A Missense Mutation in *PRPF6* Causes Impairment of pre-mRNA Splicing and Autosomal-Dominant Retinitis Pigmentosa

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Retinitis pigmentosa (RP) is an inherited form of retinal degeneration that leads to progressive visual-field constriction and blindness. Although the disease manifests only in the retina, mutations in ubiquitously expressed genes associated with the tri-snRNP complex of the spliceosome have been identified in patients with dominantly inherited RP. We screened for mutations in *PRPF6 (*NM_012469.3), a gene on chromosome 20q13.33 encoding an essential protein for tri-snRNP assembly and stability, in 188 unrelated patients with autosomal-dominant RP and identified a missense mutation, c.2185C>T (p.Arg729Trp). This change affected a residue that is conserved from humans to yeast and cosegregated with the disease in the family in which it was identified. Lymphoblasts derived from patients with this mutation showed abnormal localization of endogenous PRPF6 within the nucleus. Specifically, this protein accumulated in the Cajal bodies, indicating a possible impairment in the tri-snRNP assembly or recycling. Expression of GFP-tagged PRPF6 in HeLa cells showed that this phenomenon depended exclusively on the mutated form of the protein. Furthermore, analysis of endogenous transcripts in cells from patients revealed intron retention for pre-mRNA bearing specific splicing signals, according to the same pattern displayed by lymphoblasts with mutations in other *PRPF* genes. Our results identify *PRPF6* as the sixth gene involved in pre-mRNA splicing and dominant RP, corroborating the hypothesis that deficiencies in the spliceosome play an important role in the molecular pathology of this disease.

Mutations in more than 50 genes cause retinitis pigmentosa (RP [MIM 268000]), a form of retinal degeneration with an estimated prevalence of 1 in ~4,000 individuals.[1,2] Patients with this condition experience a progressive decline of retinal function, usually starting with night blindness, that often leads to the complete loss of vision at the end stage.[3] RP is inherited as a dominant, recessive, or X-linked trait and very rarely as a non-Mendelian phenotype.[4] The course of the disease can be slowed with vitamin A supplementation.[5] Most genes associated with nonsyndromic RP have a retina-prevalent expression and/or produce proteins that have a clear biochemical or structural role within the retinal or the retinal pigment epithelium cells. Other RP genes, however, have a more widespread expression pattern or are present in all human tissues and nonetheless cause ocular-restricted symptoms.[1]

With respect to ubiquitously expressed genes, mutations in a few cause autosomal-dominant RP (adRP) and encode PRPF31 (MIM 606419), PRPF8 (MIM 607300), PRPF3 (MIM 607301), RP9 (MIM 607331), and hBRR2 (MIM 601664),[6–12] i.e., five out of the several dozen proteins composing the spliceosome, a machinery that is essential for pre-mRNA processing in all eukaryotes. This macromolecular structure is constituted by five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6, as well as more than a hundred other non-snRNP-specific proteins (reviewed in Wahl et al.[13]). Intron removal by the spliceosome occurs through a series of sequential reactions that involve the dynamic assembly and disassembly of snRNP subcomplexes. Within each round of splicing, the U4/U6.U5 complex (the tri-snRNP) is assembled through the interaction of the U4/U6 di-snRNP with U5, which is mediated by splicing factors PRPF31 and PRPF6.[14,15] Interestingly, all the pre-mRNA splicing factor genes associated with adRP encode components of the tri-snRNP and, with the exception of RP9, have been proven to affect the assembly and/or disassembly of this particle.[16–20] Within the tri-snRNP, PRPF6 acts as a molecular bridge between the U5 snRNP and the di-snRNP.[15] Furthermore, data from electron microscopy and protein-protein interaction analyses[21,22] indicate that PRPF6 binds to 4 out of 5 tri-snRNP proteins involved in adRP, constituting the structural heart of the particle itself (Figure 1). On the basis of previous biochemical knowledge on this protein and the involvement of other tri-snRNP components in adRP, we reasoned that *PRPF6* could be a good candidate gene for this condition.

