# Premature Truncation of a Novel Protein, RD3, Exhibiting Subnuclear Localization Is Associated with Retinal Degeneration

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The *rd3* mouse is one of the oldest identified models of early-onset retinal degeneration. Using the positional candidate approach, we have identified a C→T substitution in a novel gene, *Rd3,* that encodes an evolutionarily conserved protein of 195 amino acids. The *rd3* mutation results in a predicted stop codon after residue 106. This change is observed in four *rd3* lines derived from the original collected mice but not in the nine wild-type mouse strains that were examined. *Rd3* is preferentially expressed in the retina and exhibits increasing expression through early postnatal development. In transiently transfected COS-1 cells, the RD3-fusion protein shows subnuclear localization adjacent to promyelocytic leukemia-gene-product bodies. The truncated mutant RD3 protein is detectable in COS-1 cells but appears to get degraded rapidly. To explore potential association of the human *RD3* gene at chromosome 1q32 with retinopathies, we performed a mutation screen of 881 probands from North America, India, and Europe. In addition to several alterations of uncertain significance, we identified a homozygous alteration in the invariant G nucleotide of the *RD3* exon 2 donor splice site in two siblings with Leber congenital amaurosis. This mutation is predicted to result in premature truncation of the RD3 protein, segregates with the disease, and is not detected in 121 ethnically matched control individuals. We suggest that the retinopathy-associated RD3 protein is part of subnuclear protein complexes involved in diverse processes, such as transcription and splicing.

Retinitis pigmentosa (RP [MIM #268000]) encompasses a group of retinal degenerative diseases (RDs) that severely compromise the quality of life of affected individuals. RP is genetically heterogeneous, with mutations in multiple genes; to date, 168 retinal-disease loci have been mapped, and 117 retinal disease–associated genes have been identified (RetNet Web site).[1] A majority of RP-causing genes encode proteins involved in phototransduction, photoreceptor morphogenesis, trafficking, gene regulation, and/or splicing.[2] Mutations in some of the genes are suggested to cause a prolonged and/or increased phototransduction, followed by decreased $Ca^{++}$ levels, leading to photoreceptor-cell death.[3] Despite major advances, genetic defects have not been determined for a significant proportion of subjects with RP (RetNet Web site).[4–6]

One approach for identification of additional human retinopathy genes is to elucidate the molecular basis of retinal degeneration in animal models. At least 35 strains of mice exhibit retinal degenerative disease.[7,8] Some examples of retinal degeneration (rd) mice include *rd1* (*Pde6b*), *Rds* (*peripherin*), *rd7* (*Nr2e3*), and *rd16* (*Cep290/NPHP6*), and their known and putative biological functions closely correspond to genes involved in human retinal disease.[9–13] Mutations in their human orthologs are associated with retinopathies.[14–18]

The retinal degeneration 3 (*rd3*) mice were originally collected in Switzerland in 1969, were sent to the Jackson Laboratory, were bred as different lines to establish their Robertsonian translocation, and were later identified as having retinal degeneration.[19] Different strains of *rd3* have variable phenotypes, with photoreceptors starting to degenerate at age 2–3 wk and complete loss of photoreceptors at age 8–16 wk.[19] Electroretinography (ERG) studies have demonstrated that the loss of retinal response was dependent on the genetic background, with ERGs becoming extinguished at age 6–16 wk in some strains.[19] Hence, at least one modifier locus appears to slow down the rate of degeneration in the pigmented mice compared with the albino mice. A detailed morphological examination of three *rd3* strains demonstrated the degeneration of photoreceptors through apoptosis and has confirmed the slower rate of degeneration in pigmented *rd3* mice compared with albino strains.[20]

The *rd3* genetic locus was mapped to mouse chromosome 1 between markers *D1Mit292* and *D1Mit510* and was excluded as being a mouse ortholog of one of the human

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Usher syndrome loci.[21,22] Herein, we report the identification of the genes (*3322402L07Rik* and *C1orf36*—renamed "*Rd3*" and "*RD3*," respectively) responsible for the *rd3* retinopathy. We show that *Rd3* is preferentially expressed in the retina and that the *rd3* mutation leads to a truncated and relatively unstable protein in COS-1 cells. The RD3 protein is associated with promyelocytic leukemia-gene-product (PML) bodies in the nucleus. We also describe a large *RD3*-screening effort involving 881 probands with retinopathy, revealing at least one putative disease-causing mutation and several changes of uncertain significance.

## Material and Methods

### *Histological and Electroretinogram Analysis of* rd3 *Mice*

The studies involving mice were performed after approval from institutional committees was obtained. Histology and electroretinograms were performed as described elsewhere.[23]

### *Mouse Mutation Screen*

We examined genes in the published *rd3* (RBF/DnJ) critical region by direct sequencing. We additionally screened other *rd3* lines (RBJ/DnJ, STOCK Rb(11.13)4Bnr/J, and STOCK In(5)30Rk/J), as well as wild-type (WT) mouse strains, to verify the alteration. The following primers were used: for Rd3 exon 2, 5′-cgcctctctctttcttg-gtg-3′ (forward) and 5′-gcgactccagtcaccttctc-3′ (reverse); for Rd3 exon 3 5′-caagagcaaggttgggagtt-3′ (forward) and 5′-tccagcattcaagg-actcag-3′ (reverse). Exon amplification and sequencing of another gene, *Rcor3,* was initiated but was halted when the stop codon in *3322402L07Rik* was identified. PCR was performed from mouse-tail DNA with use of standard conditions, and amplified products were evaluated by agarose gel electrophoresis and were sequenced at the University of Michigan sequencing core facility.

### *RT-PCR and Northern-Blot Analysis*

RT-PCR analysis was performed using WT mouse retinal RNA at time points ranging from embryonic day 12 (E12) to age 4 mo and from postnatal day 2 (P2) and 4-wk-old (4W) adult $Nrl^{-/-}$ and $Crx^{-/-}$ mice.[24,25] The primers used were Hprt 5′-caaactttgctttccctggt-3′ (forward), Hprt 5′-caagggcatatccaaacaaca-3′ (reverse), Rd3rt 5′-gagagaggtggagcgacaac-3′ (forward), and Rd3rt 5′-cacatcctccgagatggttc-3′ (reverse). A human multiple-tissue northern blot (BD Biosciences Clontech) and a mouse retina northern blot were probed with radiolabeled full-length *Rd3* cDNA or $\beta$-actin cDNA, as described elsewhere.[26]

