Otx2 loss arrests photoreceptor differentiation in CRX-associated dominant congenital blindness

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Inventory

Supplemental data: 6 figures and 3 Tables

Figure S1, related to Figure 2; Figure S2, related to Figure 5; Figures S3 and S4 related to Figure 6; Figure S5 and S6, related to Figure 7; Table 1, related to Figure 6

Supplemental experimental procedures

Supplemental references

Supplemental Data

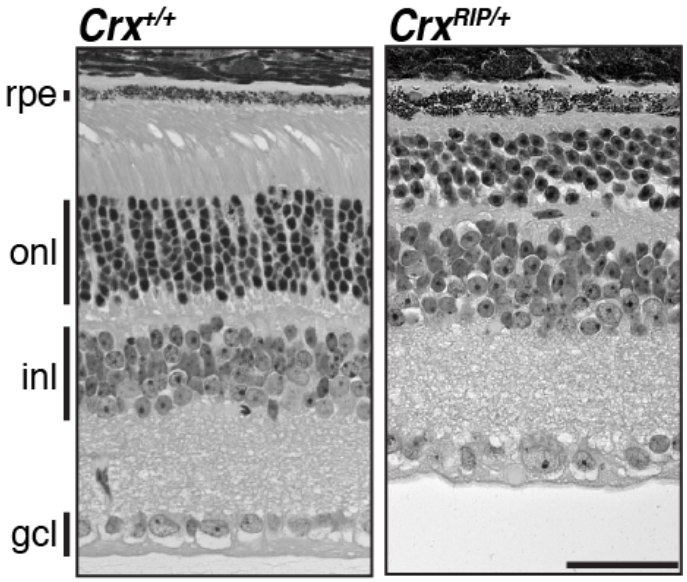

Figure S1:

Long-term preservation of the outer nuclear layer in *Crx*$^{RIP/+}$ mutant retina.

H&E staining of methacrylate sections of 18-month old WT and *Crx*$^{RIP/+}$ retina demonstrates that more than half of the photoreceptors are still present despite no visual function since birth. *Rpe*, retinal pigment epithelium; *onl*, outer nuclear layer; *inl*, inner nuclear layer; *gcl*, ganglion cell layer. Scale bar, 40 μm.

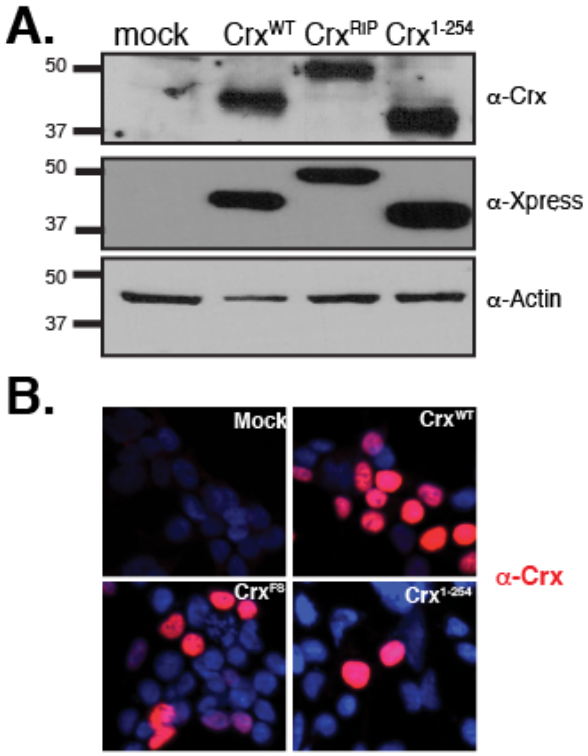

Figure S2: Crx^{RIP} and Crx$^{1-2254}$ are produced in transfected cells and localized in the nucleus

(A) Immunoblot analysis of protein extracts from HEK293T cells transfected with pcDNA4c control vector (Mock) or constructs including Xpress-tagged Crx^{WT}, Crx^{RIP}, or Crx$^{1-254}$. The blots were probed with anti-Xpress or anti-Crx antibody. Anti-actin antibody was used as loading control. (B) Immunocytochemistry of HEK293T cells with anti-Crx antibody (*red*). Nuclei were counter-stained with DAPI (*blue*). The following constructs were used: empty pcDNA4c (mock), Crx^{WT}, Crx^{RIP}, or Crx$^{1-254}$.