We screened for mutations in all 21 exons of this gene on chromosome 20q13.33 in index patients from 188 families with adRP. This study involved human subjects, was carried out in accordance with the tenets of the declaration of Helsinki, and was approved by the institutional review boards of the University of Lausanne, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary. All participants gave written, informed consent before they donated 10–30 ml of blood for genomic DNA extraction or

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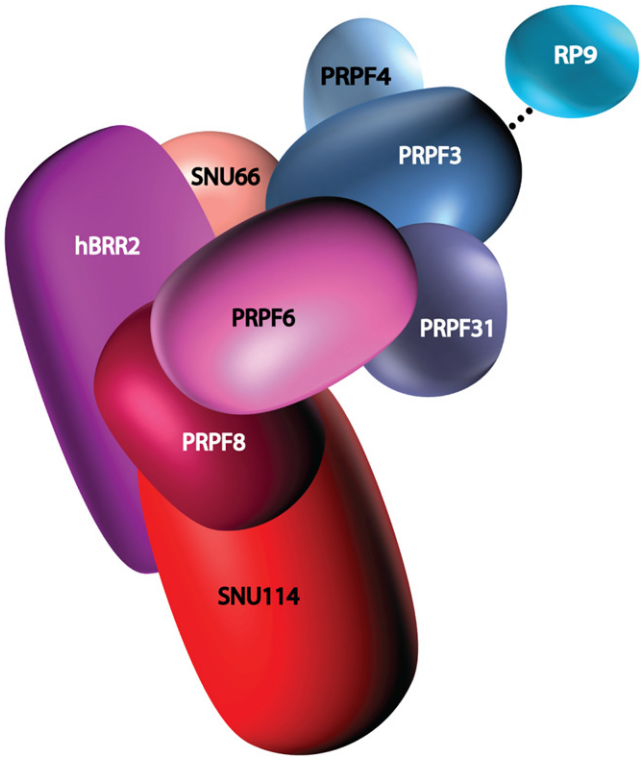

Figure 1. Schematic and Partial Representation of the Spliceosomal Tri-snRNP Particle and of Proteins Associated with adRP
This image graphically depicts current data on protein-protein interactions and the electron-microscopy-based physical structure of tri-snRNP components. Elements of the U5 snRNP are in different shades of red, and those belonging to the U4/U6 snRNP are in shades of blue. Names of proteins found to be associated with adRP are in white ink. The dotted line indicates that RP9 interacts with PRPF3, although it is not a core component of the tri-snRNP.

