal reporter co-immunolabels with the ganglion cell marker Brn3b in a subset of cells in the adult *Irx6*$^{+/lacZ}$ mouse retina. The dashed lines indicate 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.

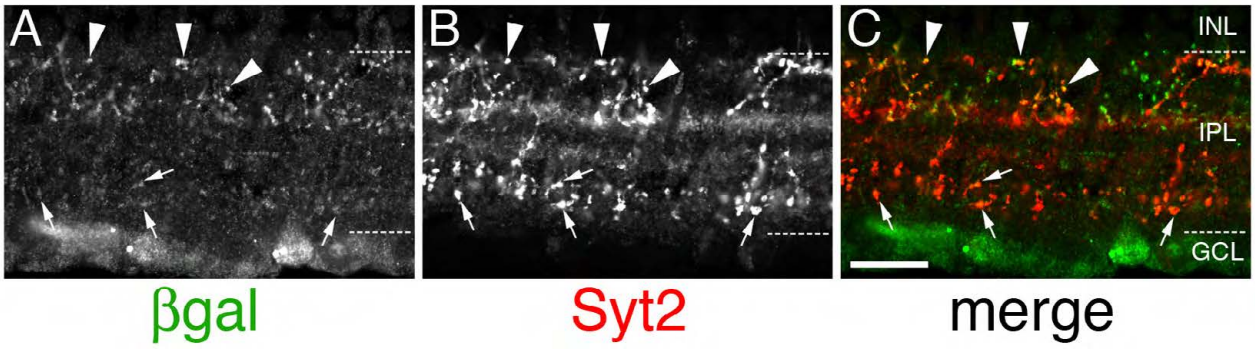

Fig. S3. *Irx6*:βgal is strongly expressed in the *Irx6*$^{lacZ/lacZ}$ mouse and can be visualized in the inner plexiform layer. Irx6:βgal (**A**) co-immunolabels with Synaptotagmin 2 (Syt2) (**B**,**C**) in both the upper and lower zones of the inner plexiform region, corresponding to the OFF and ON 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.