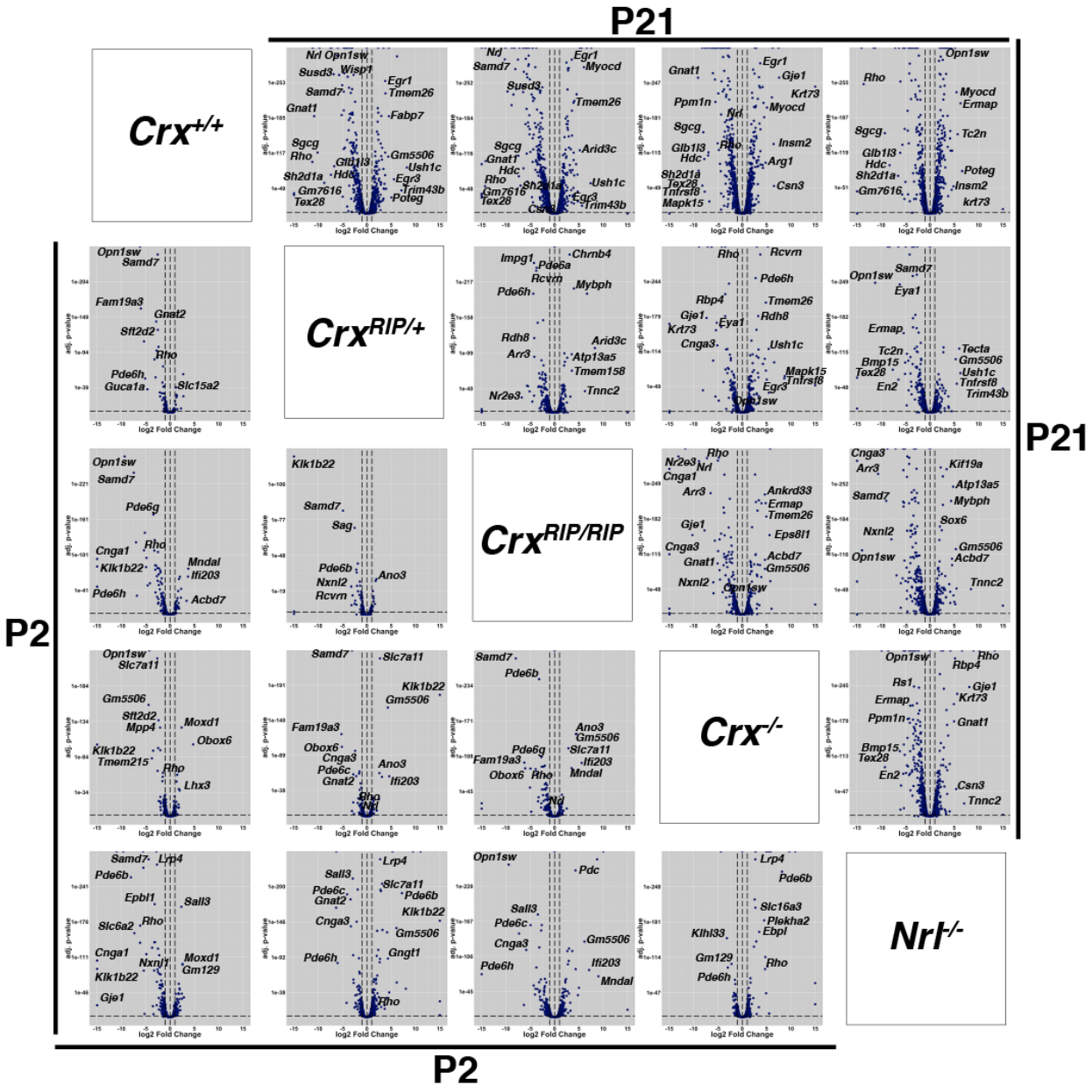

Figure S3: Volcano plots of genes differentially expressed between WT and Crx mutant retina at P2 and P21.

Volcano plots represent the FC and significance correlation (adjusted p-value (APV)) between *Crx*$^{+/+}$, *Crx*$^{RIP/+}$, *Crx*$^{RIP/RIP}$, *Crx*$^{-/-}$, and *Nrl*$^{-/-}$ retina at two different stages of development – P2 (peak of rod birth) and P21 (mature rods). Dashed lines represent the threshold for FC ≥ 2 and APV ≤ 0.05.

