HHS Public Access

Author manuscript

Adv Exp Med Biol. Author manuscript; available in PMC 2024 April 12.

Published in final edited form as:

Adv Exp Med Biol. 2023 ; 1415: 135–141. doi:10.1007/978-3-031-27681-1_21.

Gene augmentation for autosomal dominant *CRX*-associated retinopathies

Chi Sun[1], **Shiming Chen**[1,2]

[1]Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri, United States

[2]Department of Developmental Biology, Washington University, St. Louis, Missouri, United States

Abstract

The Cone-rod homeobox (CRX) protein is a key transcription factor essential for photoreceptor function and survival. Mutations in human *CRX* gene are linked to a wide spectrum of blinding diseases ranging from mild macular dystrophy to severe Leber Congenital Amaurosis (LCA), Cone-Rod Dystrophy (CRD) and Retinitis Pigmentosa (RP). These diseases are still incurable and mostly inherited in an autosomal dominant form. Dysfunctional mutant CRX protein interferes with the function of wildtype CRX protein, demonstrating the dominant negative effect. At present, gene augmentation is the most promising treatment strategy for hereditary diseases. This study aims to review the pathogenic mechanisms of various *CRX* mutations and propose two therapeutic strategies to rescue sick photoreceptors in *CRX*-associated retinopathies, namely, *Tet-On-hCRX* system and *Adeno-associated Virus* (*AAV*)-mediated gene augmentation. The outcome of proposed studies will guide future translational research and suggest guidelines for therapy evaluation in terms of treatment safety and efficacy.

Keywords

CRX mutations; dominant negative effects; gene augmentation; *Tet-On* ; AAV gene therapy

1 Pathogenic mechanisms of *CRX* mutations

The human cone-rod homeobox gene *CRX* (OMIM #602225) is located on chromosome 19q13.33. There are four exons in *CRX*, specifically, the first noncoding and three coding exons. *CRX* codes for a 299 amino acid transcription factor which is predominantly expressed in photoreceptors and the pineal gland, regulating photoreceptor development and maintenance [1, 2]. The protein consists of three major domains: the homeodomain at residues 39-99 facilitates the DNA binding; the activation domain at residues 113-284, including a WSP motif at residues 158-170, contains binding sites for other transcription coregulators; a conserved carboxyl terminus motif, also known as the OTX tail, is found at resides 284-295 [1, 3].

Corresponding Author: Chi Sun, 660 South Euclid Avenue, Campus Box 8096, St. Louis, MO 63110, USA; sunchi@wustl.edu.

To date, 93 disease-causing *CRX* mutations have been identified in human patients (www.hgmd.cf.ac.uk). Pathogenic *CRX* variants are associated with a complex group of inherited retinal diseases (IRDs) such as macular dystrophy [4], cone-rod dystrophy (CRD) [1], retinitis pigmentosa (RP) [5], and Leber congenital amaurosis (LCA) [6, 7]. Mutations within *CRX* are known to occur de novo or to be inherited mostly in an autosomal dominant pattern, consisting of substitution (nonsense and missense mutations) and frameshift mutations (deletions and insertions) [8]. Our understanding of these mutations has significantly expanded, as a great variety are being analyzed with the advent of cutting-edge tools from the sequencing and analytic technologies. However, a tremendous challenge for the pathogenic analysis of *CRX*-associated retinopathies, particularly of the autosomal dominant disorders, is to explore the phenotype-genotype correlations between disease severity and *CRX* mutations.

CRX mutations could cause dominant disorders by two possible mechanisms, namely, the haploinsufficiency of the functional CRX, and/or various dominant negative or gain-of-function effects of the mutant CRX. To the extent of data published, haploinsufficiency may not cause severe phenotypes. The study on $Crx^{+/-}$ mice corroborates this claim, as they do not develop any detectable functional defects up to 6 months [9]. A rare case of human patients with the heterozygosity of *CRX* also supports these observations, as only the patients with the nullizygosity of *CRX* develop LCA [10]. However, in the autosomal dominant disorders, it remains unknown if the mutant *CRX* allele could partially abrogate the production of a functional CRX from the normal allele. Further studies are needed to address this question in detail. Alternatively, dominant negative activities of various CRX mutant proteins have been demonstrated in animal models [9, 11]. The reported dominant-negative mutations that arise in the homeodomain are mostly missense mutations, and those identified in the activation domain are largely frameshifts [7, 9]. Pathogenic mechanisms thus are constituted by altered DNA binding properties and/or transactivation activities of CRX mutant proteins.