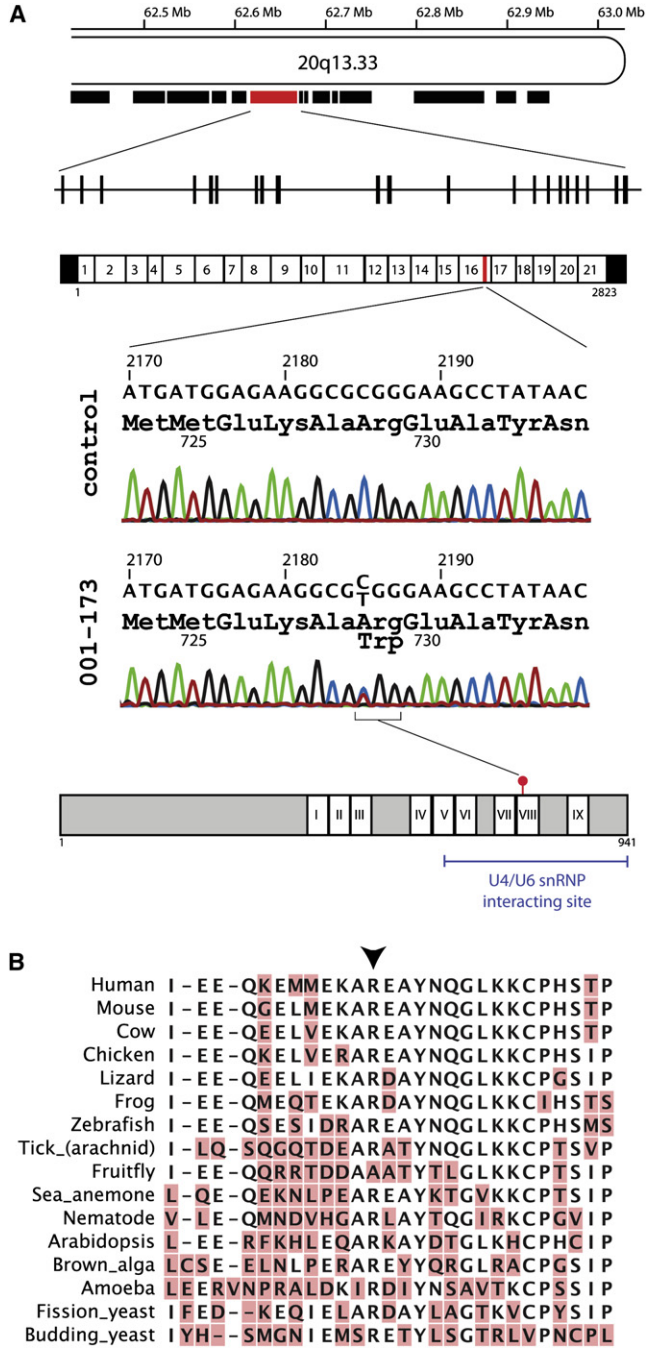

Figure 2. Relative Position, DNA Sequence, and Effects of c.2185C>T in *PRPF6*
(A) *PRPF6* (red box) is a 21 exon gene located at the end of the long arm of chromosome 20. The c.2185C>T mutation (red vertical line within the cDNA representation) lies in exon 16 and causes the Arg>Trp substitution at codon 729. Arg729 (red pin) lies within the eighth HAT (Half-a-TRP) domain of the encoded protein, in the middle of the PRPF6 moiety (blue bar) responsible for its interaction with the U4/U6 snRNP.
(B) Phylogenetic alignment of part of the HAT domain containing Arg729, indicated by the arrowhead. Non-conserved residues are shaded.

lymphoblast immortalization. DNA regions to be analyzed were amplified by PCR with the primers reported in Table S1, purified, and subsequently sequenced by standard Sanger sequencing with a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Among the many DNA changes detected (Table S2), one was a missense variation causing an arginine-to-tryptophan (positively charged to hydrophobic amino acid) substitution (p.Arg729Trp [c.2185C>T]) at codon 729 in exon 16 (Figure 2A). This variant, which was not reported in public sequence repositories (including the 1000 Genomes project database), affected a PRPF6 residue that seems to be invariant in all eukaryotic kingdoms, with the exception of the class of insects (Figure 2B). Arginine 729 is part of one of the HAT (half a TPR) repeats that are present in the C-terminal part of PRPF6. Such domains are characterized by the presence of three aromatic residues with conserved spacing and are involved in protein-protein interactions.[23] The missense mutation was present in a male patient of European descent (ID: 001-173, IV-1 in Figure 3A) who reported a family history of RP but was absent in 1,078 chromosomes from ethnically matched control individuals.

Additional genetic screening revealed the same DNA change in the patient's affected brother; no other members of this family were available for further investigation (Figure 3A).

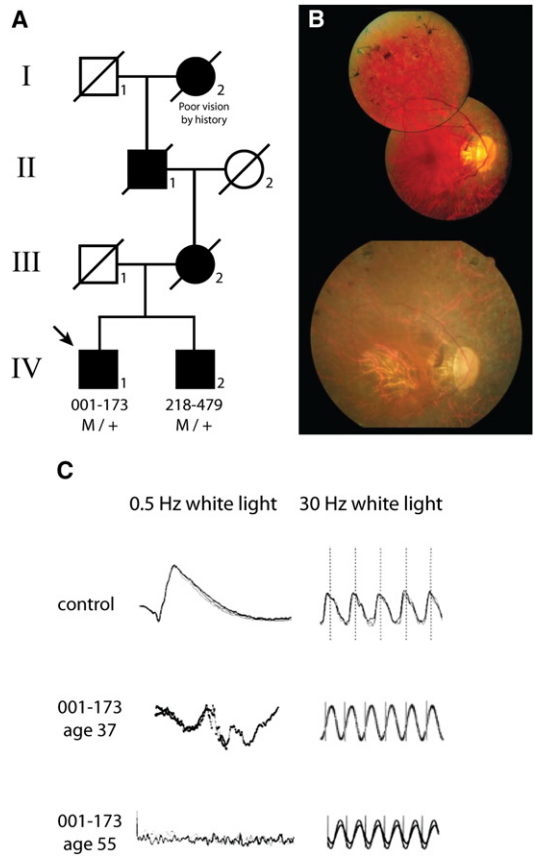

Figure 3. Pedigree, Fundus Photographs, and Electroretinograms of Patient 001-173

(A) The family tree of the index patient indicates dominant inheritance of RP over three, possibly four, generations.