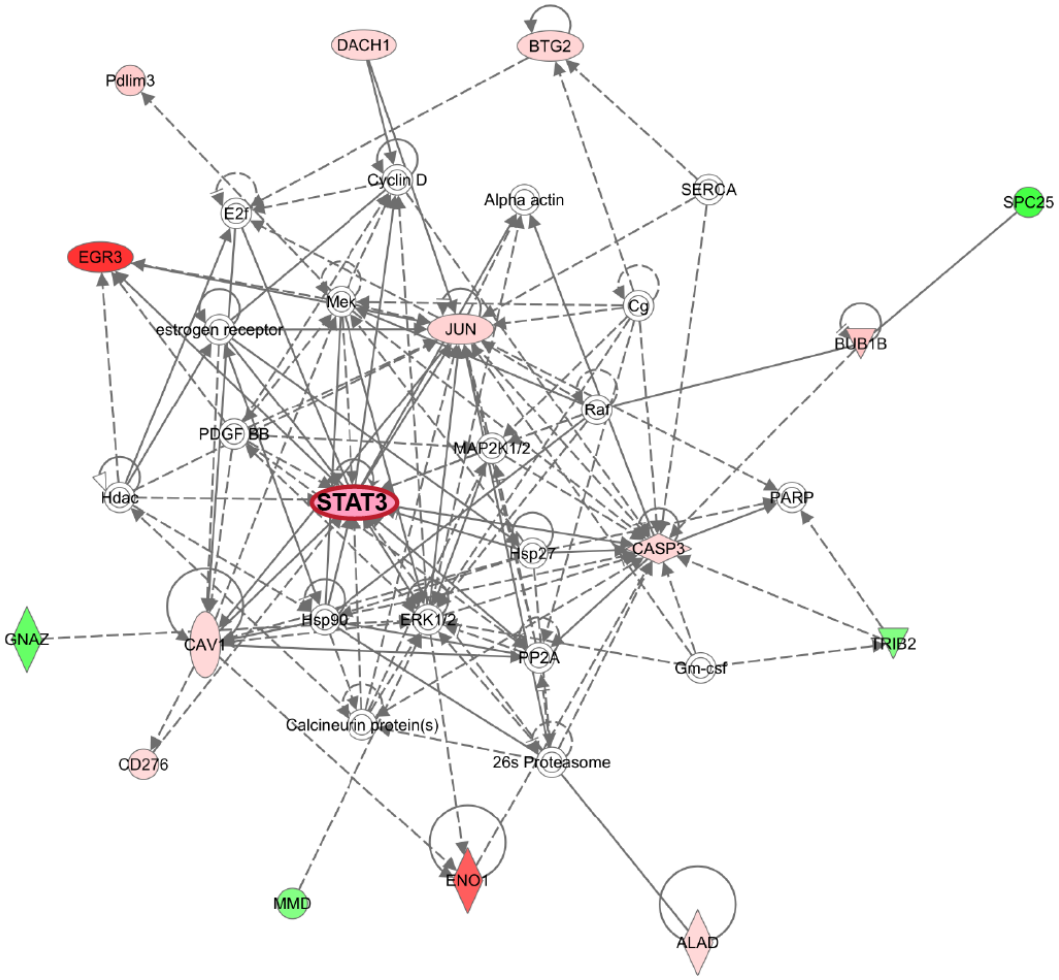

Figure S4: A cluster of genes showing differentially expression only between *Crx*$^{RIP/+}$ and *Crx*$^{+/+}$ retina at P21 belongs to cell death and survival network involving STAT3 as a central node.

Network reconstruction was done using 69 genes differentially expressed between P21 *Crx*$^{RIP/+}$ and *Crx*$^{+/+}$ retina. The expression of these genes was unchanged between *Crx*$^{-/-}$ or *Nrl*$^{-/-}$ and *Crx*$^{+/+}$ retina. Up-regulation of the gene of interest between *Crx*$^{RIP/+}$ and *Crx*$^{+/+}$ mouse retina is indicated by red and down-regulation by green.

