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Autosomal-Dominant Retinitis Pigmentosa Caused by a Mutation in *SNRNP200*, a Gene Required for Unwinding of U4/U6 snRNAs

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Mutations in genes associated with the U4/U6-U5 small nuclear ribonucleoprotein (snRNP) complex of the spliceosome are implicated in autosomal-dominant retinitis pigmentosa (adRP), a group of progressive retinal degenerative disorders leading to visual impairment, loss of visual field, and even blindness. We recently assigned a locus (*RP33*) for adRP to 2cen-q12.1, a region that harbors the *SNRNP200* gene encoding hBrr2, another U4/U6-U5 snRNP component that is required for unwinding of U4/U6 snRNAs during spliceosome activation and for disassembly of the spliceosome. Here, we report the identification of a missense mutation, c.3260C>T (p.S1087L), in exon 25 of the *SNRNP200* gene in an *RP33*-linked family. The c.3260C>T substitution showed complete cosegregation with the retinitis pigmentosa (RP) phenotype over four generations, but was absent in a panel of 400 controls. The p.S1087L mutation and p.R1090L, another adRP-associated allele, reside in the "ratchet" helix of the first of two Sec63 domains implicated in the directionality and processivity of nucleic acid unwinding. Indeed, marked defects in U4/U6 unwinding, but not U4/U6-U5 snRNP assembly, were observed in budding yeast for the analogous mutations (N1104L and R1107L) of the corresponding Brr2p residues. The linkage of hBrr2 to adRP suggests that the mechanism of pathogenesis for splicing-factor-related RP may fundamentally derive from a defect in hBrr2-dependent RNA unwinding and a consequent defect in spliceosome activation.

Introduction

Recent progress in disease-gene identification for retinitis pigmentosa (RP [MIM 268000]) has established the involvement of nuclear pre-messenger RNA (pre-mRNA) splicing as one important mechanism in the disease etiology and has shed light on the spliceosome machinery itself. RP is one of the most common inherited causes of blindness, with a prevalence of 1 in 3500 live births.[1] The disease process involves pigmentation and atrophy in the mid-periphery of the retina, leading to symptoms of night blindness, restriction, and gradual loss of the peripheral visual field. The disease primarily affects rod photoreceptors, whereas impairment of the cone-receptor function is secondary to the disease process.[2] RP has a strong genetic background,[1] with 19 disease genes identified for the autosomal-dominant forms (adRP) (RetNet database). Notably, four of these genes are involved in the pre-mRNA splicing process, including precursor mRNA processing factor 8 (*PRPF8* [MIM 607300]),[3] *PRPF31* (MIM 606419),[4] *PRPF3* (MIM 607301),[5] and PIM1-associated protein (*RP9* or *PAP1* [MIM 607331]).[6]

We previously assigned an adRP locus, *RP33* (MIM 610359), to chromosomal region 2cen-q12.1 by linkage analysis in a large family.[7] On the basis of recombinant mapping, the critical interval was defined as a 9.46 Mb region,[7] with five candidate genes (Figure 1), of which *SEMA4C* (MIM 604462), *CNGA3* (MIM 600053), and *HNK1ST* (MIM 603376) were previously excluded.[7]

The U5 snRNP 200 kDa helicase gene *SNRNP200* (MIM 601664; also known as *ASCC3L1*), which lies within the *RP33* locus, encodes a 2136 amino acid protein, known as hBrr2,[8] composed of two DExD/H box ATPase domains,[9] each followed by a Sec63 domain.[10,11] hBrr2 plays a central and conserved role in nuclear pre-mRNA splicing,[8,12–14] the process by which introns are removed and flanking exons are ligated.

Pre-mRNA splicing is catalyzed by the spliceosome, which consists of the small nuclear RNAs (snRNAs) U1, U2, U4, U6, and U5, together with approximately 80 conserved proteins.[15] During the spliceosome cycle, the snRNAs undergo structural rearrangements that are critical for intron recognition, catalysis, and recycling.[16] In particular, a base-paired duplex of U4/U6 snRNAs, which is required for assembly of the spliceosome on a pre-mRNA substrate, unwinds in a key step during the catalytic activation of the spliceosome.[17–19] Unwinding of U4/U6 permits formation of the mutually exclusive U2/U6 base-paired interaction, which makes up a key component of the catalytic core.[20–23] After splicing, U2/U6 is unwound during