CRX R90W mutation presents a hypomorphic missense mutation located in the homeodomain [3, 12], and is associated with a dominant late-onset mild CRD and recessive LCA. The mutant protein has reduced DNA binding activity, and thus malfunctions to transactivate *CRX*-downstream target genes such as *Rhodopsin* [3, 12]. The transactivation activity of wild-type (WT) CRX seems normal in the heterozygous model [3], which proves the limited dominant negative effect of the *CRX R90W* mutation through the process of retinal development. On the other hand, *CRX E80A* and *K88N* mutations represent distinct antimorphic missense mutations located in the homeodomain [13–15]. Both mutations manifest LCA in human patients [1, 14]. Interestingly, some LCA-associated *CRX* homeodomain mutations are located at non-conserved residues in human homeodomain paralogues [4]. These mutant proteins are predicted to bind discrete DNA sequences and show different transactivation activities from the WT protein, although this has not yet been studied in mammalian models. Hence the dominant negative effects of the mutant proteins, if any, remain to be proven.

The frameshift deletion *CRX E168d2* presents an antimorphic mutation located in the activation domain [3], and is associated with dominant LCA in human patients [6, 16].

This mutation results in the early truncation of the activation domain, producing a protein that retains the ability to bind target DNA but fails to transactivate *CRX*-downstream target genes [3]. In addition, *CRX E168d2* allele overproduces the mutant protein at about 4 times more than the WT protein in heterozygous mice, which exacerbates the dominant-negative effect on the binding competition [3]. As a result, the heterozygous mice have impaired visual functions at 1 month-old (MO) [3] and limited electroretinography (ERG) response at 3MO (data not shown). Cone degeneration occurs prior to rod degeneration in the heterozygous mice. As compared to the WT samples, the heterozygous mice have only about 30% survived cones at 1 month-old (MO) and no detectable cones at 3MO (data not shown); mutants rods are functional with shorter outer segments (OS) at 1MO but undergo progressive degeneration till complete lost at 6MO [3]. Interestingly, the ratio of mutant to WT CRX proteins directly correlates with the disease phenotype severity and age of onset [3]. In addition, truncation at the last exon by frameshift results in premature terminations [7, 8], hence the production of shortened but stable mutant mRNA that can avoid nonsense-mediated decay [17], which partially contributes to the dominant negative effect. On the other hand, Crx^{Rip} presents the c.763del1 mutation located in the last exon, which results in a skipping of the OTX tail and a non-homologous extension of 133 residues [18]. The mutant protein does not transactivate *CRX*-downstream target genes, suggesting its antimorphic activity [18]. More notably, the mutant protein does not bind target DNA [18]. Similar to the retinal manifestation in patients, $Crx^{Rip/+}$ mice show a LCA-like phenotype [18]. Photoreceptors in $Crx^{Rip/+}$ mice do not form outer segments, due to impaired photoreceptor gene expression and incomplete differentiation at early development [18]. In addition, the mutant protein is not overexpressed in $Crx^{Rip/+}$ mice. Taken together, the dominant negative effect of Crx^{Rip} mutation does not signify a competition between the mutant and WT proteins, but likely arises from the disruption of the photoreceptor transcription factor network.

2 Gene augmentation as a strategy to treat *CRX*-associated retinopathies

There are currently limited therapeutic options for *CRX*-associated retinopathies. Fortunately, retinal gene therapy recently shines promise for treatment of various inherited blindness [19]. In particular, the gene augmentation approach has been developed to deliver a healthy gene to a diseased retina to rescue the defective phenotypes and visual functions without replacing the mutant allele. At present, successes of gene augmentation are largely restricted to recessive mutations [20]. Five important questions need to be answered in order to apply gene augmentation to treat *CRX*-associated autosomal dominant disorders. Firstly, how much of augmented *CRX* is needed to overcome the dominant negative effect of a given mutation? If a mutant protein competes with WT CRX proteins for binding sites, how much is augmented *CRX* needed to balance the ratio of mutant to WT CRX proteins in each photoreceptor? Can altering this ratio rescue the mutant phenotypes? Secondly, considering CRX is an early photoreceptor transcription factor that activates the downstream gene regulatory network in the retinal development, could gene augmentation impose overexpression toxicity in a treated retina? Thirdly, when is augmented *CRX* needed in the treatment? Fourthly, as human patients with *CRX*-associated retinopathies are often diagnosed at different stages of disease progression, what are the windows of

opportunities for gene augmentation to a given disease? Do diseased photoreceptors possess the neuroplasticity for gene augmentation outside the development window? Lastly, can the treated photoreceptors retain their fated cell identity and functional capability upon gene augmentation?