(B) Fundus photographs of patient 001-173's right eye at age 37 (top) and 55 (bottom). Photographs reveal progression of retinitis pigmentosa over this period (see text).

(C) Full-field ERGs from a normal control and patient 001-173 at age 37 and age 55; patient's responses (right eye, illustrated) to 0.5 Hz white light flashes under dark-adapted conditions and 30 Hz white flicker at age 55 are substantially smaller than responses at age 37 (see text). Vertical lines indicate time of stimulus onset.

At an age of 37 years, patient 001-173 was first examined by one of us (E.L.B.) in 1988 for night deficiency and loss of side vision. At this initial examination he had a best-corrected visual acuity of 20/30 O.D. (right eye) and 20/40 O.S. (left eye). Slit-lamp examination showed central posterior subcapsular cataracts O.U. (in both eyes). Fundus examination of each eye showed a normal disc, some atrophy just temporal to the disc, clear macula, attenuated retinal arterioles, and bone spicule pigment 360° around the periphery (Figure 3B, top). His final dark adaptation threshold was elevated 3.0 log units. His Goldmann visual field in a V-4e white test light was constricted to a 25° diameter with a peripheral crescent in each eye. His full-field electroretinograms (ERGs) were substantially reduced, and he had dark-adapted mixed cone-rod responses to 0.5 Hz white light of 5.6 μV O.D. and 4.9 μV O.S. (lower norm = 350 μV) and cone responses to 30 Hz white flicker of 0.4 μV O.D. and 0.7 μV O.S. (lower norm = 50 μV) (Figure 3C). He was last examined in 2007 at age 55. At that time he reported that he had cataract surgery O.S. in 1999 and O.D. in 2000 and a reoperation O.D. in 2001. After the reoperation in 2001, he had extensive inflammation and never recovered acuity O.D. The acuity O.S. gradually decreased from 1999 to 2007. He also noted further loss of peripheral vision O.U. during this period. His best-corrected vision was hand motions O.D. and 20/100 O.S. Slit-lamp examination showed pseudophakia O.U. Fundus examination of each eye showed waxy pallor of each disc, atrophy of the retinal pigment epithelium and prominent choroidal vessels in each macula, attenuated retinal vessels, and bone spicule pigment visible in the midperiphery (Figure 3B, bottom). The final dark-adaptation threshold was elevated 5.0 log units. His visual field was constricted by confrontation to about a 10° diameter O.D. and a 20° diameter O.S. His full-field ERGs (Figure 3C) in response to 0.5-Hz white light were nondetectable (i.e., less than 1.0 μV) and were reduced to 0.26 μV O.D. and 0.15 μV O.S in response to 30 Hz white flicker. (NB: virtual blindness, i.e., loss of the ability to walk independently in a well-lighted room, occurs when 30 Hz cone flicker ERG responses are $\leq$0.05 μV). The decline in visual acuity, visual fields, and full-field ERG amplitudes showed considerable progression of his retinitis pigmentosa over 18 years. His brother 218-479 (IV-2 in Figure 3A) reported to us that he had had night deficiency since age 16 and loss of side vision in his 40s. His retinitis pigmentosa was diagnosed around age 16. Although he is two years younger than 001-173, his vision was always worse than that of his brother and gradually decreased to light perception in both eyes by age 54. Patient 218-479 has not had a recent eye examination, and therefore no clinical records were available for this study.

