Crx-L253X mutation produces dominant photoreceptor defects in TVRM65 mice

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**Crx-L253X Mutation Produces Dominant Photoreceptor Defects in TVRM65 Mice**

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**Purpose.** The cone-rod homeobox (CRX) transcription factor is essential for photoreceptor gene expression, differentiation, and survival. Human CRX mutations can cause dominant retinopathies of varying onset and phenotype severity. In animal models, dominant frameshift CRX mutations introduce a premature termination codon (PTC), producing inactive truncated proteins that interfere with normal CRX function. Previously, a mutant mouse, TVRM65, was reported to carry a recessive late PTC mutation, Crx-L253X. More detailed phenotype analysis of the pathogenicity of Crx-L253X sheds new light on the variability of CRX-linked diseases.

**Methods.** Homozygous (L253X/X); heterozygous (L253X/+); Crx+/− and control C57BL/6J (WT) mice were analyzed at various ages for changes in retinal function (ERG), morphology (histology) and photoreceptor gene expression (qRT-PCR).

**Results.** At 1 month, L253X/X mice lack visual function, show greater reductions in retinal thickness, and distinct gene expression changes relative to Crx+/−, suggesting that the phenotype of L253X/X is more severe than Crx+/−. L253X/+ mice have reduced rod/cone function, but normal retinal morphology at all ages tested. qRT-PCR assays described a complex phenotype in which both developing and mature photoreceptors are unable to maintain proper gene expression. L253X mRNA/protein is overexpressed relative to normal Crx, suggesting a pathogenic mechanism similar to early PTC mutations. However, the overexpression is less pronounced, correlating with a relatively mild dominant phenotype.

**Conclusions.** The L253X mouse provides a valuable model for CRX-associated retinopathy. The pathogenicity of CRX frameshift mutations depends on the position of the PTC, which in turn determines the degree of mutant mRNA/protein overproduction.

Keywords: CRX mutations, animal models, inherited photoreceptor degeneration, cone-rod dystrophy, gene expression

The cone-rod homeobox protein (CRX) is a transcription factor (TF) that regulates expression of many photoreceptor genes essential for the development and maintenance of both rod and cone photoreceptors.1–4 Human CRX mutations are associated with various diseases, including retinitis pigmentosa (RP); cone-rod dystrophy (CoRD); and Leber congenital amaurosis (LCA).5,6 CRX-linked diseases are largely inherited in an autosomal dominant fashion or arise de novo, and vary widely in severity and age of onset.5,7–10 Since CRX mutations can be detected with early genetic testing, we need to be able to predict their effects and to define effective treatment and gene therapy regimens.7

Previous research has divided disease causing CRX mutations into four classes.11 Classes I and II are missense mutations that fall within or near the region coding for the DNA-binding homedomain; class I mutations reduce the binding of CRX to its DNA targets.12 Previous work has shown by a variety of metrics that heterozygous mice carrying the class I R90W mutation suffer from a mild form of CoRD, similar to human patients with such mutations, while homozygotes exhibit an LCA-like condition.13 Class III and IV mutations represent frameshift mutations caused by insertions or deletions in the region coding for the transactivation domains of CRX.12 Class IV mutations, modeled by the RIP mouse, cause translation of a much longer peptide sequence due to a frameshift and extension of the open reading frame (ORF) into the 3’ untranslated region (3’UTR).14 In contrast to this extended peptide sequence, Class III mutations truncate the ORF of CRX with a premature termination codon (PTC) that results in a CRX protein with a shortened transactivation domain and leads to a severe dominant degenerative phenotype (LCA or CoRD).13 This phenotype has been modeled by the E168d2 mouse15 and Rdy (A182dI) cat16 (Fig. 1A). In vitro DNA binding and transactivation experiments determined that the E168d2 protein has a similar affinity for CRX target DNA sequences as normal CRX, but is unable to activate transcription on its own or in combination with other retinal TFs that normally synergize with CRX.12,13 Furthermore, when tested together with normal CRX, E168d2 protein interfered with normal CRX transactivation in a dominant-negative manner.13

Class III PTC-causing CRX mutations also result in a novel untranslated region between the PTC and normal stop codon, which becomes a part of the mutant mRNA transcript’s 3’UTR (Fig. 1A).13 In the E168d2 mouse and the Rdy (A182dI) cat, longitudinal studies showed gradual accumulation of mutant
protein and mRNA.\textsuperscript{13,15} The mechanism for this accumulation is unknown, but it was postulated to arise from decreased mutant RNA degradation,\textsuperscript{13} possibly due to the presence of cryptic stabilizing elements within the PTC-expanded mutant protein and mRNA.\textsuperscript{13,15} The specificity of the two allele-specific ddPCR assays was maintained with L253X/+ mRNA results are presented as percent of total Crx mRNA (mutant plus normal transcripts; mean ± SEM, n ≥ 3; **** P < 0.0001; unpaired t test for mutant mRNA level relative to L253X/+ DNA control; n ≥ 3).

which correlates with the abundance of mutant protein products. These findings have implications for predicting human CRX disease severity and progression.