### *Immunoblot Analysis*

*Rd3* cDNA was amplified and cloned into the pEGFPN1 vector (BD Biosciences Clontech) with use of primers N1 5′-gacgacaagctt-atagtggccctgaaggaggt-3′ (forward) and N1 5′-cagcagggatccgagtcgg-cctggggcgccctgaa-3′ (reverse). Mutagenesis was performed, as described elsewhere,[27] with primers W6R 5′-atgtccctcatcccgcggctccg-gtggaacg-3′ (forward), W6R 5′-cgttccaccggagccgcgggatgagggacat-3′ (reverse), E23D 5′-cggaccccggccgacatggtgctggagacg-3′ (forward), E23D 5′-cgtctccagcaccatgtcggccggggtccg-3′ (reverse), K130M 5′-gccc-tggagaagatgatgcaggaggaggaggcc-3′ (forward), and K130M 5′-ggcct-cctcctcctgcatcatcttctccagggc-3′ (reverse). Primers used to transfer *Rd3* into pcDNA4c (Invitrogen) were Rd3pc 5′-gacgacggatccatgtcc-ctcatcccgtgg-3′ (forward) and Rd3pc 5′-cagcaggcggccgccttgatgggtc-tcctggttg-3′ (reverse). Primers (Rd3mut 5′-gctgctggctgaatgagagccc-gaggtg-3′ [forward] and Rd3mut 5′-cacctcgggctctcattcagccagcagc-3′ [reverse]) were used to introduce the *rd3* mutation into the mouse *Rd3* cDNA. The *rd3* cDNA construct was then used as a PCR template with Rd3pcF and Rd3pcR primers. The amplified PCR product was digested and cloned into pcDNA4. Each *Rd3* mutant cDNA clone was completely sequenced. Mouse *Mef2c* in pcDNA4 was used as a transfection control in some experiments. Expression constructs were transfected into COS-1 cells with Fugene 6 (Roche), according to the manufacturer's instructions. Cells were harvested after 48 h, with use of PBS and protease inhibitors (Roche), and extracts were fractionated by SDS-PAGE and were transferred to nitrocellulose membrane. The blots were probed with rabbit anti-Green Fluroescent Protein (GFP) polyclonal antibody (1:2,000) and mouse anti-beta tubulin antibody (1:10,000) or with mouse-anti Xpress antibody (1:4,000) (Invitrogen) and were visualized using appropriate secondary antibodies and luminescence (Pierce).

### *Localization in COS-1 Cells*

Cells were washed with PBS 48 h after transfection, were fixed using 4% paraformaldehyde/PBS for 15 min, and were washed again in PBS. Cells were permeabilized using 0.05% Triton X-100/PBS (PBS-T) for 10 min. After washing, a 5% BSA/PBS solution was applied, and the cells were blocked for 30 min. The primary antibodies used were anti-coilin antibody ab11822 (1:50) (Abcam), anti-SC-35 antibody ab11826 (1:50) (Abcam), anti-PML antibody sc-5621 (1:50) (Santa Cruz Biotechnology), and anti-Xpress antibody (1:400) (Invitrogen). The cells were incubated for 1 h in 1% BSA/PBS with primary antibody, were washed with 1% BSA/PBS, and were incubated with the appropriate secondary: anti-rabbit IgG Alexa fluor 546, anti-mouse IgG Alexa fluor 546, or anti-mouse IgG Alexa fluor 488 (Molecular Probes) (1:750 dilution). Cells were washed with 1% BSA/PBS, were counterstained with bisbenzimide to stain nuclei, and were examined by fluorescent microscopy.

### *Human-Mutation Screen*

All human studies were performed in a manner consistent with the Declaration of Helsinki and were approved by the respective institutional review boards. Appropriate informed consent was obtained from study subjects. We performed a three-continent (North America, Asia [India], and Europe [United Kingdom and Scandinavia]) human-mutation screen of the *RD3* gene. Clinical phenotypes observed among the North American patient pool included simplex RP, multiplex RP, juvenile (early-onset) RP, recessive RP, cone-rod dystrophy (CRD), Leber congenital amaurosis (LCA [MIM #204000]), partial cone degeneration, fundus albipunctatus, atypical pattern dystrophy, macular degeneration, Stargardt disease, autoimmune retinopathy, achromatopsia, congenital stationary night blindness, and enhanced S-cone syndrome. For the North American and European screen, the following primers were used: for exon 2, RD3 5′-ctgttagggcagagcaggtc (forward) and RD3 5′-ctctagtcctggtggcctca (reverse); for exon 3, RD3 5′-ggactaattggccatgagga (forward) and RD3 5′-ctttgtggccagag-gaagag (reverse). The screen in India used exon 2 5′-ttcccaggttcc-ccactctg-3′ (forward) and 5′-ccactgcagccacctttcct-3′ (reverse) and exon 3 5′-gacgcccggggtgccagac-3′ (forward) and 5′-gttccagggcccgg-cgct-3′ (reverse). PCR was performed using standard conditions, and products were subjected to direct sequencing. Sequencing

was performed at the University of Michigan sequencing core, at Bangalore Genei (Bangalore, India), and at the Institute of Ophthalmology (London). We performed RFLP analysis to determine segregation of a splice-site change within the LCA-affected family and to examine 121 unrelated ethnically matched unaffected controls. This change leads to the abolition of a restriction site for *Hph*1. The normal allele in a 436-bp amplified PCR product is digested into 387 bp and 49 bp fragments, whereas the homozygous mutated allele shows a 436-bp product.

## Results

### *rd3 Mouse Strains and Visual-Function Analysis*

The *rd3* mutation has been bred into different strains of mice and now exists in five separate strains—RBF/DnJ, STOCK In(1)Rk/J, STOCK In(5)30Rk/J, RBJ/DnJ, and STOCK Rb(11.13)4Bnr/J (hereafter termed "4Bnr"). An updated pedigree of *rd3*-bearing mice is shown in figure 1.[19] Histologically, STOCK In(5)30Rk/J mice have a modest reduction in outer nuclear layer (ONL) thickness, with 3–5 rows of nuclei left at age 35 d. In contrast, the RBF/DnJ albino strain has a fully degenerated retina at the same age. The 4Bnr albino strain had the least degenerate retina of the three assessed *rd3/rd3* mice, but some degeneration was observed at the earlier time point of 27 d (fig. 2*A*).

For comprehensive evaluation of rod and/or cone photoreceptor dysfunction, we performed dark-adapted (rod photoreceptor derived) and light-adapted (cone photoreceptor derived) ERG tests on STOCK In(5)30Rk/J, 4Bnr, and RBF/DnJ *rd3*/*rd3* strains, with C57BL/6J mice as controls. In a previous study, rod derived ERG showed progressive retinal functional loss in *rd3* mice; no cone ERG was reported.[19,28] We found that the amplitude of the loss of dark-adapted ERG varied, depending on the mouse strain. The In(5)30Rk/J (pigmented) strain had dark-adapted ERG closer to the WT at both age 24 d and age 32 d. The 4Bnr (albino) strain had a large response at day 17, but ERG was reduced at day 24. Lastly, the ERG in the RBF/DnJ (albino) strain was almost undetectable at age 24 d and was completely abolished by age 31 d (fig. 2*B*, top panel). All three strains of *rd3*/*rd3* mice had greatly reduced or nondetectable light-adapted ERGs by age 24 d (fig. 2*B*, bottom panel, and data not shown). These results demonstrate that both rod and cone photoreceptors in the *rd3* homozygotes are functionally affected.