Mutations in splicing-factor genes associated with adRP affect the amount and composition of the tri-snRNP and thus lead to defects in its function[11,16,17,20] and impair the process of intron excision for specific pre-mRNA substrates.[20,24] To evaluate the pathogenicity of the identified DNA change and gain insights into the possible consequences of this heterozygous missense, we immortalized lymphoblast cells from both patients 001-173 and 218-479 upon transformation with the Epstein-Barr virus and first analyzed the cellular localization of endogenous PRPF6. Lymphoblasts from controls and patients were grown on coverslips, fixed, and costained with an anti-PRPF6 antibody that is specific to its N-terminal moiety (Santa Cruz Biotechnology, Santa Cruz, CA) and an antibody (Sigma-Aldrich, St. Louis, MO) recognizing the SC35 protein, a marker for splicing speckles. Confocal microscopy revealed that in all cell lines PRPF6 localized in the nucleoplasm and splicing speckles (not shown), similar to what has been observed in HeLa cells.[15] However, lymphoblasts from patients revealed additional accumulation of PRPF6 in round and sharply defined subnuclear structures, which were identified as Cajal bodies after specific staining with the anti-p80-coilin

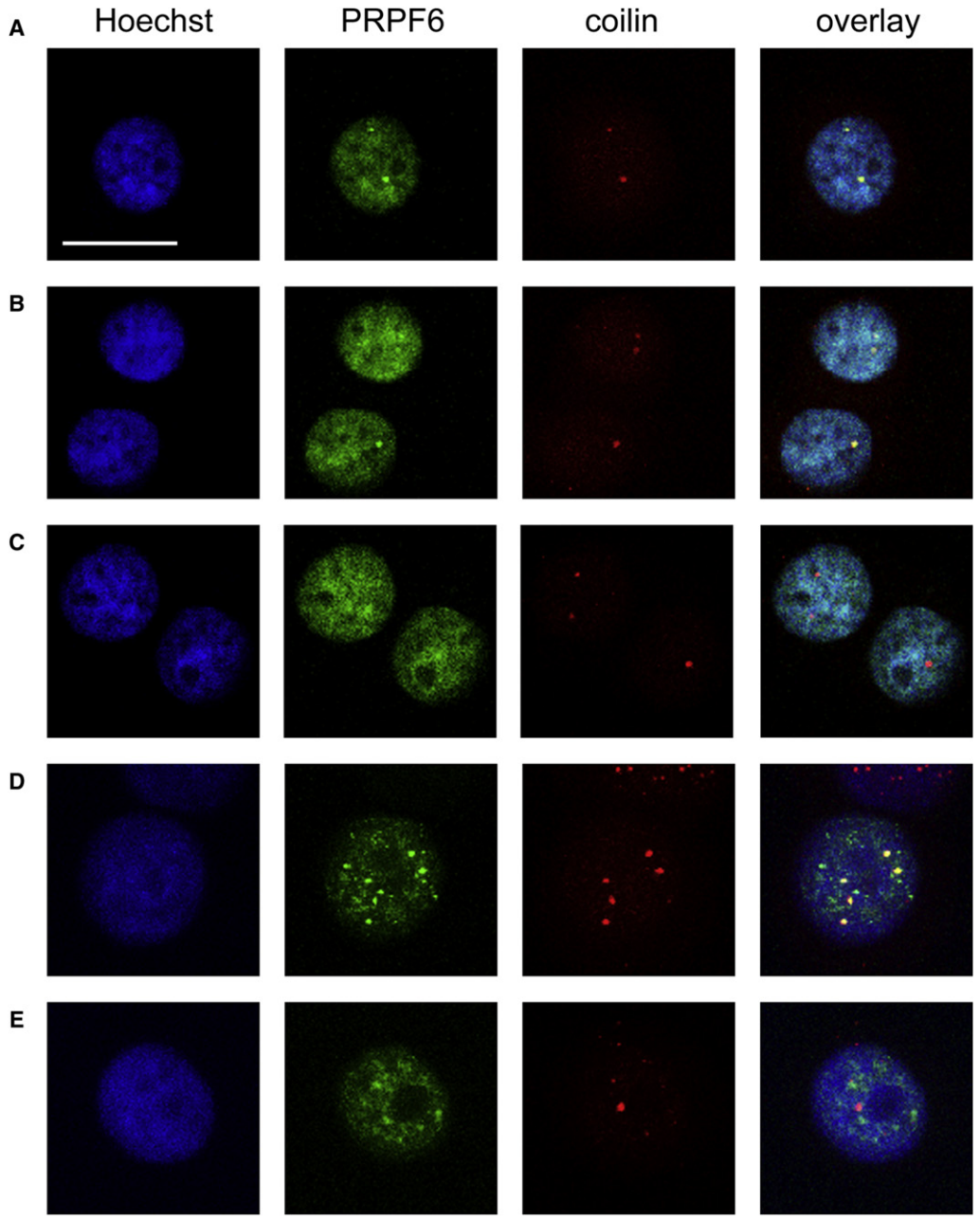

Figure 4. Confocal-Microscope Images of PRPF6 Localization in Lymphoblasts and HeLa Cells
Endogenous PRPF6 in lymphoblasts from patients 001-173 and 218-479 with p.Arg729Trp (A and B, respectively) and from a control individual (C) is stained in green (rabbit anti-PRPF6 and donkey anti-rabbit FITC), and the p80-coilin is shown in red (mouse anti-p80-coilin and donkey anti-mouse Texas red). In transfected HeLa cells, fluorescence originating from AcGFP-PRPF6-Arg729Trp (D) and AcGFP-PRPF6-wt (E) are shown in green. p80-coilin (red) was visualized with the same antibodies as above. The white bar corresponds to 10 μm; all images are at the same magnification. Yellow signals in the overlay images indicate accumulation of PRPF6 in Cajal bodies.