### Methods

#### Nomenclature for the TVRM65 Mutation

There are several annotated transcripts for the murine Crx locus. The previously published amino acid change in the TVRM65 mouse was based on isoform 2 (NM001115330.1) that encodes a CRX protein with 329 amino acids.\textsuperscript{16,19} However, the major transcript in the retina is isoform 1 (NM_007770.4), which encodes a CRX protein homologous to human CRX, with 299 amino acids. Thus, here we refer to the TVRM65 mutation as p.L253X, based on isoform 1 numbering.

#### Mice

Crx-L253X (TVRM65) mice\textsuperscript{16} were obtained from The Jackson Laboratory (MGI ID: 4867395). Mice were mated and maintained with C57BL/6J (JAX Stock number 000664), and
confirmed free of rd1 and rd8 mutations by PCR genotyping. Crx null mice (Crx<sup>L253X</sup>) were provided by Constance Cepko, Harvard University (Boston, MA, USA) and back-crossed onto C57Bl/6J for more than 10 generations. All mice were housed in a barrier facility in the Division of Comparative Medicine of Washington University School of Medicine. All procedures involving the use of mice were approved by Washington University's Animal Care and Use Committee and followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

PCR genotyping, RNA purification and reverse transcription, and qRT-PCR were performed as previously described. Primer sets for genotyping and RT-PCR are listed in Supplementary Table S1. For qRT-PCR, all primers were tested for proper amplification efficiency prior to use. Relative gene expression was normalized to the retinal constitutively expressed genes Ubb, Tubat1B, and Gapdh. Data for 5 biologic replicates were then analyzed using the Delta Cq method in qPCR analysis software (QBase; Biogazelle, Ghent, Belgium). The results are presented by the heatmap.2 function of the R gplots package (v3.0.1).

**Droplet Digital PCR (ddPCR)**

Confirmatory genotyping and allelic expression data were generated using the droplet digital PCR system (BioRad, Hercules, CA, USA). A 20 μl ddPCR reaction mixture containing custom-designed normal and mutant primer/probe sets (see Supplementary Table S2) was prepared according to manufacturer's directions, droplets generated, and nanoreactions cycled on a thermal cycler (C1000 Touch; Bio-Rad Laboratories). The average number of allelic transcripts per biological replicate (n = 5) was then determined with a droplet reader and analytical software (QX 200 with QuantaSoft Analysis Pro; Bio-Rad Laboratories).

**Transient Transfection Luciferase Reporter Assays**

HEK293T cells (ATCC CRL-11286) were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum and penicillin/streptomycin. Cells were transfected using a conventional CaCl<sub>2</sub> and boric acid buffered saline method in 6-well plates as previously described. Experimental plasmids and transfection amounts included 2 μg Br130-Luc (a Rbo promoter-luciferase reporter) and 1 ng pRL-CMV (transfection normalization control); and protein expression vectors pED-NRL (100 ng), pcDNA3.1-hCRX, CRX1-254, and CRX-E168D2. Cells were harvested 48 hours posttransfection and assays performed using the dual-luciferase reporter assay system (Promega Corp., Madison, WI, USA).

**ERG and Statistical Analyses**

These were performed as previously described. Briefly, tests were performed on a visual electrodiagnostic system (UTAS-VS; Microsystems GmbH, Buffalo Grove, IL, USA). Mouse 800 nm fluorescent dye (1:10,000 IRDyes; LI-COR, Inc., Lincoln, NE, USA), then imaged and quantified using commercial software (Odyssey Infrared Imager and ImageStudio 6; LI-COR, Inc.).