### *Mutation Screen of* rd3 *Mice*

To identify the *rd3* mutation, we screened candidate genes in the mapped *rd3* region (fig. 3*A*) that had been defined elsewhere.[21] The critical genomic region was ~1 Mb and contained 13 genes or expressed sequences (National Center for Biotechnology Information [NCBI] Map Viewer build 36.1). To select candidates for mutation analysis, we first examined the expression profile of all genes *in silico* and through our microarray profiling, as reported elsewhere.[29] The Riken cDNA *3322402L07* showed a twofold increase in expression from P2 to P10 in purified rod photoreceptors[29] (A. Swaroop, M. Akimoto, and H. Cheng, unpublished data). Hence, we selected it as a candidate gene for the *rd3* mutation screen. Complete sequencing of all exons and intron-exon boundaries (fig. 3*B*) revealed a homozygous c.319C→T transition in the third exon. This change alters codon 107 from CGA to TGA, thus converting arginine to a stop codon, and is predicted to result in premature truncation of the RD3 protein. The mutation was detected in all *rd3* lines tested (RBF/DnJ, RBJ/DnJ, 4Bnr, and STOCK In(5)30Rk/J) but not in the nine control WT mouse lines (C57BL/6J, A/J, AE/J, BALB/cJ, C3H/HeJ, CBA/J, DBA/2J, MOLC/RkJ, and NON/LtJ) (fig. 3*C*). To further confirm whether this is indeed the causative mutation in *rd3*-containing strains, we generated haplotypes using markers spanning the *rd3* locus.[21] The three markers—

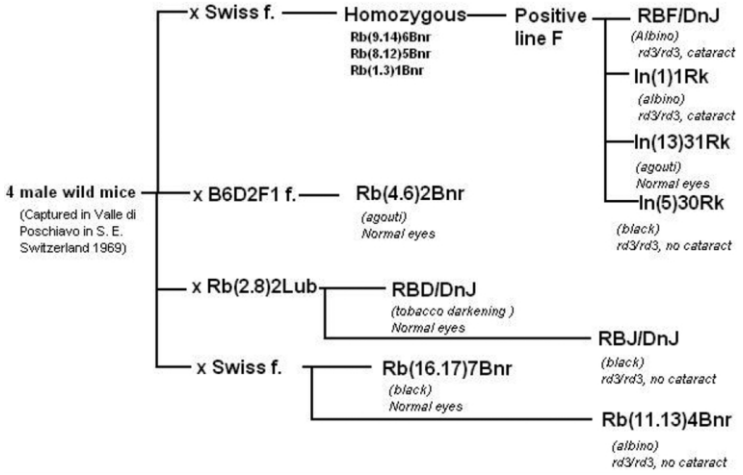


**Figure 1.** Pedigree of the *rd3* mouse strains. Four male mice were collected in Switzerland in 1969 and were genetically fixed to several lines. The mouse strains are currently housed at the Jackson Laboratory. This pedigree was updated from an earlier version,[19] with the kind permission of Springer Science and Business Media.

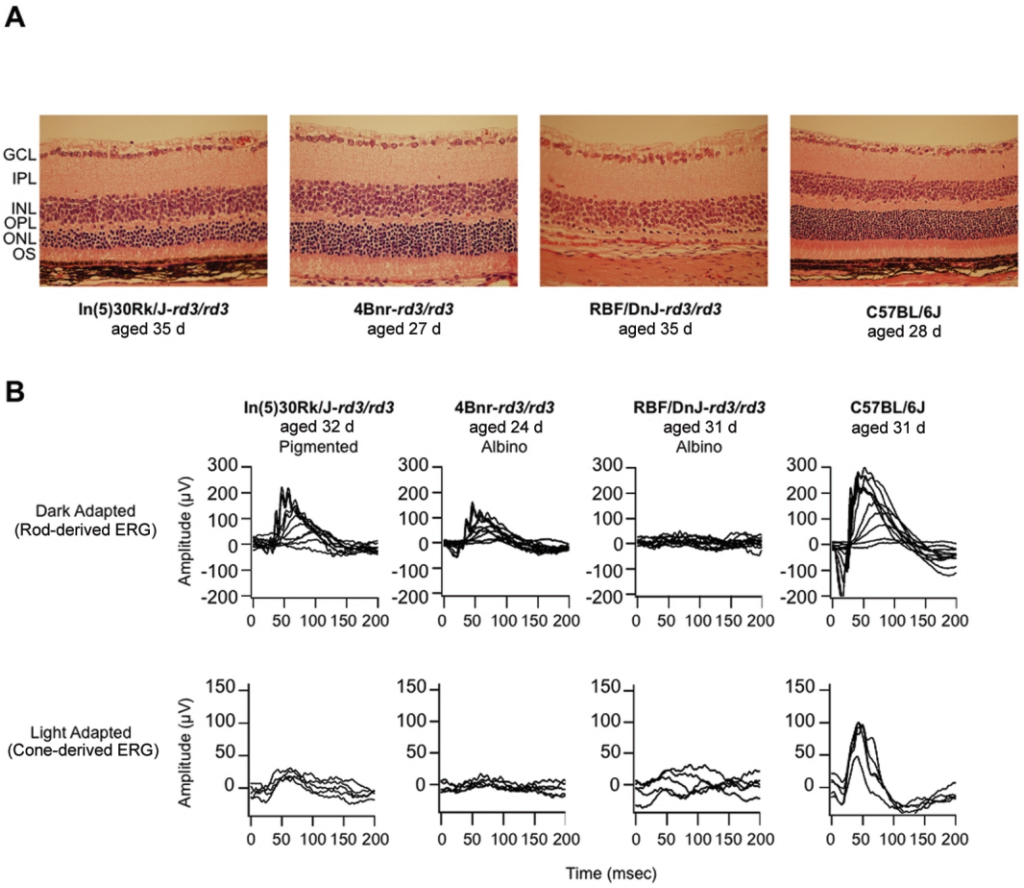


**Figure 2.** *A,* Histology of *rd3/rd3* mice. We examined STOCK In(5)30Rk/J, 4Bnr, RBF/DnJ (all *rd3/rd3*), and control C57BL/6J mice at ages 35, 27, 35, and 28 d, respectively. *B, top,* ERG responses under dark-adapted conditions: STOCK In(5)30Rk/J(*rd3/rd3*) mice (aged 32 d), 4Bnr(*rd3/rd3*) mice (aged 24 d), RBF/DnJ(*rd3/rd3*) mice (aged 31 d), C57BL/6J mice (aged 31 d). *Bottom,* Light-adapted ERG responses of the same mice.

*D1Mit292.1, D1Mit462.2,* and *D1Mit511*—revealed identical alleles in the three tested *rd3* lines (data not shown), which is consistent with the presence of the *rd3* mutation in this genetic background.

### *Analysis of the* Rd3 *Gene*

To determine the expression profile of the *Rd3* transcript, we performed northern-blot and RT-PCR analysis using RNA from human and mouse tissues. The *Rd3* transcript of 1.9 kb was enriched in adult-mouse retinal tissues and had no detectable expression in nonretinal adult-human tissues, as revealed by human multiple-tissue northern-blot analysis (data not shown). We then examined its expression using WT, $Nrl^{+/-}$, and $Nrl^{-/-}$ mouse retinal RNA by northern-blot analysis. WT mice and $Nrl^{+/-}$ mice have a rod/photoreceptor–dominated retina, whereas $Nrl^{-/-}$ mice lack rods and instead have a cone-dominant retina.[24] *Rd3* is transcribed as a single RNA in these strains, with no obvious difference (data not shown).