Answers to these questions could inform the feasibility and strategies to treat *CRX*-associated retinopathies. To address these questions at least partially, a new transgenic mouse model, *Tet-On-hCRX*, has been developed to allow both quantitative and temporal control of augmented human *CRX* (*hCRX*) gene expression under a given *CRX* mutant background (Figure 1). Upon binding of doxycycline, a conformation change of the reverse tetracycline-controlled transcriptional activator (rtTA) is triggered [21], which allows the resulted complex bind to tetracycline-responsive element (*TRE*) (*Tet-On*) [22]. FLAG-tagged human CRX protein will hence be produced (Figure 1A). Moreover, the photoreceptor-specific induction can be achieved by the combination of two transgenes, namely, a Cre-dependent *pCAG-LSL-rtTA* (Figure 1B) and a *Crx* promoter-driven *Cre, pCrx-Cre* [23] (Figure 1C). Proof-of-concept validation of gene augmentation by *Tet-On-hCRX* system shows that *CRX* expression can be induced and detected in *Crx*−/− mouse retinae at all tested ages throughout the entire developmental window (postnatal (P) day 5, 14, 21 and 35) (Figure 2), confirming the success of *Tet-On-hCRX* system. Doxycycline-treated samples also display significant upregulation of *CRX*-downstream *Rho* expression. Furthermore, the comparisons between treated mice with different starts of the treatments suggests the ‘early the better’ effect on induced *CRX* and *Rho* expression at P35, as samples with P0-35 treatment display significantly higher induced *CRX* and *Rho* expression than those with P14-35 or P21-35 treatments (Figure 2A, 2B). However, as compared to the WT samples, treated mice still have significantly lower *CRX* and *Rho* expression at P35 under current treatment schemes, implying a partial rescue effect. These results suggest that *Crx*-null photoreceptors retain the machinery for *CRX*-mediated gene expression and neuroplasticity for therapeutic intervention.

As compared to *Tet-On-hCRX* system, a clinically applicable approach is *Adeno-associated Virus* (*AAV*)-mediated gene augmentation. The retina is surgically accessible, which facilitates intravitreal or subretinal administration of the viral vector [24, 25]. Since the retina is a relatively immune privileged tissue [24] and photoreceptors are nondividing and differentiated [26], a small amount of the non-integrating AAV viral vector can be tolerated to generate a therapeutic response [24, 25] with minimal risk of stimulating a severe inflammatory immune response [25]. Yet, two immediate challenges emerge during testing *AAV*-mediated *CRX* gene augmentation with animal models, i.e., choice of AAV vectors and cell-type specificity. To date, the *AAV2/5* vector yields efficient transduction and good tropism for photoreceptors in P0 mice [27]. And the size of human or mouse *CRX* cDNA well fits into the AAV packaging capacity. Also, photoreceptor-specific promoters, including *CRX* or *GRK*, can be employed to achieve desirable sensitivity and precision.

However, there are still several safety precautions of applying *AAV*-mediated gene augmentation. Although subretinal administration is currently the most efficient route for targeting photoreceptors [28], it causes a transient detachment of the RPE from photoreceptors, which might damage photoreceptors at the injection site. A possible solution

is the use of low-volume and low-concentration multiple blebs to reduce the danger of the RPE detachment and systemic exposure of high concentration by a single bleb, despite of associated minor risks [29, 30]. In addition, immunity and tolerance to AAV is untested in human photoreceptors at early and middle childhood [31], and multiplicity of infection (MOI) of viral vectors per cell may vary at different stages of the disease, all of which require further trials on human patients with early-onset diseases.