**RESULTS**

**L253X Overproduces Mutant mRNA/Protein in Affected Retinas**

A hallmark of class III CRX frameshift mutations is the overexpression of the mutant allele. To determine if L253X retinas accumulate mutant protein, we performed quantitative Western blots on total protein extracts from retinas of WT and heterozygous L253X/+ mice at postnatal day 10 (P10), 1 and 2 months old, and homozygous L253X/X mice at P10 only (because photoreceptors degenerate before later ages). Immunoblotting with an anti-CRX antibody revealed a truncated L253X protein that ran faster than the normal full-length CRX protein in samples from both heterozygous and homozygous mutant retinas (Fig. 1B). We quantified the total amount of CRX protein and of each isoform. Both L253X/+ and L253X/X displayed a 1.5- to 2.2-fold increase in total CRX protein compared to the WT retinas (Fig. 1C). The increases can be attributed to an accumulation of mutant protein (black bar), since normal CRX protein levels (gray bar) in L253X/+ retinas with one normal allele were roughly half of the total CRX protein present in WT retinas with two alleles.

To determine if the increased mutant protein corresponded with an increase in mutant mRNA, we quantified the relative allelic expression of L253X and normal Crx mRNA using droplet digital PCR (ddPCR). First, using mutant and WT mouse DNA, we developed a ddPCR assay that clearly distinguished the L253X and normal Crx sequences (Fig. 1D). qRT-PCR was then used to quantify allele-specific mRNA species. At all ages tested, the mutant transcript was
overrepresented, accounting for 57% of total Crx mRNA (Fig. 1D). Total Crx transcript levels quantified by conventional qRT-PCR (Supplementary Fig. S1) showed a statistically significant total Crx mRNA increase in 1-month L253X/+ retinas relative to WT, although levels were comparable to WT retinas during development at P10 and by 3 months.

Overall, L253X/+ retinas develop and maintain an elevated level of mutant mRNA that translates into an imbalance of the two protein forms, but this misregulation is not as severe as observed in other class III mutants. These results identify Crx-L253X as a bona fide class III mutation, but suggest that the position of the PTC determines the toxicity of the allele.

**L253X/+ Mice Have No Detectable Rod and Cone Function**

Homozygous animals carrying class III or null Crx mutations lack measurable photoreceptor function. To confirm that L253X/+ photoreceptors are functionally compromised, we quantified rod/cone light responses in L253X/+ and WT control mice at 1 month using ERG. Even at the highest light intensity stimuli, L253X/+ retinas displayed very little...
response (Figs. 2A–C). These results confirm that, like other class III models, homozygous L253X/X animals lack photoreceptor visual responses, resembling human LCA.

**L253X/X Photoreceptors Degenerate Earlier Than Crx−/−**

Other class III mutations show a greater impact on retina morphology and function than seen in Crx−/− mice. The original report noted that degeneration was more rapid in L253X/X than Crx−/− retinas, but suggested differences in mouse background might be a factor. To directly compare the impact of the L253X mutation with that of complete loss of Crx, we collected retinas from 1- and 3-month WT, L253X/X, and Crx−/− mice (all backcrossed 10 generations onto C57BL/6J) and assessed morphologic changes in H&E-stained sagittal sections through the optic nerve head (Figs. 2D–K). At 1 month, both L253X/X and Crx−/− retinas lacked photoreceptor outer segments (OS) and had thinner outer nuclear layers (ONL) than the WT control (Figs. 2E, 2F versus 2D). However, thinning was more pronounced in L253X/X than Crx−/−: L253X/X retina had only four to five rows of ONL nuclei, while Crx−/− maintained 7 to 8 rows at the same age (Fig. 2E versus 2F). Quantitative morphometry measures showed that the ONL of L253X/X was significantly thinner than that of Crx−/− at multiple positions (Fig. 2G, black versus solid gray). By 3 months, both models had similarly degenerated retinas with only 2–3 rows of ONL cells left (Fig. 2I versus 2J; 2K). Since both mutants showed normal ONL thickness at P7 through P10 (Supplementary Figs. S2A, S2B, and Tran et al.), these results suggest that ONL degeneration occurs earlier or at a faster pace in L253X/X mice than in Crx−/−.

In addition to progressive ONL loss, L253X/X also underwent age-dependent thinning of the outer plexiform layer (OPL) (Figs. 2E, 2F, Supplementary Figs. S2D, S2I, S2N, and Won et al.). In contrast, nonphotoreceptor cell layers (e.g., inner nuclear layer, ganglion cell layer) were largely unchanged in L253X/X compared to WT controls at various ages (Supplementary Figs. S2C–Q). These results suggest that the L253X mutation mainly affects photoreceptor structural integrity and survival.