To elucidate developmental expression of *Rd3,* we performed RT-PCR experiments using mouse retinal RNA from E12 to age 4 mo (fig. 4*A*). The *Rd3* transcript is detected at very low levels at E12 but was more noticeable beginning at E18, with subsequent increase in expression at P2 and P6. After P6, the levels of *Rd3* transcript remained high. Additional RT-PCR experiments were performed using retinal RNA from $Nrl^{-/-}$ (aged P2 and 4W), $Crx^{-/-}$ (aged P2 and 4W), *rd1* (adult), and *rd16* (adult) mice. The $Nrl^{-/-}$ mouse lacks rod photoreceptors, whereas the $Crx^{-/-}$ mouse exhibits a lessening of rod and cone gene expression at P10 and exhibits photoreceptor degeneration at age 2 mo.[24,25] The *rd1* and *rd16* homozygotes exhibit early-onset degeneration of photoreceptors.[9,13] The detection of the *Rd3* transcript in all mutant mouse strains suggests that it is also expressed in inner retina cell types (fig. 4*B*). Our data are consistent with the expression profile for C1orf36 (Hs.632495 [NCBI Expression Profile Viewer]) and previous in situ hybridization experiments,[30,31] which demonstrate high expression of *3322402L07Rik* cDNA in the ONL in addition to the other cellular layers of the retina.

Clustal analysis of the RD3 protein orthologs suggests its strong conservation in vertebrates, from human to ze-

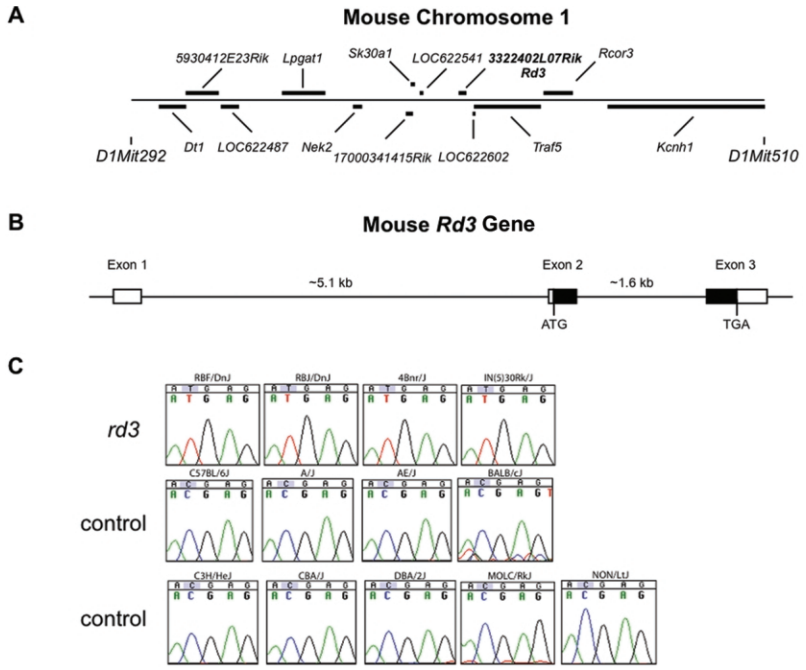


**Figure 3.** *A,* Critical genomic region encompassing the *rd3* mutation, between markers *D1Mit292* and *D1Mit510*.[21] Genes within the critical region are indicated. *B,* Genomic structure of the mouse *Rd3* gene. *C,* Chromatogram showing the region of the *Rd3* (*3322402L07Rik*) molecular defect in *rd3/rd3* strains RBF/DnJ, RBJ/DnJ, STOCK Rb(11.13)4Bnr/J, STOCK In(5)30Rk/J, and WT control strains C57BL/6J, A/J, AE/J, BALB/cJ, C3H/HeJ, CBA/J, DBA/2J, MOLC/RkJ, and NON/LtJ. The homozygous C→T change in the *rd3*-bearing mice is indicated. It generates a stop codon after aa 106.

brafish (fig. 4*C*). An *in silico* analysis of the mouse and human RD3 proteins reveals a coiled-coil domain at aa 22–54 and another weaker coiled-coil domain at aa 121–141.[32] The RD3 protein also has several putative protein kinase C and consensus Casein kinase II phosphorylation sites and one predicted sumoylation site. The mouse and human RD3 orthologs are basic proteins, with isoelectric points of 8.9 and 7.7, respectively.

Immunoblot analysis of COS-1 cells transfected with an *Rd3*-expression construct in the EGFPN1 vector demonstrated an expected RD3-GFP–fusion protein of ~45 kDa (predicted molecular mass of RD3 is ~22 kDa and that of GFP is ~23 kDa) (fig. 4*D*).

### *Localization of the RD3 Protein in Transfected COS-1 Cells*

In transfected COS-1 cells, the RD3-GFP–fusion protein primarily exhibited nuclear or nuclear/cytoplasmic localization (fig. 5*A*–5*D* and data not shown). Some cells also contained a putative aggresome-like staining adjacent to the nucleus—likely because of the overexpression of the RD3 protein (data not shown). The most striking pattern was the presence of subnuclear spots, henceforth named "RD3 bodies," of different sizes (fig. 5*A*–5*C*). We examined whether RD3 bodies colocalized with other subnuclear proteins, such as coilin (Cajal body marker), SC-35 (Nuclear Speckle marker), and promyelocytic leukemia gene product (PML body marker). Coilin staining did not localize near RD3 bodies in a consistent manner, suggesting that they are not associated (fig. 5*A*). SC-35 staining tended to be diffuse and localized near RD3 bodies (fig. 5*B*). PML staining also existed near RD3 bodies, with an occasional overlap (fig. 5*C* and 5*G*). To further confirm close PML proximity, we performed the same experiment using an Xpress-tagged *Rd3* plasmid. The Xpress-RD3 protein demonstrated a more highly varied localization pattern than did RD3-GFP, suggesting that the size of the tag may impact the distribution of transfected RD3 protein (fig. 5*E* and 5*F*). Nonetheless, we observed many cells with similar RD3 bodies in close proximity to PML bodies in Xpress-*Rd3*–transfected cells (fig. 5*E* and 5*H*).