3 Unanswered questions and challenges

One-step gene augmentation may not rescue all *CRX*-associated autosomal dominant disorders. For examples, gain-of-function activities of a mutant protein with altered DNA binding specificity, such as CRX K88N, may generate the irreversible but detrimental effects on the developmental program. In such cases, expression from the mutant allele needs to be silenced to prevent the disease progression. Similar approaches of treating *RHO*-associated autosomal dominant RP in the mouse and canine models have shown encouraging outcomes [32, 33]. In addition, the CRISPR-Cas9/gRNA technique [34–37] can also be used to knockout the mutant allele.

Since gene augmentation utilizes the transcription machinery of the host cells to express the functional protein, cellular conditions of the host cells require close monitoring. In a developing retina, *CRX* gene augmentation may not fully rescue mutations that block early differentiation in photoreceptors, due to an insufficient but early window of opportunity. On the contrary, when the host cells are at the late stage of degeneration or the degeneration tends to progress too fast, gene augmentation may not succeed. Prerequisite anti-apoptotic or neuroprotective therapies can be combined to target the defective photoreceptors in order to gain a required time window for gene augmentation [38, 39].

Maculopathy has been reported in human patients of different *CRX* mutations with large variability in disease progression and symptoms [4, 40–42]. In addition, *CRX* may be involved in foveal development, as the haploinsufficiency of *CRX* results in subclinical foveal abnormalities in human patients [10]. None of these observations can be validated and studied in non-primate models.

The phenotypic and onset variability between patients sharing the same *CRX* mutant allele has constantly been reported [4, 7, 10, 43, 44]. It may be due to, firstly, possible polymorphisms in the *CRX* promoter region; secondly, the impacts of possible epigenetic modifications in coding regions; thirdly, any differential expression of other transcription factors such as NRL and NR2E3; fourthly, variable expression levels of the WT and/or mutant alleles; lastly, sexual dimorphisms [42], suggesting CRX may interact with specific targets on the sex chromosomes. None of these hypotheses has systematically been tested in animal models. In addition, the stochastic effects of *CRX* mutations and overexpression toxicity on the retinal degeneration are currently unknown, which appends uncertainty on the application of gene augmentation.

4 Methods

4.1 Transgenic mouse lines.

All male and female mice in this study were on the genetic background of *C57BL/6J*. *TRE-hCRX* line was generated for this study. The transgene had a *TRE* promoter upstream of full-length human *CRX* cDNA sequence, all inserted into the *H11* locus. *pCAG-LSL-rtTA* (*Rosa26:pCAG-LSL-rtTA*) line was obtained from the Jackson Laboratory (Stock No: 029617). *pCrx-Cre* (BAC-Tg *Crx-Cre*) line was obtained from a published colony [23]. Doxycycline diets (200mg/kg, Bio-Serv, NJ) were provided to mice (> P14) and breeding dams. Intraperitoneal (IP) injection of 100μL of 20mg/mL doxycycline solution was provided to mice (> P21) once a week to maximize the treatment effect. All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee.

4.2 Quantitative PCR (qPCR).

Each RNA sample was extracted from 2 retinae of a mouse using the NucleoSpin RNA Plus kit (Macherey-Nagel, PA). 1μg of RNA was used to produce cDNA using First Strand cDNA Synthesis kit (Roche, IN). Technical triplicates were run for each gene. Primers (5' to 3') used in this study were *Crx* (F) TGTCCCATACTCAAGTGCCC, (R) TGCTGTTTCTGCTGCTGTCG; *Rho* (F) GCTTCCCTACGCCAGTGTG, (R) CAGTGGATTCTTGCCGCAG; *Ubb* (F) CAACATCCAGAAAGAGTCAACC, (R) ATGTTGTAATCAGAGAGGGTGC. The reaction master mix consisted of EvaGreen polymerase (Bio-Rad Laboratories, CA), 1μM primer mix, and diluted cDNA samples. Samples were run using a two-step 40-cycle protocol on a Bio-Rad CFX96 Thermal Cycler (Bio-Rad Laboratories, CA). The statistical analysis is done by Student's t-test with $p<0.05$, CI:95%.

Acknowledgements

The authors are grateful to Dr. Tom Glaser for *pCrx-Cre* mice as a gift, to Dr. Ke Jiang for critical evaluation of the manuscript. Funding was provided by R01 EY012543 and R01 EY032136 (SC), RPB unrestricted fund (WU-DOVS), EY002687 (WU-DOVS), and McDonnell Center for Cellular and Molecular Neurobiology Fellowship (CS).