**L253X/X Mice Exhibit Photoreceptor Gene Misregulation Distinct From Crx−/−**

To further examine if the more severe morphologic phenotype of L253X/X retinas reflected greater changes in CRX target gene expression, we collected RNA from L253X/X and Crx−/− retinas at P10 during photoreceptor terminal differentiation prior to cell death. Transcripts of 14 known CRX-dependent genes were quantified by qRT-PCR and compared both with WT levels (Supplementary Fig. S2R) and between the two models (Fig. 3A). Although most of these genes were dysregulated in both mutants compared to WT mice (Supplementary Fig. S2R), half of them showed statistically significant expression differences between L253X/X and Crx−/− (Fig. 3A). Three of these, Gnat1, Grk1, and especially Rho differed markedly (>2-fold) between the two models (Fig. 3A). Such differences in essential photoreceptor gene expression likely contribute to the morphologic and phenotype differences between the two models.

To confirm qRT-PCR results at the protein level, we performed immunofluorescent staining for RHO on retinal sections of 1-month-old L253X/X, Crx−/− and WT control mice. RHO was chosen because it displayed the largest difference in the RNA level between L253X/X and Crx−/−. As expected, in WT retina, RHO was predominantly localized to the rod outer segments (OS) with faint staining seen in ONL cell bodies (Fig. 3B). In contrast, L253X/X retina displayed no detectable RHO immunostaining (Fig. 3C), while Crx−/− retina still had weak and spotty RHO signals localized to individual ONL cell bodies and inner segments (Fig. 3D). These results supported the RNA expression data and suggest that L253X mutant protein negatively affects transcription.

Overall, the comparison between L253X/X and Crx−/− indicates that L253X disease is more severe than that
resulting from loss of Crx, supporting a dominant effect of the mutation.8,13 The more severe photoreceptor deficits in L253X/X thus presumably result from antimorphic activity of the truncated CRX protein.

Heterozygous L253X+/Mice Show Mild Decreases in Rod and Cone Function

The L253X (TVRM65) mutation was originally reported to cause recessive retinal disease.16 However, if the mutant protein possesses antimorphic activity, we would hypothesize that one copy would produce a photoreceptor phenotype. To test this, we investigated photoreceptor function in L253X/+ retinas by assessing electrical responses to whole retina light stimulation. ERGs recorded at 1, 2, 3, and 5 months revealed L253X/+ mice exhibit mild but significant reductions in ERG amplitudes compared to WT mice (Fig. 4). At all ages tested, dark-adapted A-waves were reduced in L253X/+ mice, especially at high stimulus intensities (Figs. 4A-D, black line versus gray line). Dark-adapted B-waves of L253X/+ mice were comparable to WT at 1 month (Fig. 4E), but significantly reduced at 2, 3, and 5 months (Figs. 4F-H, black line versus gray line). The degree of A-wave or B-wave reductions appeared to be constant, suggesting that the defects were unlikely a result of progressive degeneration. Reduction in cone function was also observed: Light-adapted B-waves were significantly decreased in L253X/+ mice at 2, 3 and 5 months (Figs. 4J-L), but not at 1 month (Fig. 4I). These results suggest that L253X/+ mice have deficits in both rod and cone function, resembling a mild retinopathy.

L253X/+ Retinas Do Not Degenerate

To test whether the functional defects correspond to morphologic changes, we measured retinal thickness in H&E stained retinal sections, comparing L253X/+ and L253X/X with WT mice at three ages (Fig. 5). Despite the progressive retinal degeneration seen in L253X/X mice (Figs. 5C, 5D, 5G, 5H, 5K, 5L; Supplementary Figs. S2C–Q), L253X/+ showed no degeneration or morphologic abnormalities compared to WT controls up to 3 months of age (Figs. 5B, 5D, 5F, 5H, 5J, 5L; Supplementary Figs. S2C–Q).

L253X/+ Retinas Display Dynamic Changes of Photoreceptor Gene Expression

To examine developmentally regulated photoreceptor gene expression in L253X/+ versus WT mice, we performed qRT-PCR analyses at two early ages: P10, when rods/cones are still terminally differentiating, and 1 month when rods/cones are fully mature in normal retinas. The genes tested were previously shown to dramatically change in expression during WT rod and cone development.22 Figure 6A shows expression changes of these genes in L253X/+ relative to WT mice at both ages. Gene expression conformed to three general trends in addition to the slight increase in total Crx expression (dashed black line) noted above: Gnat1, Rbo, and Sop (solid black lines), markers of mature photoreceptors, were significantly decreased in L253X/+ at P10 but attained WT levels by 1 month. Rxrg (dotted gray line), which in WT retina loses expression over development,22 was elevated in L253X/+ at P10 but decreased closer to WT
levels by 1 month. The other genes tested, which increase over development in WT retina, showed similar relative expression levels at both ages that did not vary considerably from WT (solid gray lines). Together, these gene expression trends suggest that L253X/+/retinas exhibit altered gene expression early in photoreceptor development, similar to other Crx mutant models.