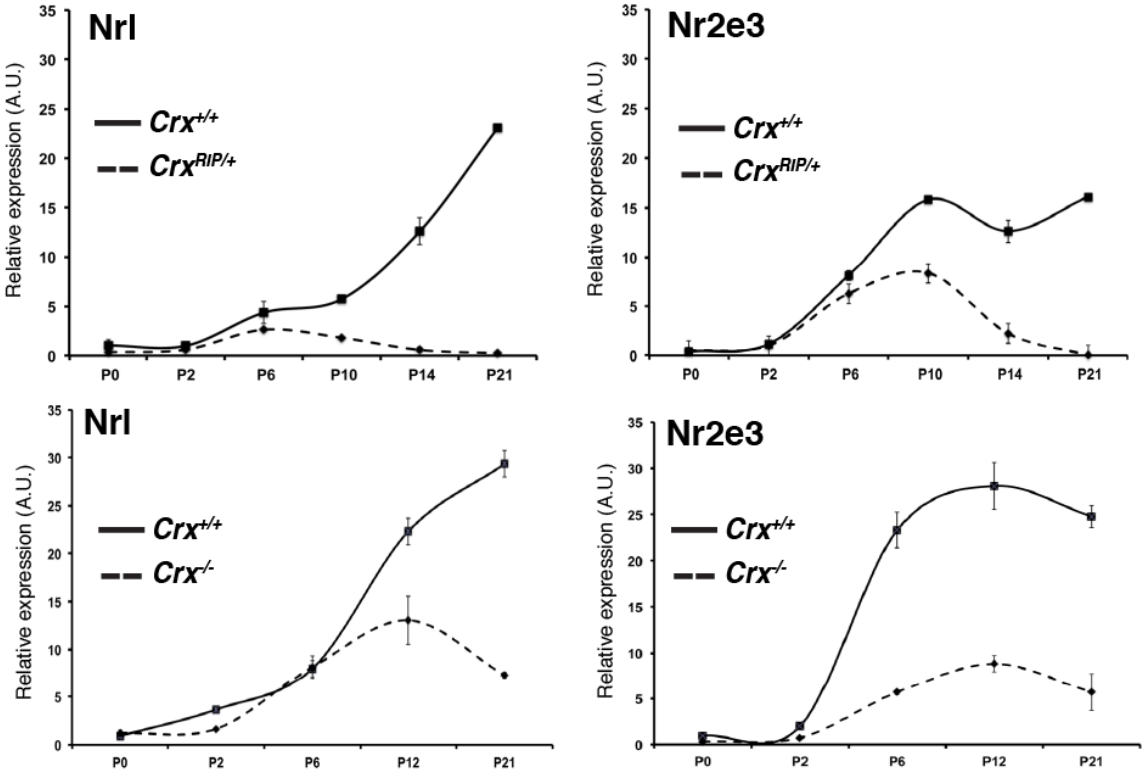

Figure S5: *Nrl* expression rapidly declines in *Crx*^{Rip} mutant retina.

Differential expression analysis by qPCR of *Nrl* and *Nr2e3* expression in *Crx*$^{RIP/+}$ (*top panel*) and *Crx*$^{-/-}$ retina (*bottom panel*) compared to littermate controls at P0 and P21. All values are expressed as mean ± SEM from 3 biological replicates and compared to mRNA expression at P0. The data was normalized against the average of two housekeeping genes: *Actβ* and *Hprt*. Solid lines, *Crx*$^{+/+}$; dashed lines, *Crx*$^{RIP/+}$ or *Crx*$^{-/-}$.

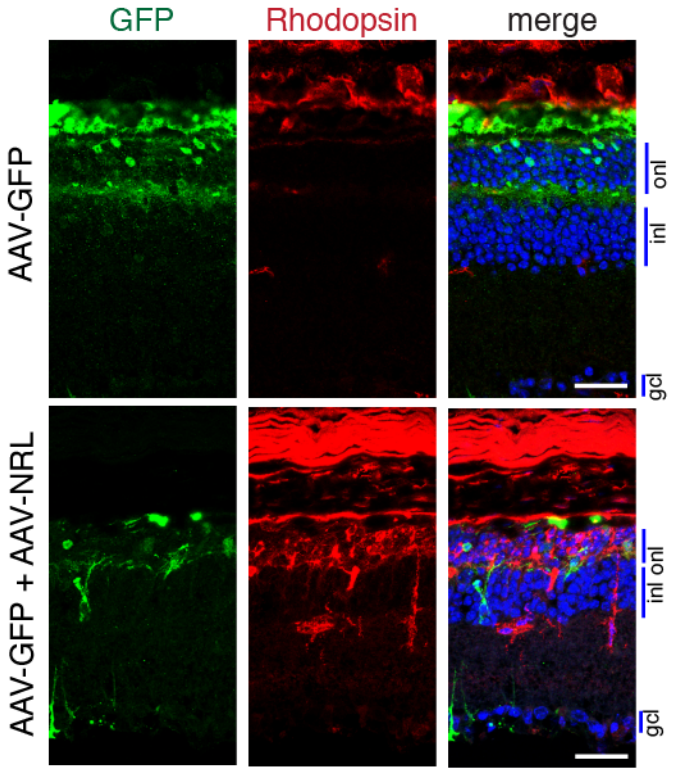

Figure S6: Expression of *Nrl* using AAV in adult Crx$^{Rip/+}$ mutant restores Rho expression.