### *Analysis of the* rd3 *Mutation*

To determine the consequence of the *rd3* mutation, we introduced this sequence alteration into the *Rd3*-pcDNA4 construct. Immunoblot analysis of COS-1–transfected cells revealed a truncated mutant RD3 protein (~11 kDa + ~4 kDa Xpress epitope), which was consistently detected at reduced levels compared with the WT protein (~22 kDa + ~4 kDa Xpress epitope) (fig. 6*A*). The mutant truncated protein therefore appears to be unstable and prematurely degraded. The mouse *Mef2c*-pcDNA4 construct was used as an internal control for transfection and was detected in roughly equal amounts in all experiments (fig. 6*A*). By immunocytochemistry, Xpress-RD3 mutant protein could

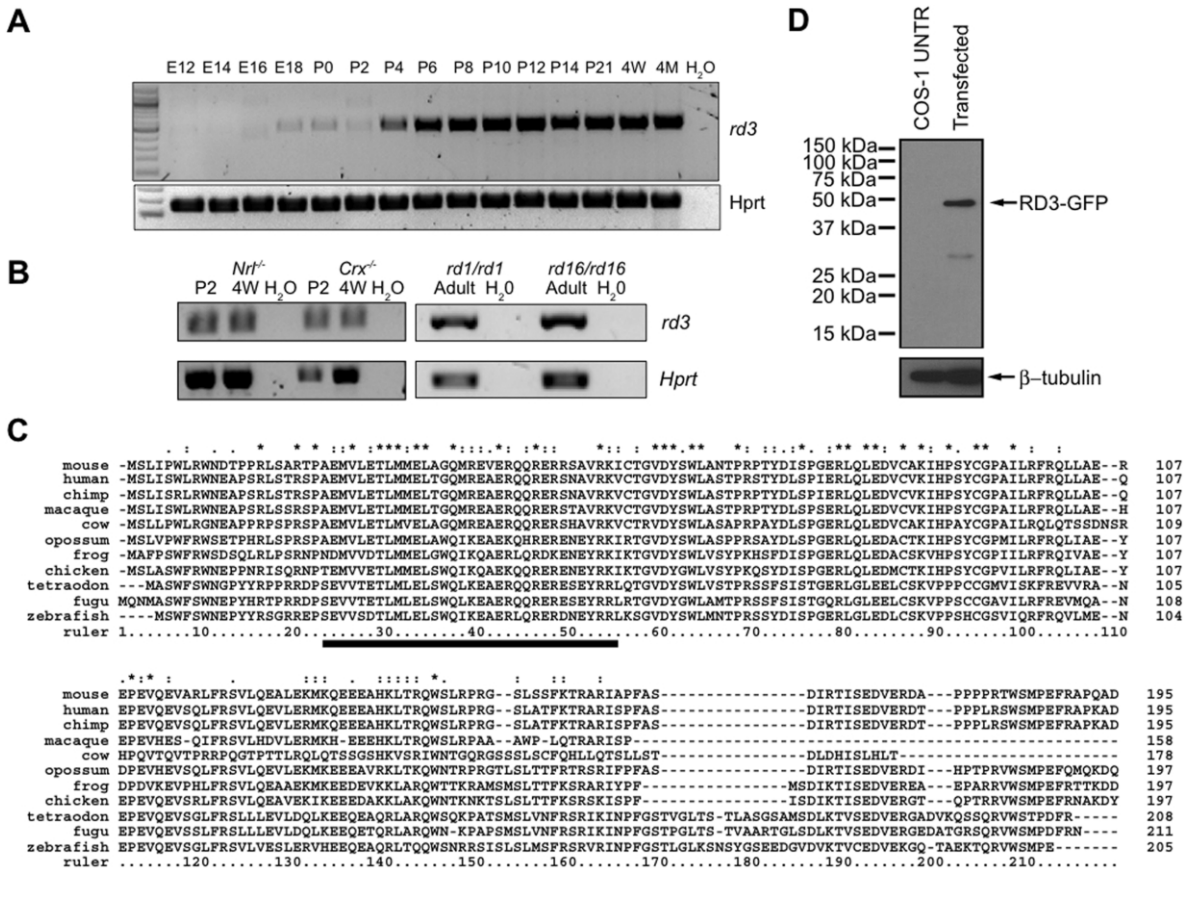


**Figure 4.** Expression of the *Rd3* gene. *A, Rd3* transcripts in mouse retina (aged E12 to 4 mo), present from at least E18, with a large increase from age P2 to age P6. *Hprt* was used to evaluate RNA quality and to normalize for quantity. *B,* Expression of *Rd3* in $Nrl^{-/-}$, $Crx^{-/-}$, *rd1/rd1,* and *rd16/rd16* mouse retina cDNA from P2, 4W, or adult. *Hprt* was used as a positive control. *C,* Alignment of RD3 protein orthologs. The amino acid sequence of mouse RD3 protein is aligned with those of human, chimpanzee, macaque, cow, opossum, frog, chicken, tetraodon, fugu, and zebrafish. Amino acid residues conserved in all orthologs are indicated by an asterisk (*), and reduced identity is shown using either a colon (:) or a dot (•). The long bar denotes a highly conserved coiled-coiled domain. *D,* Immunoblot expression of RD3-GFP fusion protein. An expression construct containing *Rd3*-GFP cDNA was transfected into COS-1 cells and was examined by immunoblot with use of anti-GFP antibody. The observed band is approximately the size estimated for the fusion protein (~50 kDa). A lower band of 30 kDa may represent a putative degradation product. Beta-tubulin was used to normalize for loading. UNTR = untransfected.

be observed in COS-1 cells, but at much lower levels compared with the WT protein at similar exposure times (fig. 6*B*–6*D*).

### *Human* RD3-*Mutation Screen*

To examine whether mutations in the human *RD3* gene are associated with retinal disease, we performed mutation screening of 461 probands with retinopathy from North America (including at least 30 with cone dystrophy or CRD and 15 probands with LCA), 103 probands of Indian descent who had retinal dystrophy, 302 probands (177 with autosomal dominant RP, 96 with autosomal recessive RP, and 29 with cone dystrophy or CRD) from the United Kingdom, and 15 probands with CRD from Scandinavia. We sequenced all coding exons, including the flanking intron/exon boundaries (fig. 7*A*).

Of particular interest were two siblings from India who had LCA (fig. 7*B*). Both probands have had poor vision since birth: nystagmus and atrophic lesions in the macular area, with pigment migration. Fundus photographs of the affected siblings are shown in figure 7*C*. A homozygous G→A transition in the donor splice site at the end of exon 2 (c.296+1G→A) was observed in both siblings (fig. 7*D*). When the splice site is removed, the 99th codon still encodes arginine and is followed by a stop codon, suggesting that the protein is prematurely truncated. RFLP analysis of other family members demonstrated that the homozygous change was present only in the affected members of the family, with unaffected members heterozygous for the mutation (fig. 7*E*). We examined 121 unrelated ethnically matched controls by RFLP and did not observe this nucleotide substitution. The G→A alteration was not ob-