Reference

1. Freund CL, et al. , Cone-Rod Dystrophy Due to Mutations in a Novel Photoreceptor-Specific Homeobox Gene (CRX) Essential for Maintenance of the Photoreceptor. Cell, 1997. 91(4): p. 543–553. [PubMed: 9390563]
2. Chen S, et al. , Crx, a Novel Otx-like Paired-Homeodomain Protein, Binds to and Transactivates Photoreceptor Cell-Specific Genes. Neuron, 1997. 19(5): p. 1017–1030. [PubMed: 9390516]
3. Tran NM, et al. , Mechanistically distinct mouse models for CRX-associated retinopathy. PLoS genetics, 2014. 10(2): p. e1004111–e1004111. [PubMed: 24516401]
4. Hull S, et al. , The Phenotypic Variability of Retinal Dystrophies Associated With Mutations in CRX, With Report of a Novel Macular Dystrophy Phenotype. Investigative Ophthalmology & Visual Science, 2014. 55(10): p. 6934–6944. [PubMed: 25270190]
5. Sohocki MM, et al. , Prevalence of mutations causing retinitis pigmentosa and other inherited retinopathies. Human mutation, 2001. 17(1): p. 42–51. [PubMed: 11139241]

6. Freund CL, et al. , De novo mutations in the CRX homeobox gene associated with Leber congenital amaurosis. Nature Genetics, 1998. 18(4): p. 311–312. [PubMed: 9537410]
7. Rivolta C, et al. , Novel frameshift mutations in CRX associated with Leber congenital amaurosis. Human Mutation, 2001. 18(6): p. 550–551.
8. Stenson PD, et al. , The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Human genetics, 2014. 133(1): p. 1–9. [PubMed: 24077912]
9. Tran NM and Chen S, Mechanisms of blindness: animal models provide insight into distinct CRX-associated retinopathies. Developmental dynamics : an official publication of the American Association of Anatomists, 2014. 243(10): p. 1153–1166. [PubMed: 24888636]
10. Ibrahim MT, et al. , A complete, homozygous CRX deletion causing nullizygosity is a new genetic mechanism for Leber congenital amaurosis. Scientific Reports, 2018. 8(1): p. 5034. [PubMed: 29568065]
11. Ruzycki PA, et al. , Crx-L253X Mutation Produces Dominant Photoreceptor Defects in TVRM65 Mice. Investigative ophthalmology & visual science, 2017. 58(11): p. 4644–4653. [PubMed: 28903150]
12. Swaroop A, et al. , Leber Congenital Amaurosis Caused by a Homozygous Mutation (R90W) in the Homeodomain of the Retinal Transcription Factor CRX: Direct Evidence for the Involvement of CRX in the Development of Photoreceptor Function. Human Molecular Genetics, 1999. 8(2): p. 299–305. [PubMed: 9931337]
13. Chen S, et al. , Functional analysis of cone–rod homeobox (CRX) mutations associated with retinal dystrophy. Human Molecular Genetics, 2002. 11(8): p. 873–884. [PubMed: 11971869]
14. Nichols LL II, et al. , Two novel CRX mutant proteins causing autosomal dominant Leber congenital amaurosis interact differently with NRL. Human Mutation, 2010. 31(6): p. E1472–E1483. [PubMed: 20513135]
15. Terrell D, et al. , OTX2 and CRX rescue overlapping and photoreceptor-specific functions in the Drosophila eye. Developmental Dynamics, 2012. 241(1): p. 215–228. [PubMed: 22113834]
16. Jacobson SG, et al. , Retinal degenerations with truncation mutations in the cone-rod homeobox (CRX) gene. Investigative Ophthalmology & Visual Science, 1998. 39(12): p. 2417–2426. [PubMed: 9804150]
17. Lejeune F and Maquat LE. Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Current Opinion in Cell Biology, 2005. 17(3): p. 309–315. [PubMed: 15901502]
18. Roger JE, et al. , OTX2 loss causes rod differentiation defect in CRX-associated congenital blindness. The Journal of clinical investigation, 2014. 124(2): p. 631–643. [PubMed: 24382353]
19. Campa* C, et al. , The Role of Gene Therapy in the Treatment of Retinal Diseases: A Review. Current Gene Therapy, 2017. 17(3): p. 194–213. [PubMed: 29149824]
20. Vazquez-Dominguez I, Garanto A, and Collin RWJ, Molecular Therapies for Inherited Retinal Diseases-Current Standing, Opportunities and Challenges. Genes (Basel), 2019. 10(9).
21. Das AT, Tenenbaum L, and Berkhout B, Tet-On Systems For Doxycycline-inducible Gene Expression. Current gene therapy, 2016. 16(3): p. 156–167. [PubMed: 27216914]
22. Gossen M, et al. , Transcriptional activation by tetracyclines in mammalian cells. Science, 1995. 268(5218): p. 1766–1769. [PubMed: 7792603]
23. Prasov L and Glaser T, Pushing the envelope of retinal ganglion cell genesis: context dependent function of Math5 (Atoh7). Developmental biology, 2012. 368(2): p. 214–230. [PubMed: 22609278]
24. Öner A, Recent Advancements in Gene Therapy for Hereditary Retinal Dystrophies. Turkish journal of ophthalmology, 2017. 47(6): p. 338–343. [PubMed: 29326851]
25. Gupta PR and Huckfeldt RM, Gene therapy for inherited retinal degenerations: initial successes and future challenges. Journal of Neural Engineering, 2017. 14(5): p. 051002. [PubMed: 28829759]
26. Patrício MI, et al. , Inclusion of the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances AAV2-Driven Transduction of Mouse and Human Retina. Molecular therapy. Nucleic acids, 2017. 6: p. 198–208. [PubMed: 28325286]