Immunolabeling analysis of Rho (*red*) in 4-month old *Crx*$^{Rip/+}$ mice after injection of AAV GFP as control or AAV-GFP and AAV-NRL in 2 month-old animals. The infected cells are visualized with GFP (*green*). *onl*, outer nuclear layer; *inl*, inner nuclear layer; *gcl*, ganglion cell layer. Scale bar, 40 μm.

Gene Symbol	Entrez Gene Name	P2					P21				
		Crx$^{+/+}$	*Crx*$^{RIP/+}$	*Crx*$^{RIP/RIP}$	*Crx*$^{-/-}$	*Nrl*$^{-/-}$	*Crx*$^{+/+}$	*Crx*$^{RIP/+}$	*Crx*$^{RIP/RIP}$	*Crx*$^{-/-}$	*Nrl*$^{-/-}$
Transcription Factor											
Thrb	thyroid hormone receptor beta	6	4	2	4	4	14	13	11	12	18
Rxrg	retinoid X receptor gamma	9	5	3	7	5	3	17	7	32	19
Crx	cone-rod homeobox containing gene	204	211	167	112	153	347	293	197	170	321
Neurod1	neurogenic differentiation 1	196	211	201	180	114	153	231	212	234	166
Otx2	orthodenticle homolog 2 (Drosophila)	329	396	460	441	259	81	153	276	199	117
Neurod4	neurogenic differentiation 4	205	240	253	219	180	51	76	154	107	72
Pias3	protein inhibitor of activated STAT 3	32	26	19	27	28	34	23	13	21	31
Rorb	RAR-related orphan receptor beta	55	54	63	61	56	30	13	17	24	13
Rora	RAR-related orphan receptor alpha	7	7	7	7	7	24	29	25	20	27
Rxra	retinoid X receptor alpha; similar to retinoid X receptor-alpha	13	10	11	10	10	9	6	4	5	6
Neurod2	neurogenic differentiation 2	3	3	3	3	2	3	3	3	3	2
Rorc	RAR-related orphan receptor gamma	3	2	3	4	2	1	2	3	4	1
Neurog2	neurogenin 2	14	13	14	11	8	1	1	1	1	1
Nrl	neural retina leucine zipper gene	75	68	40	77	0	447	1	0	85	0
Nr2e3	nuclear receptor subfamily 2, group E, member 3	132	137	66	123	0	211	1	0	114	0
Esrrb	estrogen related receptor, beta	1	1	1	1	1	83	2	0	6	2
Mef2c	myocyte enhancer factor 2C	0	0	0	0	0	17	1	1	1	1
Visual transduction											
Opn1sw	opsin 1, short-wave-sensitive	42	1	0	2	40	153	1	0	3	2400
Gnb3	guanine nucleotide binding protein, beta 3	104	39	19	116	147	121	257	126	338	728
Arr3	arrestin 3, retinal	1	0	0	0	0	120	9	1	44	693
Opn1mw	opsin 1 (cone pigments), medium-wave-sensitive	0	0	0	0	0	101	1	1	1	255
Pde6h	phosphodiesterase 6H, cGMP-specific, cone, gamma	8	0	0	2	13	82	94	5	14	562
Gnat2	guanine nucleotide binding protein, alpha transducing 2	11	2	1	7	14	76	160	91	127	785
Pde6c	phosphodiesterase 6C, cGMP specific, cone, alpha	2	1	0	4	8	32	98	60	45	427
Cngb3	cyclic nucleotide gated channel beta 3	7	3	2	7	6	5	16	26	13	92
Cnga3	cyclic nucleotide gated channel alpha 3	3	1	0	3	5	4	0	0	6	50
Prph2	peripherin 2	50	31	8	34	14	1519	334	178	276	350
Pdc	phosducin	143	121	62	178	3	1295	228	196	715	538
Rbp3	retinol binding protein 3, interstitial	149	128	106	138	101	758	832	525	760	981
Rcvrn	recoverin	7	2	0	3	5	526	96	7	7	246
Guca1a	guanylate cyclase activator 1a	4	0	0	1	2	383	127	58	116	446
Rs1	retinoschisis (X-linked, juvenile) 1	2	0	0	0	0	366	78	37	22	198
Grk1	G protein-coupled receptor kinase 1	2	2	1	1	1	295	119	81	99	184
Gnb5	guanine nucleotide binding protein , beta 5	19	17	15	17	18	229	130	111	128	194
Rgs9	regulator of G-protein signaling 9	11	7	6	13	12	101	46	38	81	83
Gucy2f	guanylate cyclase 2f	0	0	0	0	0	45	1	0	1	1
Rho	rhodopsin	8	1	0	3	0	7683	4	1	68	1
Gnat1	guanine nucleotide binding protein, alpha transducing 1	2	1	1	1	1	4363	3	0	9	0
Gnb1	guanine nucleotide binding protein, beta 1	193	175	154	188	142	1318	67	68	116	59
Pde6g	phosphodiesterase 6G, cGMP-specific, rod, gamma	35	12	3	21	27	1296	213	72	99	516
Rom1	rod outer segment membrane protein 1	74	66	40	84	12	849	121	82	274	191
Pde6b	phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide	16	9	2	17	0	539	30	7	177	15
Guca1b	guanylate cyclase activator 1B	1	0	0	0	0	538	36	10	10	50
Pde6a	phosphodiesterase 6A, cGMP-specific, rod, alpha	1	0	0	0	0	488	21	2	45	38
Cnga1	cyclic nucleotide gated channel alpha 1	5	0	0	1	0	236	0	0	57	0
Pde6d	phosphodiesterase 6D, cGMP-specific, rod, delta	29	31	28	27	16	57	68	56	46	69
Cngb1	cyclic nucleotide gated channel beta 1	0	0	0	0	0	6	0	0	1	0