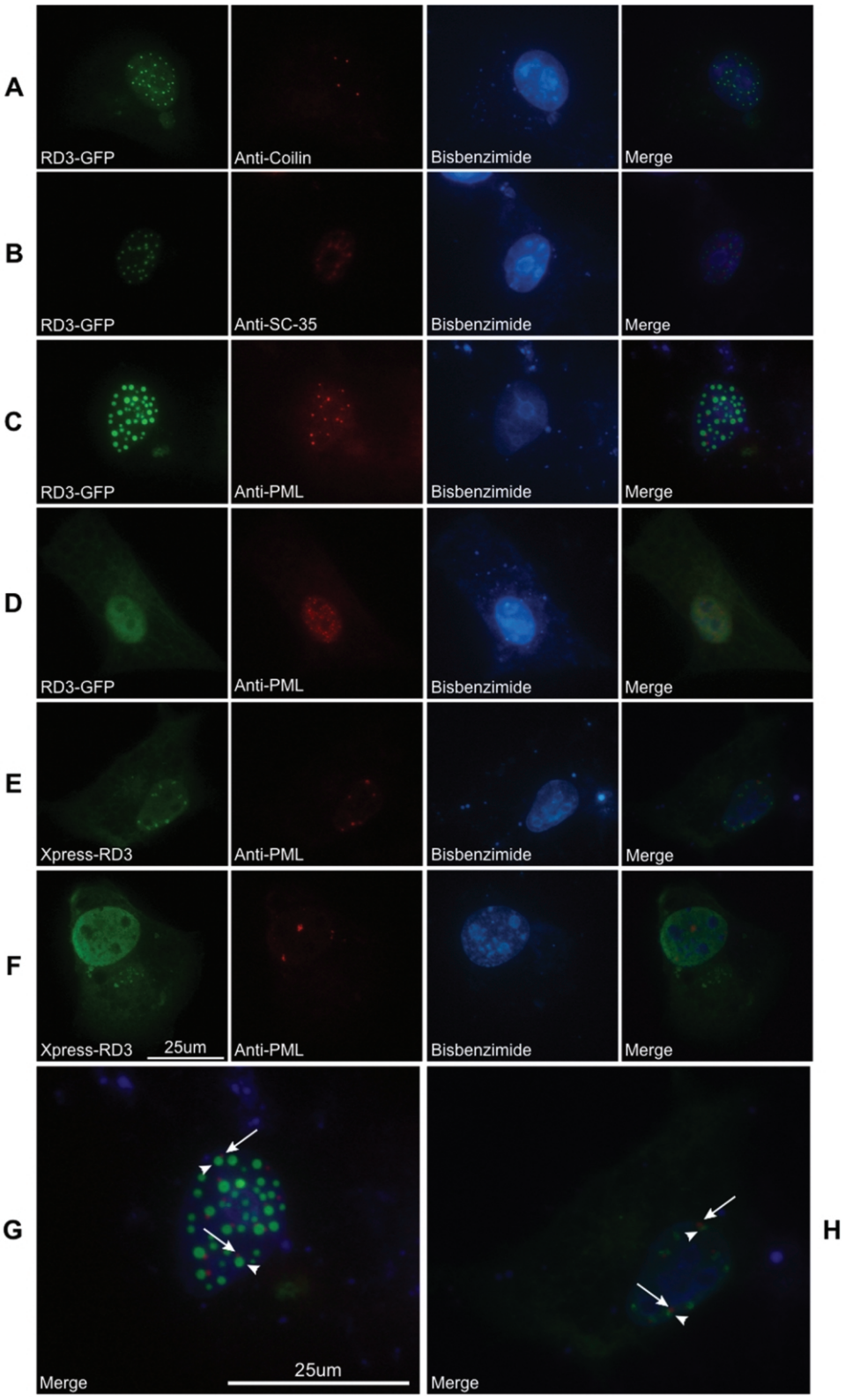


**Figure 5.** Immunolocalization of the RD3 protein. Mouse *Rd3* cDNA was placed in either the pEGFPN1 or the pcDNA4 vector and was expressed in COS-1 cells. The cells were observed directly via GFP or with use of an anti-Xpress antibody (*green*). Cells were also stained with subnuclear markers (*red*), to test for colocalization. Bisbenzimide was used to stain the nuclei (*blue*). *A,* RD3-GFP and Cajal body marker anti-coilin. *B,* RD3-GFP and nuclear speckle marker anti-SC-35. *C* and *D,* RD3-GFP and PML body marker anti-PML. *E* and F, Xpress-RD3 and anti-PML antibody. *G* and *H,* Higher magnification of the merged images from panels C and E, respectively. Close localization of RD3 and PML bodies are marked with an arrowhead and an arrow, respectively.

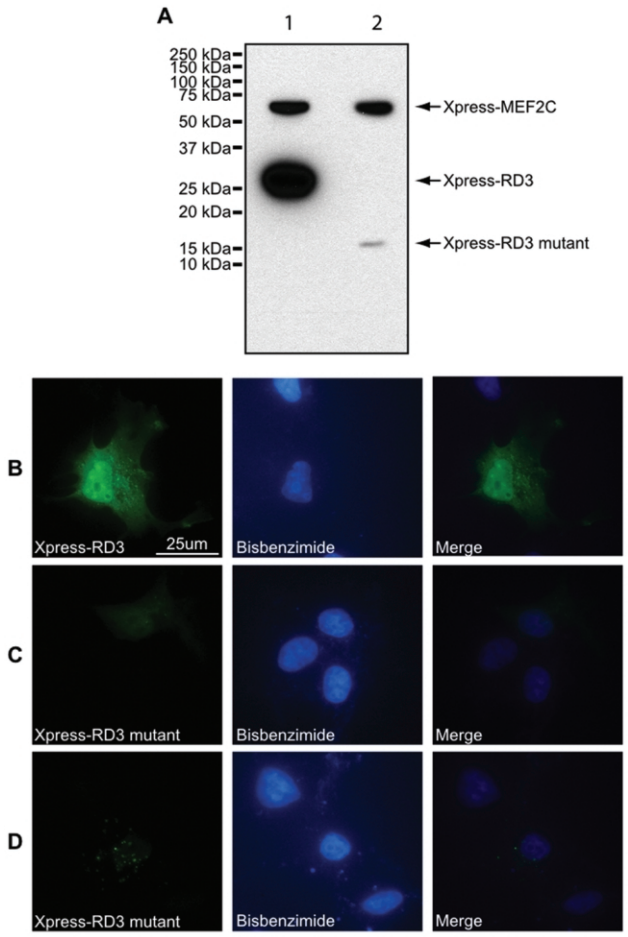


**Figure 6.** *A,* Examination of the mouse *rd3* mutation in COS-1 cells. Equal amounts of WT (*lane 1*) and mutant (*lane 2*) *Rd3*-pcDNA4 vectors were cotransfected with *Mef2c*-pcDNA4 into COS-1 cells. Cells were examined by immunoblot with use of anti-Xpress antibody. Roughly equal intensity of MEF2C signal shows that equal amounts of protein were loaded on the gel. WT RD3 protein is of expected molecular mass (~26 kDa), whereas the mutant RD3 protein is truncated (~15 kDa) and is expressed at lower levels. *B,* Immunolocalization of the RD3 protein in transfected cells, with use of WT *rd3*-pcDNA4 construct. Bisbenzimide was used to stain the nuclei (*blue*). *C* and *D,* Immunolocalization of the RD3 protein in transfected cells, with use of mutant *rd3*-pcDNA4 plasmid. Cells were examined as in panel B. Image exposures were performed in a consistent manner.

served in any other probands or controls from North America or Europe. To directly examine the impact of this change on RD3 transcript, we performed RT-PCR analysis of RNA from control lymphocytes, but no product could be amplified (data not shown).