27. Watanabe S, et al. , Tropisms of AAV for Subretinal Delivery to the Neonatal Mouse Retina and Its Application for In Vivo Rescue of Developmental Photoreceptor Disorders. PLOS ONE, 2013. 8(1): p. e54146. [PubMed: 23335994]
28. Planul A and Dalkara D, Vectors and Gene Delivery to the Retina. Annual Review of Vision Science, 2017. 3(1): p. 121–140.
29. Peng Y, Tang L, and Zhou Y, Subretinal Injection: A Review on the Novel Route of Therapeutic Delivery for Vitreoretinal Diseases. Ophthalmic Research, 2017. 58(4): p. 217–226. [PubMed: 28858866]
30. Xue K, et al. , Technique of retinal gene therapy: delivery of viral vector into the subretinal space. Eye, 2017. 31(9): p. 1308–1316. [PubMed: 28820183]
31. Verdera HC, Kuranda K, and Mingozzi F, AAV Vector Immunogenicity in Humans: A Long Journey to Successful Gene Transfer. Molecular Therapy, 2020. 28(3): p. 723–746. [PubMed: 31972133]
32. Millington-Ward S, et al. , Suppression and Replacement Gene Therapy for Autosomal Dominant Disease in a Murine Model of Dominant Retinitis Pigmentosa. Molecular Therapy, 2011. 19(4): p. 642–649. [PubMed: 21224835]
33. Cideciyan AV, et al. , Mutation-independent rhodopsin gene therapy by knockdown and replacement with a single AAV vector. Proceedings of the National Academy of Sciences, 2018. 115(36): p. E8547.
34. Yu W and Wu Z, In Vivo Applications of CRISPR-Based Genome Editing in the Retina. Frontiers in cell and developmental biology, 2018. 6: p. 53–53. [PubMed: 29868583]
35. Hung SSC, et al. , AAV-Mediated CRISPR/Cas Gene Editing of Retinal Cells In Vivo. Investigative Ophthalmology & Visual Science, 2016. 57(7): p. 3470–3476. [PubMed: 27367513]
36. Trapani I and Auricchio A, Seeing the Light after 25 Years of Retinal Gene Therapy. Trends in Molecular Medicine, 2018. 24(8): p. 669–681. [PubMed: 29983335]
37. Suzuki K, et al. , In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature, 2016. 540(7631): p. 144–149. [PubMed: 27851729]
38. Petit L and Punzo C, Gene therapy approaches for the treatment of retinal disorders. Discovery medicine, 2016. 22(121): p. 221–229. [PubMed: 27875674]
39. Chinskey ND, Besirli CG, and Zacks DN, Retinal cell death and current strategies in retinal neuroprotection. Current Opinion in Ophthalmology, 2014. 25(3).
40. Ozdek S, et al. , Bilateral Disc Edema and Unilateral Macular Hole in a Patient with Retinitis Pigmentosa. European Journal of Ophthalmology, 2006. 16(3): p. 487–490. [PubMed: 16761258]
41. Griffith JF, DeBenedictis MJ, and Traboulsi EI, A novel dominant CRX mutation causes adult-onset macular dystrophy. Ophthalmic Genetics, 2018. 39(1): p. 120–124. [PubMed: 28945142]
42. Gonzalez-Gonzalez LA, et al. , Novel clinical presentation of a CRX rod-cone dystrophy. BMJ Case Reports, 2021. 14(4): p. e233711.
43. Paunescu K, et al. , Genotype–Phenotype Correlation in a German Family with a Novel Complex CRX Mutation Extending the Open Reading Frame. Ophthalmology, 2007. 114(7): p. 1348–1357.e1. [PubMed: 17320181]
44. Lu Q-K, et al. , A novel CRX mutation by whole-exome sequencing in an autosomal dominant cone-rod dystrophy pedigree. International journal of ophthalmology, 2015. 8(6): p. 1112–1117. [PubMed: 26682157]