Table S1. A summary of selected expression changes based on RNA-seq analysis of P2 and P21 retina from various mutants compared to WT control.

Global gene expression profiles were generated from *Crx*$^{+/+}$, *Crx*$^{RIP/+}$, *Crx*$^{RIP/RIP}$, *Crx*$^{-/-}$, and *Nrl*$^{-/-}$ retina by RNA-seq. Normalized expression values are shown for transcription factor genes associated with photoreceptor development and genes involved in visual transduction. Dark and light grey areas indicate normalized expression values of rod- and cone-specific genes, respectively.

Primary Antibodies	*Type*	*Source*
Nrl	Rabbit polyclonal	Swain et al., 2001
Crx, clone H-120	Rabbit polyclonal	Santa Cruz
Rho4D2	Mouse monoclonal	R. Molday
Opn1sw	Rabbit polyclonal	C.M. Craft
Opn1mw	Rabbit polyclonal	C.M. Craft
Cone Arrestin	Rabbit polyclonal	Millipore
Recoverin	Rabbit polyclonal	Millipore
Peanut Agglutinin (PNA)	Conjugated to Alexa Fluor 568	Life Technologies
β-Actin	Mouse monoclonal	Millipore
Secondary Antibodies	*Type*	*Source*
Anti-rabbit Alexa 488	Goat polyclonal	Life Technologies
Anti-mouse Alexa 568	Goat polyclonal	Life Technologies
Anti-rabbit light chain, HRP conjugate	Goat polyclonal	Jackson ImmunoResearch Laboratories
Anti-Mouse light chain, HRP conjugate	Goat polyclonal	Jackson ImmunoResearch Laboratories

Table S2. List of antibodies used for immunoblotting and immunohistochemistry.

Gene	Forward Primer	Reverse Primer
Rho	CGCGAGATATCATACTAACGCTAATC	GCCCCTCTGCAAGCCAAT
Gnat1	GGACAGGTGAAGCTCAGTCTCCTA	GGTGCCAAGTCAGCAGTTGA
Gnat2	GGATGGGTGTCCATAAAGAACCT	GCCTGGGTCCAGCTTACAGAT
Gnb1	CAGAGGACAACCTGAAGGAGTTC	CCAGAAGGTATCTGCTGTCAAACC
Opn1mw	CGATGTCAGCATCCTTTTTTCTG	GACATCTAATCTTCCAGGCTTCCA
Opn1sw	GCCTGCCCTTCAGACACATC	GAGCAGAAGGGAAGAAAGAGCTT
Nrl	ACCGCGCCCAGACAGA	GTTGTGGTGTTCAGGACAAGGA
Nr2e3	CCCGCCAGGCTCACCTA	ACGACTATGACTCCTCAATGGTTAGAT
Rorb	GCTCACATAATCCACAACAGCTTT	TGAAGCAGAGCGGTGTCACT
Thrb	CCCTTTTGAGGTCCCCATCT	ATACATGCAGGGTGTCTGGAGTAC
MyoD	TCCAGCAACCCGAACCA	CAGCAGAGCCTGCAGACCTT

Table S3. List of primers used for ChIP-qPCR.