Our mutation screening uncovered changes of uncertain significance in three female probands. The first affected individual (from North America) revealed four homozygous alterations (c.16T→C, 69G→C, 84G→A, and 235T→C) leading to two predicted amino acid changes (p.W6R, E23D, T28T, and L79L, respectively). She exhibited an atypical late-onset form of RP. The second proband (also from North America) carried a heterozygous c.389A→T alteration encoding a predicted p.K130M. This girl presented in 1996, then aged 11 years, with a history of poor vision beginning at age 3 years but with no family history of eye problems or blindness. Fundus examination showed round atrophic lesions in both maculae, and there was a cellophane-like sheen and atrophy in the other regions of the posterior poles. By ERG, it was determined that she had a CRD pattern, with cone ERG b-wave amplitudes at about one-third of normal and rod ERG amplitudes at ~80% of normal levels. The third proband (from Scandinavia) had a heterozygous c.170G→T change encoding p.G57V. She had no family history of visual handicap but had visual problems since age 6 years. Her eye disorder was diagnosed by repeated full-field ERG, at ages 9 years and 19 years, as cone-rod degeneration with reduced but still-remaining rod and cone function. At age 19 years, fundus examination revealed macular changes with atrophy and spicular pigment in the midperiphery. Visual acuity was reduced to 20/200, with central scotoma in both eyes.

Several other alterations were observed in the heterozygous form, both in probands and in controls, or did not segregate with the disease in affected families; these include c.103G→A (p.G35R), c.139C→T (p.R47C), c.202C→T (p.R68W), c.500G→A (p.R167K), and c.584A→T (p.D195V). The population group and frequency of each alteration are listed in table 1.

To examine whether missense alterations observed in the human mutation screen affected subcellular localization of the RD3 protein, we generated p.W6R, p.E23D, p.K130M, and p.W6R;E23D mutations in the RD3-GFP–expression construct. These changes were selected because of their absence in normal controls and their presence in individual probands. The GFP-fused mutant RD3 proteins did not appear to have a substantive difference from the control in immunoblot analysis or in COS-1 localization (data not shown). Overall, our data suggest that *RD3* mutations represent a rare cause of retinopathy.

## Discussion

RDs are a significant cause of untreatable blindness in the United States. Delineation of precise genetic defects in RDs and of cellular pathways that lead to photoreceptor-cell death are critical for developing knowledge-based design of treatment and therapies. In our continuing effort to identify retinal-disease genes, we have uncovered genetic defects in the *RD3* gene that are responsible for photoreceptor degeneration in the *rd3* mouse and some patients with retinopathies. Notably, RDs can be caused by mutations in photoreceptor-specific (such as rhodopsin, *RP1, CRX,* and *NRL*) or ubiquitously expressed (such as *RPGR, CHM,* and *PRPF31*) genes. The *RD3* gene exhibits an interesting expression profile: whereas it is preferentially (and highly) expressed in the retina and in photorecep-

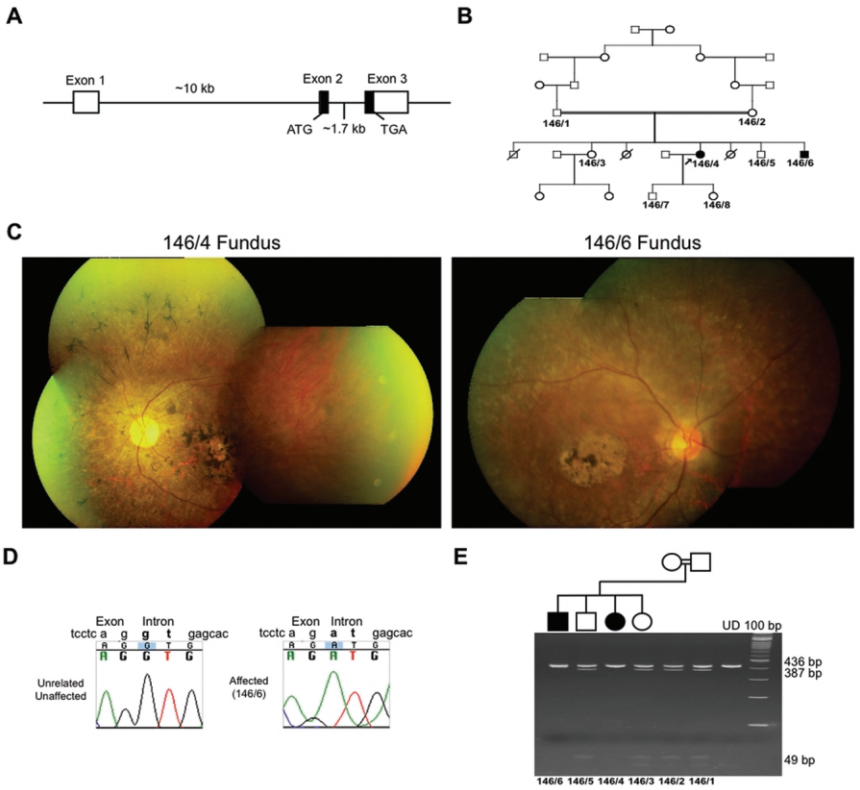


**Figure 7.** Findings for the LCA-affected family (from India), with a homozygous change in *RD3*. *A,* Genomic structure of the human *RD3* gene. *B,* Pedigree of family 146. Proband 146/4 is marked with an arrow. *C,* Fundus photographs of affected individuals 146/4 and 146/6. *D,* Chromatograms of unrelated unaffected subject and affected proband 146/6 at the exon2/intron2 junction. Individual 146/6 has a homozygous G→A change, which is predicted to remove the donor splice site of exon 2 and result in a stop codon after aa 99. *E,* Pedigree of a portion of family 146 and RFLP analysis of the mutation. Probands 146/4 and 146/6 show only a 436-bp band, whereas all other members show 49-bp and 387-bp bands in addition to the 436-bp band. UD = undigested.

tors, *Rd3* transcripts can be detected in mouse retinas that lack rod and cone photoreceptors.

The RD3 protein has a relatively low molecular mass (22 kDa) and includes a number of conserved sites for protein modification (specifically, phosphorylation and sumoylation). Additionally, the putative coiled-coil domain(s) might serve as protein-interaction site(s). The mutation identified in the *rd3* mouse generates a stop codon in the *Rd3* gene, thereby producing a truncated protein (of 11 kDa) that appears to be less stable, at least in transfected COS-1 cells. Hence, our data strongly implicate partial or complete loss of RD3 protein function as a possible mechanism of retinal degeneration in the *rd3* mouse. Although the *rd3* mutation is in the last exon, nonsense-mediated decay of the mutant transcript cannot be ruled out. In any event, the detection of a truncated RD3 mutant protein in COS-1 cells further validates the causal relationship of this mutation to retinopathy.