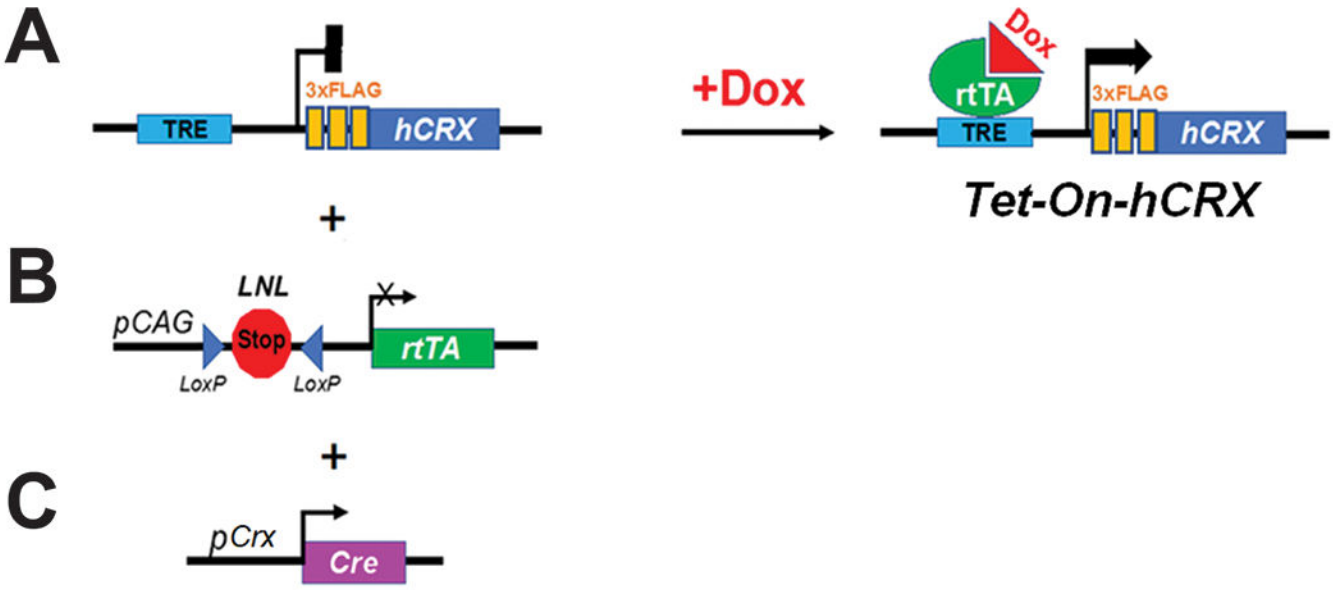

Figure 1.
Components of *Tet-On-hCRX* system. (A) *TRE-hCRX* (B) *pCAG-LSL-rtTA* (C) *pCrx-Cre*.