Supplemental experimental procedures

Animals and tissue collection

Mouse eyeballs were fixed in 4% paraformaldehyde for one hour on ice, incubated in an increasing concentration of PBS/sucrose (10%, 20%, 30%), embedded in Tissue-Tek CRYO-OCT compound (Thermo Fisher Scientific), frozen using cold acetone and sectioned at 10 mm. For methacrylate sections, eyeballs were first fixed for 30 min in 2% glutaraldehyde before overnight fixation in 4% paraformaldehyde. After embedding in methacrylate, 5 mm-thick sections were cut and stained with hematoxylin and eosin (H&E). For RNA and protein extraction, the retina was quickly isolated and frozen at -80°C.

Plasmid Constructions and Site-directed mutagenesis

Mouse wild-type *Crx* (Crx^{WT}) (900 nucleotides), Crx^{1-254} (768 nucleotides) or Crx^{RIP} mutant (1164bp) DNA was amplified by PCR from adult retinal cDNA using a common forward primer, 5'-AAATTTTGATATCCAATGATGGCATATATGAACCCGGG-3' including a *EcoRV* restriction site and the following reverse primers containing a *XhoI* restriction site: 5'-ATATATCTCGAGCTACAAGATCTGAAACTTCCAG-3' for Crx^{WT}; 5'-ATATATCTCGAGCTATGACAAGGAGGTGGGGGACTG-3' for Crx^{1-254} and 5'-ATATATCTCGAGTCAGTCATGACCTGTACTGACTG-3' for Crx^{RIP}. PCR products were sub-cloned into pcDNA4c His/MaxC vector (Life Technologies) using EcoRV and XhoI restriction sites. For *in vivo* electroporation, human CRX^{WT}, CRX^{L237}, CRX^{G255}, and CRX^{P263} DNA were amplified by PCR from

human retinal cDNA using the forward primer, 5'-TGGTACCCACCATGATGGCGTATATGAACCCG-3' and the reverse primer 5'-AGCGGCCGCCTAGGACCTGTGAGCTGTC-3'. The PCR products were sub-cloned at *KpnI-NotI* sites of Ub-GFP vector after removing GFP cassette (1). In this vector, the ubiquitin (Ub) promoter is transcriptionally active in all retinal cells. The different mutants were generated using QuickChange XL site directed mutagenesis kit (Stratagene). Rho-dsRed and Ub-GFP have been previously described (1).

Electrophoretic mobility shift assays

The following modifications were made in the previously described method (2): HEK293T cells were transiently transfected with pcDNA4c-Crx^{WT}, pcDNA4c-Crx^{RIP}, pcDNA4c-Crx^{1-254} or with the empty vector pcDNA4c. Nuclear extracts from transfected cells were prepared according to manufacturer instructions (NE-PER, Thermo Scientific). The oligonucleotides were: mouse rhodopsin promoter forward, 5′-ATCTCGCGGATGCTGAATCAGCCTCTGGCTTAGGGAGAGAAGGT-3′, mouse rhodopsin promoter reverse, 5′-ACCTTCTCTCCCTAAGCCAGAGGCTGATTCAGCATCCGCGAGAT-3′; mouse S-Opsin promoter forward, 5'-GGTGAGAACTAAGAGATCTCTAATCTGGGACTTTGTCTTTGGGA-3', mouse S-Opsin promoter reverse, 5'-TCCCAAAGACAAAGTCCCAGATTAGAGATCTCTTAGTTCTCACC-3'; mouse