antibody (Sigma-Aldrich) (Figures 4A and 4B). This did not occur in control cells, for which colocalization of PRPF6 with p80-coilin was never observed (Figure 4C). Cajal bodies are membraneless organelles involved in the final stages of nuclear snRNP maturation[14,25–27] and possibly harbor the tri-snRNP regeneration steps that follow the splicing process.[28] When tri-snRNP assembly is impaired, accumulation of immature snRNP complexes within these structures is observed.[14] Specific accumulation of PRPF6 in the Cajal bodies within nuclei of lymphoblasts from patients with PRPF6 p.Arg729Trp therefore suggests an impairment in tri-snRNP assembly or recycling; this impairment is similar to what has been shown by other methods for cells derived from patients with *PRPF31*, *PRPF3*, and *PRPF8* mutations.[20]

To gain further insights into this process, we cloned both wild-type and mutant *PRPF6* full-length cDNA into the mammalian expression plasmid pAcGFP-C1 (Clontech,

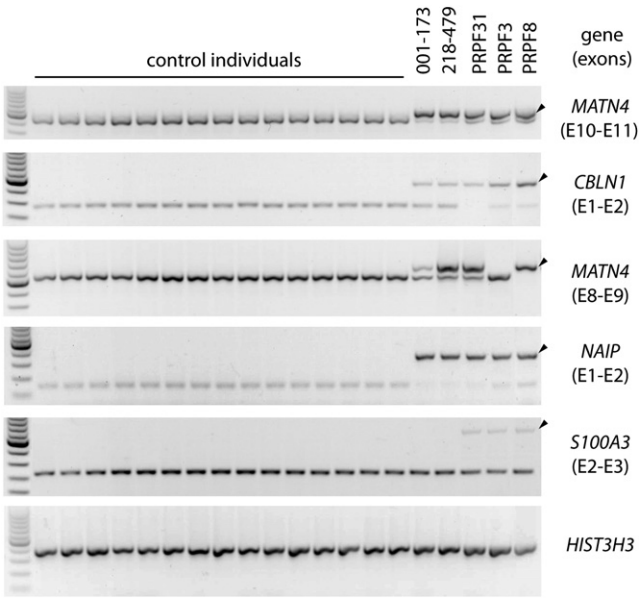

Figure 5. RT-PCRs of Selected Endogenously Expressed Transcripts in Lymphoblasts from Controls, Patients with p.Arg729Trp, and Patients with Other *PRPF31*, *PRPF3*, and *PRPF8* Mutations

A panel of RT-PCRs encompassing five introns previously found to be partially unspliced in lymphoblasts from patients with *PRPF31*, *PRPF3*, and *PRPF8* mutations is shown. Lane 1, molecular size marker (Promega, Cat #G2101); lanes 2–16, lymphoblasts from 15 control individuals; lane 17, lymphoblasts from patient 001-173; lane 18, lymphoblasts from patient 218-479; lanes 19–21, lymphoblasts from patients with mutations in *PRPF31*, *PRPF3*, or *PRPF8* (positive controls). RT-PCRs performed on *HIST3H3* represent technical negative controls because this gene is intronless. E#: exon harboring the forward or reverse RT-PCR primer. Arrowheads indicate unspliced products.