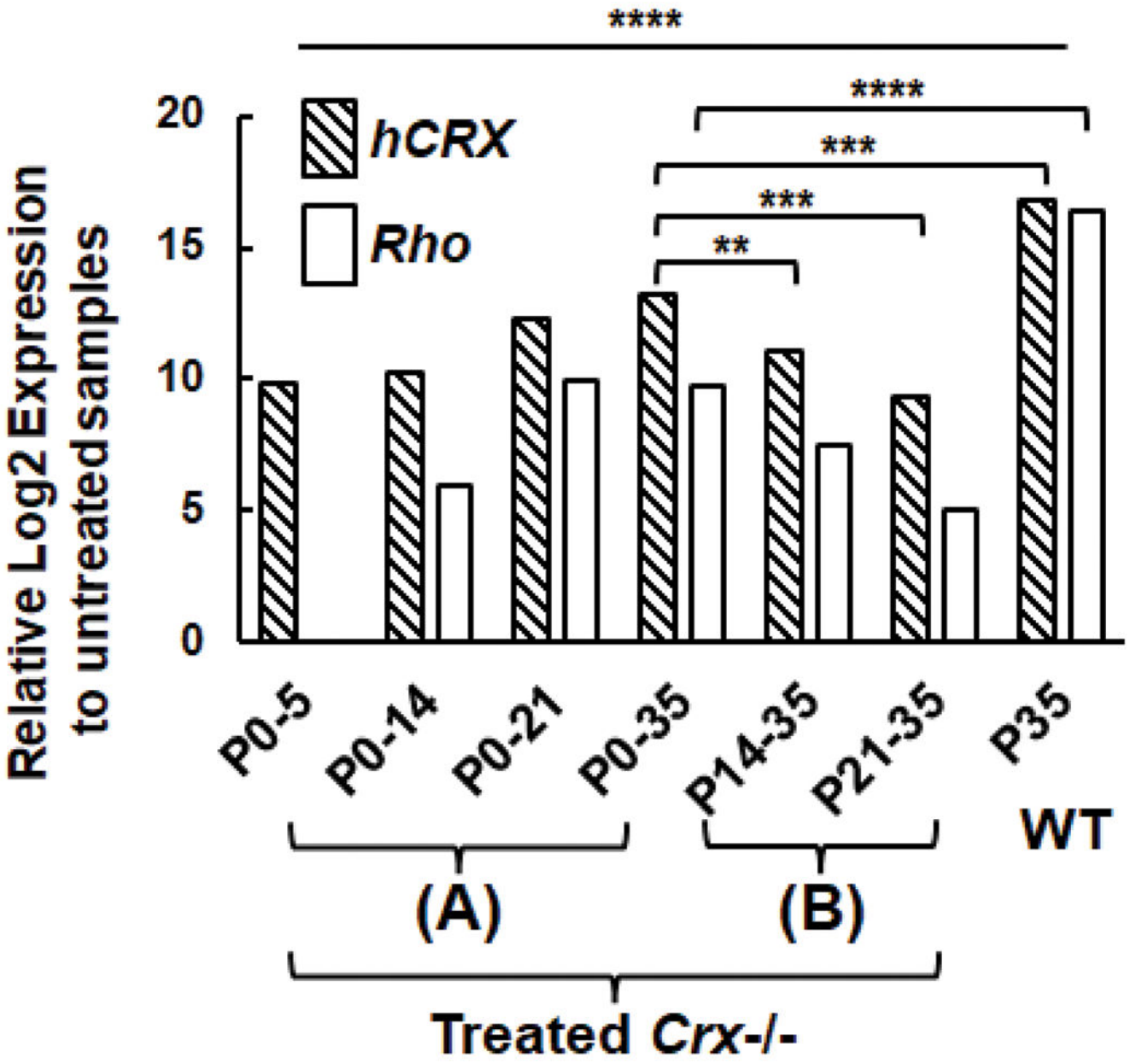

Figure 2.

Quantitative PCR (qPCR) analysis of *hCrx* and *Rho* expression in untreated *Crx−/−, Crx-BAC-Cre*$^{+}$, *Tet-On-hCRX*$^{+}$; doxycycline-treated *Crx−/−, Crx-BAC-Cre*$^{+}$, *Tet-On-hCRX*$^{+}$; WT mice. (A) Treatments started at P0, samples were harvested at P5, P14, P21 or P35. (B) Treatments started at P14 or P21, samples were harvested at P35. Results are plotted as relative Log2 expression to untreated samples (n≥4). Asterisks (**, ***, ****) denote $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ respectively by T-test.