To determine if L253X/+ retinas maintain photoreceptor gene expression after differentiation, we used qRT-PCR to examine expression of 17 essential rod and cone genes in WT and L253X mutants at other adult ages. The results at 1, 2, 3 and 5 months (presented in a heatmap in Fig. 6B) suggest a complex phenotype. Genes coding for transcription factors Crx, Rxrg, Otx2, and Nrl were upregulated at many of the time points tested. The expression of essential rod and cone phototransduction genes showed a less clear pattern across ages, but in general all exhibit phases of reduced expression relative to WT.

Late PTC-Caused C-Terminal Truncation Reduces Transactivation Function of CRX

As an initial step to understand the molecular mechanism underlying misregulation of gene expression in L253X mutant retinas, we asked whether a short C-terminal truncation caused by a late PTC affects the ability of CRX to transactivate...
target gene expression. This was tested using transient transfection assays in HEK293T cells with a Rhodopsin (Rho) promoter-luciferase reporter (BR130-Luc). Vectors expressing either the full-length (normal) or truncated CRX 1-254 were cotransfected with an NRL vector to measure their ability to transactivate the BR130-Luc reporter. CRX 1-254 did show some dose-dependent ability to activate the Rho promoter, suggesting that it does bind DNA and interacts with NRL, but the maximum activity was greatly reduced compared to the normal CRX protein (~30% of normal activity; Fig. 7).

**DISCUSSION**

**L253X Mouse Provides a New Class III Model for Mild Dominant Cone-Rod Dystrophy**

Although L253X (TVRM65) was originally identified as a recessive Crx mutation, several pieces of evidence suggest that L253X is antimorphic with dominant inheritance. First, L253X retinas overexpress the mutant gene product, a hallmark of class III mutations that amplifies the mutation's effects. Second, the photoreceptors in homozygous L253X/X mice degenerate earlier than Crx+/−, and show patterns of photoreceptor gene dysregulation that are distinct from Crx+/−. Thus, the presence of the mutant L253X protein causes more severe pathology than absence of CRX altogether, which is consistent with the antimorphic effect of other class III mutations. Furthermore, heterozygous L253X/+ mice have early gene expression differences that largely resolve by 1 month, suggesting that full maturation may be delayed in these retinas. Although gene expression changes in adults were modest, rod and cone functional deficits were detectable as early as 1 month. Previous work on class III mutants has emphasized the effects that small changes in gene expression over a large number of genes can have on photoreceptor function. Collectively, these findings identify L253X as a class III Crx mutation with the phenotypes summarized in the Table. Compared to previously reported class III mutations, pathogenicity of L253X is relatively mild. Unlike E168d2/−
Summary of L253X Related Phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Crx-L253X/X</th>
<th>Crx-L253X/+</th>
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<tbody>
<tr>
<td>Morphology (histology)</td>
<td>ONL degeneration earlier than Crx&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>No detectable abnormality up to 5 mo</td>
</tr>
<tr>
<td>Function (ERG)</td>
<td>Lack of rod and cone responses to light</td>
<td>Minor reductions in rod and cone function</td>
</tr>
<tr>
<td>Gene expression (qRT-PCR)</td>
<td>Altered gene expression distinct from Crx&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Dynamic changes from WT</td>
</tr>
<tr>
<td>CRX protein/RNA levels</td>
<td>L253X protein/RNA overproduction</td>
<td>Increased ratio of L253X versus normal</td>
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the last exon. This hypothesis further predicts that the position of the PTC influences the rate of mutant RNA accumulation. Indeed, L253X with a later PTC showed less RNA accumulation than the two early PTC alleles, E168del and A182del (Fig. 1). Thus, the earlier the PTC is, the more mutant product accumulates. These differences between early versus late PTCs may be attributed to the length of PTC-determined 3′UTR, within which multiple discrete elements could independently and additively contribute to mRNA hyperstability. Future identification of novel RNA regulatory elements shared among different PTC mutations may shed light on the mechanisms for mutant transcript hyperstability and phenotype variability in class III CRX disease. It is also notable that the accumulation of mutant protein in L253X retinas is more prominent than mutant RNA, raising the new possibility that altered protein turnover rate could also contribute to mutant protein accumulation.

In conclusion, our in-depth characterization of the CRX-L253X (TVRM65) mouse supports the classification of L253X as a dominant class III CRX mutation and provides a new animal model for understanding CRX-associated dominant retinopathies. The insights gained will hopefully lead to new treatment strategies for this complex disease.

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References