Subnuclear structures that commonly exist in cells include PML bodies, Cajal bodies, and nuclear speckles. PML bodies are implicated in diverse biological functions, including DNA repair, antiviral response, apoptosis, proteolysis, gene regulation, and tumor suppression.[33–35] PML is also suggested to be a transcriptional regulator and/or sensor of DNA damage and cellular stress.[33–35] Cajal bodies are associated with snRNPs and snoRNPs (small nuclear and nucleolar RNAs), which are involved in pre-mRNA and rRNA processing, respectively.[36] Nuclear speckles are observed close to genes with highly active transcription, are linked with pre-mRNA splicing, and are suggested to form a compartment for splicing factors and proteins involved in transcription.[37] Interestingly, a number of retinopathy genes are associated with transcription and splicing.[16,38–45] Varied localization of RD3 protein in COS-1 cells suggests its dynamic role in cellular processes and within subnuclear compartment(s). A reduced amount of mutant RD3 protein in transfected cells indicates that the loss of RD3 protein may compromise nuclear function(s). However, the precise role of RD3 in transcription, splicing, or other regulatory processes is subject to further experimentation.

LCA is a cause of early-onset (childhood) blindness, with an estimated prevalence of 1 in 50,000–100,000.[46] Known and putative functions and/or location of proteins encoded by mutant LCA genes are diverse and include pro-

**Table 1. Summary of the *RD3* Human Mutation Screen**

| Change | No. of Subjects with Exon Change | | | | | |
|---|---|---|---|---|---|---|
| | United Kingdom/ Scandinavia | | India | | North America | |
| | Proband ($n$ = 317) | Control ($n$ = 95) | Proband ($n$ = 103) | Control[a] ($n$ = 121) | Proband ($n$ = 461) | Control ($n$ = 175) |
| Homozygous c.16T→C, 69G→C, 84G→A, 235T→C (p.W6R, E23D, T28T, L79L) | 0 | 0 | 0 | NA | 1 | 0 |
| c.103G→A (p.G35R) | 0 | 0 | 1 | NA | 0 | 0 |
| c.139C→T (p.R47C) | 8 | 2 | 2 | NA | 10 | 5 |
| c.170G→T (p.G57V) | 1 | 0 | 0 | NA | 0 | 0 |
| c.202C→T (p.R68W) | 0 | 0 | 1 | NA | 0 | 0 |
| c.389A→T (p.K130M) | 0 | 0 | 0 | NA | 1 | 0 |
| c.500G→A (p.R167K) | 3 | 0 | 0 | NA | 1 | 1 |
| c.584A→T (p.D195V) | 1 | 3 | 0 | NA | 4 | 6 |
| c.296+1G→A homozygous | 0 | 0 | 1[b] | 0 | 0 | 0 |

NOTE.—Probands with RP were sequenced for changes in exon 2 and exon 3 of *RD3*.

[a] The c.296+1G→A mutation was examined in 121 Indian controls by RFLP. NA = not applicable.

[b] One proband was found in the initial screen. An affected sibling was subsequently identified during follow-up.

tein trafficking, cell-cycle progression, photoreceptor morphogenesis, transcription, phototransduction, and retinoid cycle.[4,13,18,47] *RD3* is likely responsible for a small subset of LCA, and further examination of this retinopathy might allow us to determine the proportion of *RD3* mutation–based early-onset retinopathy. We have identified two siblings carrying a mutation in the invariant G residue of the exon 2 donor splice site. Splice-site alterations are responsible for at least 15% of human mutations.[48] Mutation of the invariant G at donor splice site +1 is a commonly reported cause of human disease (Human Gene Mutation Database).[49] It is possible that a cryptic splice site within exon 2 or intron 2 is used after the invariant G is changed. Additionally, exon 2 may be skipped entirely,[50] or the mRNA may be subject to nonsense-mediated decay.[51] Within exon 2, there are two potential cryptic splice sites—c.159 and c.169—with scores of 0.50 and 0.43, respectively. After the normal exon 2 splice site, the next putative splice sites are at nucleotides c.296+219 and c.296+432 (respective scores 0.41 and 1.00). The likely splicing site for mutant allele is c.296+432, because of its high score[52] (Berkeley *Drosophila* Genome Project: Splice Site Prediction by Neural Network Web site). The splice-site mutation is predicted to result in a truncated RD3 protein, ending at aa 99. This shortened protein would be similar to the 107-aa protein in the *rd3* mice, thereby raising the possibility that the *rd3* mouse is a useful mouse model for the retinal disease in this family. Notably, the *rd3* mice exhibit a relatively quick degeneration of photoreceptors. For example, ONL loss in RBF/DnJ-*rd3/rd3* mice is complete in ~8 wk.[19] In comparison, the quite severe *Pde6b*$^{rd1}$ and *Rd4* mouse retinas show severe degeneration by age 1 mo and 2 mo, respectively.[53] Several strains of mutant mice reveal much slower photoreceptor loss than do *rd3,* with complete degeneration as late as age 30 mo.[53]

In the one proband carrying four homozygous alterations, W6 and E23 residues are conserved in almost all species. Codon 6 in chimpanzee is arginine (instead of tryptophan), and codon 23 in frog is aspartic acid (instead of glutamic acid). Since W6R and E23D alterations exist individually in chimpanzee and frog, it is difficult to conclude whether these changes cause disease in humans. Additional work would be required to fully characterize the functional effect of these alterations. For c.389A→T (p.K130M)– and c.16T→C;69G→C;84G→A;235T→C (p.W6R; E23D;T28T;L79L)–containing probands, we attempted to collect DNA samples from the respective families but were unsuccessful. Although c.389A→T (p.K130M) and c.170G→T (p.G57V) heterozygous changes are not observed in our cohort of controls, we learned that these subjects are of Chinese and mixed European–Middle Eastern decent, respectively. Screening of ethnically matched controls might be necessary to determine whether these changes are polymorphic in their respective populations. We identified several additional nucleotide changes that do not appear to be associated with disease in heterozygous state; these are c.103G→A (p.G35R), c.139C→T (p.R47C), c.202C→T (p.R68W), c.500G→A (p.R167K). and c.584A→T (p.D195V).

Since the splice–site alteration represents the only likely disease-causing mutation in 881 examined probands, we suggest that *RD3* mutations are a rare cause of retinal disease. *C1orf36* (*RD3*) was tested elsewhere as a candidate retinal-disease gene; however, no significant alterations were observed in this earlier study.[30] Recently, initial screening of coding regions in patients with retinopathy did not reveal any disease-causing mutation in *CEP290/NPHP6,* and only the lymphocyte RNA analysis established it as a major LCA gene.[18] However, unlike *CEP290, RD3* is not detected in lymphocytes.

Recent studies suggest a more dynamic environment in the nucleus than previously expected.[54] Discrete nuclear processes (such as steps in gene transcription and post-transcriptional events) appear to be mediated by association of gene regions, transcribed sequences, and/or proteins in unique subnuclear compartments. PML bodies are

but one component of the nucleus whose role(s) is not completely understood. The discovery of *RD3* and its further characterization might allow us to gain insights into fundamental regulatory events that are necessary for retinal function.


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### Web Resources

The URLs for data presented herein are as follows:

Berkeley *Drosophila* Genome Project: Splice Site Prediction by Neural Network, http://www.fruitfly.org/seq_tools/splice.html
Human Gene Mutation Database, http://www.hgmd.org/
NCBI Expression Profile Viewer, http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.632495 (for Hs.632495)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for RP and LCA)
RetNet, http://www.sph.uth.tmc.edu/Retnet/

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