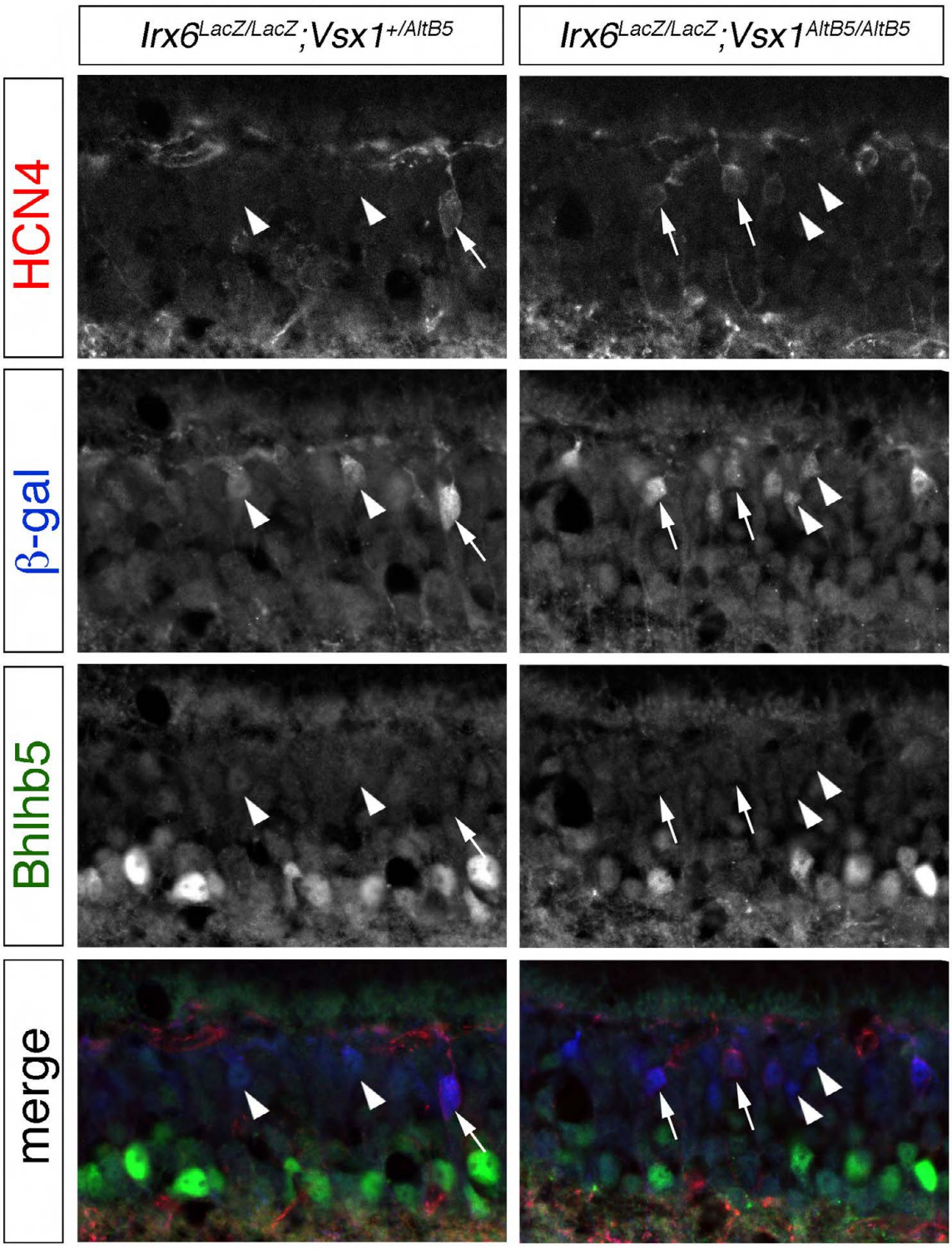

Fig. S4. In both the *Irx6*$^{lacZ/lacZ}$; *Vsx1*$^{+/AltB5}$ and *Irx6*$^{lacZ/lacZ}$; *Vsx1*$^{AltB5/AltB5}$ mouse, not all *Irx6*:βgal-positive cells express Hcn4. Arrowheads indicate *Irx6*:βgal-positive cells that do not express Hcn4 whereas arrows indicate *Irx6*:βgal-positive cells that express 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.

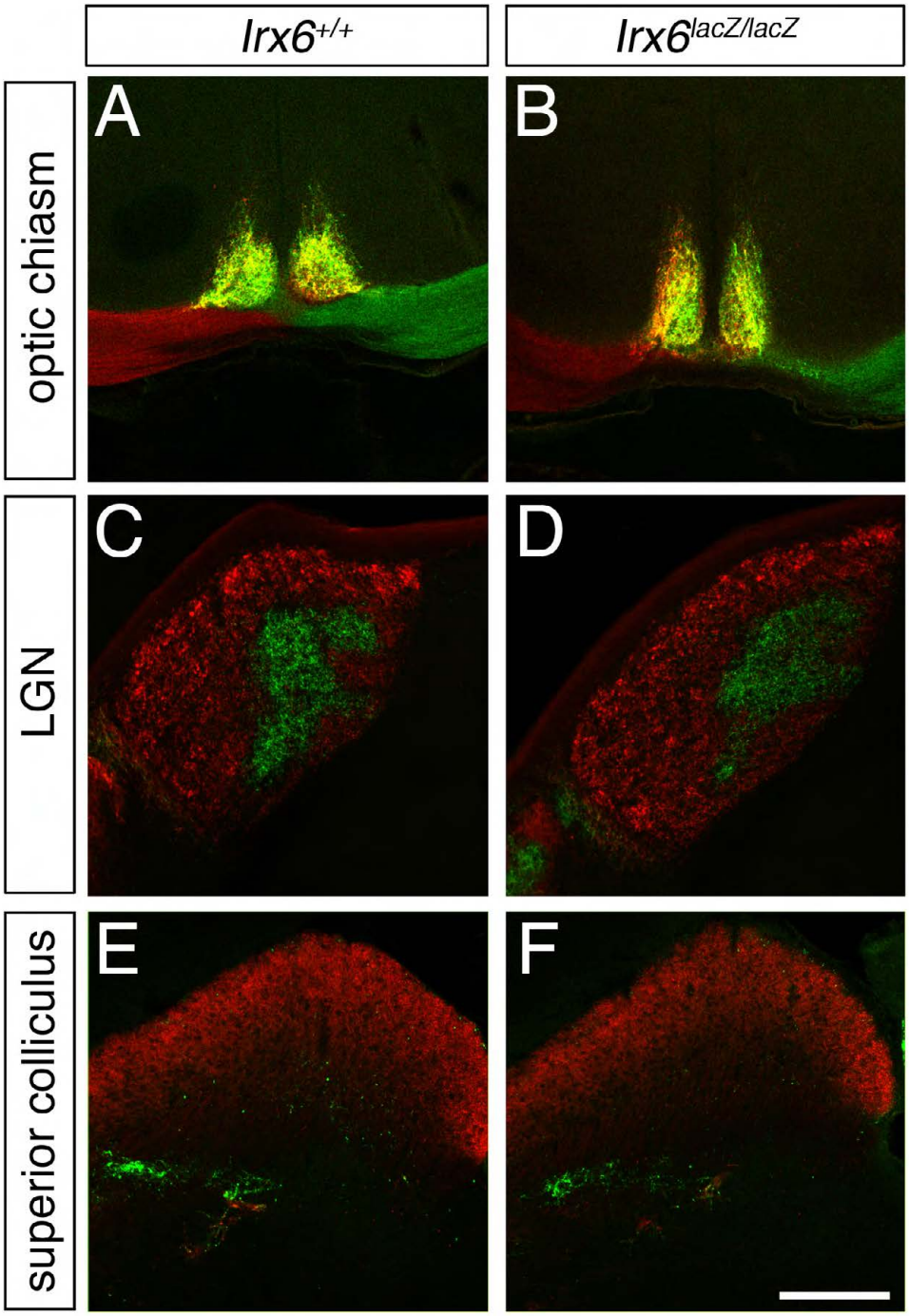

Fig. S5. Ganglion cell projections to the brain are grossly normal in *Irx6*$^{lacZ/lacZ}$ mice. As *Irx6* is expressed in a subset of retinal ganglion cells (supplementary material Fig. S2), we investigated whether *Irx6* is required for ganglion cell axon outgrowth, migration and targeting. Cholera 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