Nrl promoter forward, 5'-GCTCTAAGAGGCGTTAGGCGATTAGGCTGAAAATGTAGGTCACA-3', mouse Nrl promoter reverse, 5'-TGTGACCTACATTTTCAGCCTAATCGCCTAACGCCTCTTAGAGC-3'). All oligonucleotides contain a CRX binding element (3-5). The nuclear extract (5 μg) was first incubated with 1 μg of poly (dIdC) and 1 μg of salmon sperm DNA at 4°C for 15 min in binding buffer (12 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.9; 60 mM KCl; 4 mM MgCl2; 1 mM EDTA [ethylenediaminetetra acetic acid]; 12% glycerol; 1 mM dithiothreitol; and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). ^{32}P-Labeled double-stranded oligonucleotide (30,000 cpm) was added to the reaction and incubated at 4°C for another 20 min. In competition studies, nuclear extracts were pre-incubated with 100-fold excess of unlabeled oligonucleotide for 30 min at 4°C and then incubated with labeled oligonucleotide for an additional 20 min. To examine the presence of Crx in protein-DNA complexes, nuclear extracts were incubated with 1 µg of anti-Xpress antibody for 15 min at 4°C, followed by the addition of labeled oligonucleotide and a further incubation for 20 min at 4°C. The reaction mixtures were electrophoresed on 8% polyacrylamide gels at 80 V for 2 h and subjected to autoradiography.

Whole transcriptome sequencing (RNA-seq) and data analysis

RNA was extracted from 2 independent frozen retina samples each dissected at P2 or P21 from *Crx*$^{+/+}$, *Crx*$^{RIP/+}$, *Crx*$^{RIP/RIP}$, *Crx*$^{-/-}$, and *Nrl*$^{-/-}$ mice. Tissue was lysed

with mortar and pestle in 1 ml of Trizol (Life Technologies, Carlsbad, CA), and RNA was isolated according to manufacture's instructions. RNA quality and quantity were assessed using BioAnalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). RNA-seq transcriptome libraries were constructed from 1 µg of total RNA using a modified TruSeq RNA Sample Preparation Kit protocol as described (6). The libraries were validated using DNA 1000 Kit (Agilent) and quantitated by qRT–PCR (7). Each library (10 pM) was loaded into an independent flow-cell lane and single-read cluster generation proceeded according to manufacture's protocols using TruSeq Single-Read Cluster Generation Kit v5 (Illumina). Single read sequence-by-synthesis of 76 base length using TruSeq SBS v5 reagents was carried out on Genetic Analyzer IIx running SCS2.9 software (Illumina). RNA-seq reads passing chastity filter were analyzed using Genomatix Software Suite (Genomatix Software GmbH, Munich Germany). Sequences were aligned to NCBI mouse reference genome Build 37 on Genomatix Mining Station with Genomatix Mapper 3.5.1. Alignments were annotated on Genomatix Genome Analyzer using ElDorado 08-2011 database, and differential expression analysis was performed using DEseq (8). RefSeq transcripts having a fold change greater than 2.0, an adjusted p-value lower than 0.05, and a normalized expression value greater than 5 were kept for hierarchical clustering.

Real-time PCR

After digestion with DNaseI (Life technologies) according to the manufacturer's instruction, 1 μg of total RNA was reverse transcribed in presence of oligo-(dT)20 using Superscript II reagents (Life technologies) as previously described (9). For each qRT-PCR, 2 μl of a tenfold dilution of the cDNA was used, and the reactions were performed in triplicates on 7900HT Genetic Analyzer (Life technologies) as previously described (7). Differential expression analysis was performed using the ddCt method using *Actβ* and *Hprt* as endogenous controls (10).

Luciferase assays

HEK293 cells were seeded in 24-well plates ($5x10^4$/well) and co-transfected with 0.1 μg of bovine *Rho* promoter driving firefly luciferase (pBR130-luc (11)), 0.1 μg of pcDNA4c-Nrl (11), 0.1 μg of pcDNA4c-Nr2e3 (11), and different amount of pcDNA4c-Crx^{WT}, pcDNA4c-Crx^{RIP} and pcDNA4c-Crx^{1-254} construct. In addition, 0.1 μg of *Opn1sw* promoter driving firefly luciferase (5) was co-transfected with 0.1 μg of *CMV*-Rorβ (5) and different amount of pcDNA4c-Crx^{WT}, pcDNA4c-Crx^{RIP} and pcDNA4c-Crx^{1-254} construct. For normalization, 10 ng of *Renilla* reporter pRL-TK (Promega) was also transfected. Empty pcDNA4c was used to adjust the total amount of transfected DNA. After 48 h, cells were harvested and lysed with 100 μl of passive lysis buffer (Promega). Luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega) and measured with Modulus microplate luminometer (Promega). All experiments were performed in triplicates.

Supplemental References

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