Mountain View, CA) to produce a PRPF6 fusion protein with the green fluorescent protein from *A. coerulescens* (AcGFP). Specifically, cDNA from a control human lymphoblast library was amplified by PCR with the proofreading *Pfu* DNA polymerase (Promega, Madison, WI) and primers 5′-CGGAATTCCATGAACAAGAAGAAG-3′ and 5′-GGGGTACCTCAGAAGGTGTT-3′. These matched the *PRPF6* sequence at their 3′ moiety but carried artificially introduced restriction sites (underlined) for the EcoRI and KpnI endonucleases, respectively, at their 5′ end. We used these restriction sites to clone PCR products into the pAcGFP-C1 and produce plasmid AcGFP-PRPF6-wild-type. We introduced the p.Arg729Trp change into this latter plasmid by following the QuickChange Site-Directed Mutagenesis protocol (Agilent Technologies, La Jolla, CA) and using mutagenesis primers 5′-GATGATG GAGAAGGCGTGGGAAGCCTATAACC-3′ and 5′-GGTTA TAGGCTTCCCACGCCTTCTCCATCATC-3′ (the nucleotide carrying the mutation is underlined) to obtain plasmid AcGFP-PRPF6-Arg729Trp. The sequence of all cloned DNA was assessed by direct nucleotide sequencing, and it was verified not to contain any unwanted variants. Imagery of HeLa cells transfected with these constructs revealed that the mutant fusions produced the same phenotype displayed by cells with naturally occurring p.Arg729Trp and that AcGFP-PRPF6-mut partly avoided splicing speckles (Figure S1) and localized, unlike AcGFP-PRPF6-wild-type, within Cajal bodies (Figures 4D and 4E). These data demonstrate that mislocalized PRPF6 concerns only mutated protein and that endogenous functional copies of *PRPF6* in HeLa cannot complement this phenotype.

We have previously demonstrated that patients with mutations in *PRPF31*, *PRPF3*, and *PRPF8* fail to correctly splice introns from pre-mRNAs that probably carry specific, yet unidentified, signals in their primary sequences.[20] Specifically, cell lines from seven patients with different mutations in these RP genes were unable to completely excise five "signature" introns, out of a panel of 57 endogenously transcribed sequences that recapitulated the majority of splicing signals detected so far in humans and displaying a wide range of gene expression. According to a recently proposed model for the disease, this deficiency in mRNA maturation would lead to accumulation of defective transcripts or proteins as the first step in the molecular pathology of RP.[20] Strikingly, when analyzed for their ability to process the same panel of 57 introns, the two cell lines with p.Arg729Trp displayed incomplete excision of four out of five signature introns (Figure 5). These data indicate that this PRPF6 mutation had an effect not only in spliceosomal composition but also in general pre-mRNA processing. Furthermore, they strongly indicate that *PRPF6* is a true RP gene; cells with p.Arg729Trp are defective in excising specific introns that were found to be incorrectly processed only in patients with *PRPF*-associated RP.

In conclusion, we identify *PRPF6* as the sixth splicing-factor gene involved in autosomal-dominant retinitis pigmentosa. The mutation detected in a proband and his affected brother is a missense mutation, as are many other DNA changes that have been detected so far in RP genes associated with the tri-snRNP. It concerns a uniquely conserved residue in a HAT domain that is part of the U4/U6 snRNP interaction region[21] and therefore could affect PRPF6 binding to the di-snRNP. It is presently unclear whether mutated *PRPF6* alleles would act as haploinsufficient or dominant-negative elements.

Although many hypotheses linking defects in splicing-factor genes and RP have been proposed (e.g.[20,24,29–31]), most of them can be incorporated into two general models. The first model postulates misplicing events affecting mRNAs that are retina specific or are directly related to RP (e.g., rhodopsin). The second proposes that splicing defects could touch more widely expressed transcripts such as those that were affected in cell lines from our patients, for which no clear-cut relationship with retinal physiology has been shown. In this case, RP would be the consequence of an increased susceptibility of the retina to a general reduction of splicing activity and/or to the continuous accumulation of defective gene products. Although our results do not clearly sustain one model over the other, they seem to fit the second one better, and they certainly highlight the central role of the tri-snRNP in the molecular etiology of RP.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/Omim)

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