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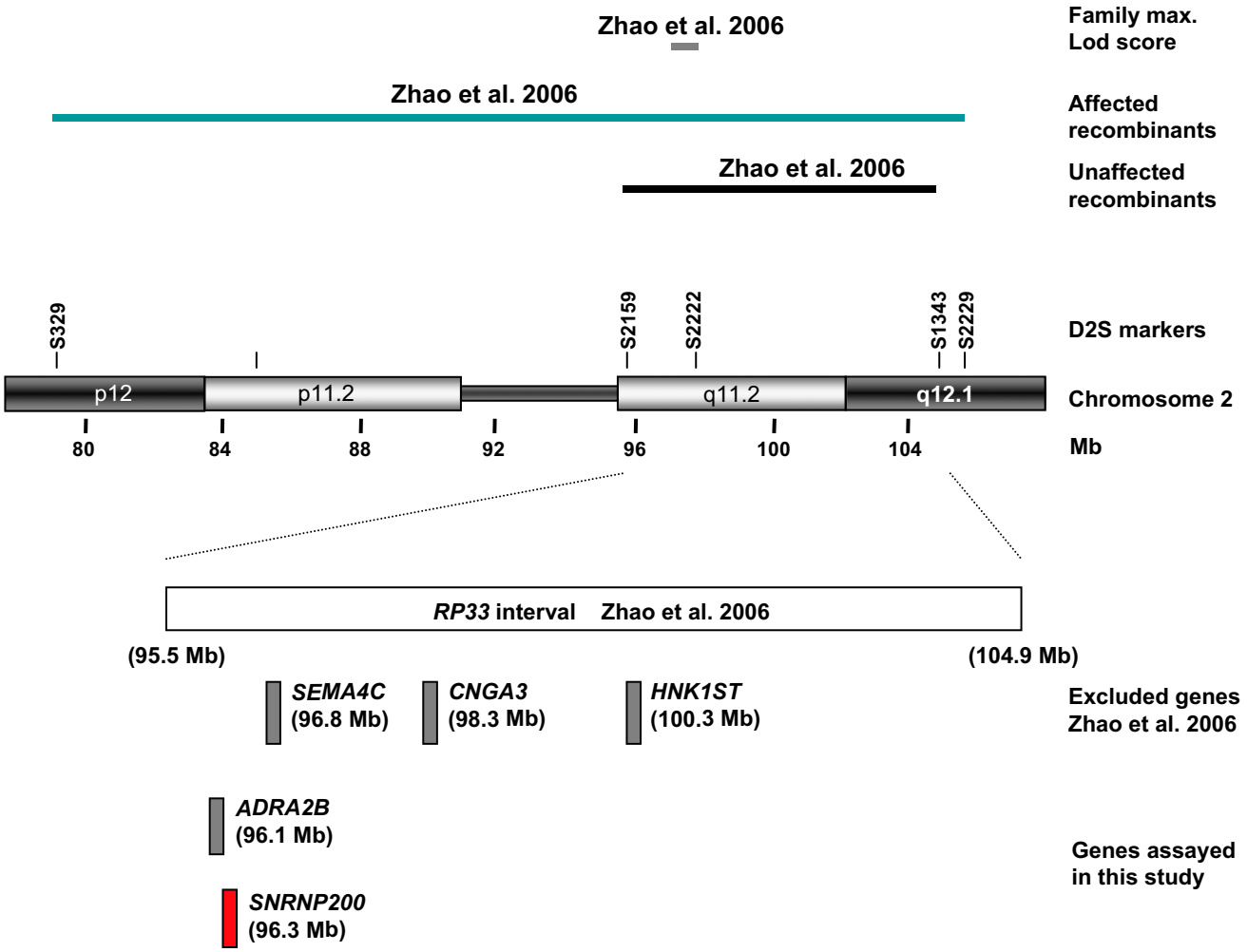

Figure 1. Schematic Representation of *RP33* Mapping and Analyzed Genes
A physical map of the *RP33* region in chromosomal region 2cen-q12.1[7] is shown in the middle, critical microsatellite markers are indicated above, and physical distances (in Mb) are shown below. The adRP-affected family was reportedly linked to the region with maximum LOD score at D2S2222,[7] as indicated at the top (gray bar). *RP33* is flanked by D2S2159 and D2S1343, on the basis of unaffected recombinants (black bar), and is flanked by D2S329 and D2S2229, on the basis of affected recombinants (green bar).[7] Candidate genes within the 9.4 Mb *RP33* region are shown below the physical map.

spliceosome disassembly and U4 and U6 are reannealed for subsequent rounds of splicing.[24]

In budding yeast, Brr2p, the ortholog of human hBrr2, is required both for U4/U6 unwinding[25] and for spliceosome disassembly.[26] Brr2 is thought to directly unwind U4/U6,[10,11,27,28] a reaction that requires the first DExD/H box ATPase domain;[25,29] such domains function in many RNA-dependent processes by binding RNA, hydrolyzing ATP, and unwinding RNA.[9] Additionally, U4/U6 unwinding requires the first Sec63 domain.[10,11,26] Brr2p is a core component of U4/U6-U5 small nuclear ribonucleoproteins (snRNPs) and remains associated with the spliceosome throughout the splicing process.[15] Presumably for timing of the activity of this central ATPase, Brr2p is regulated by the GTPase Snu114p,[26] ubiquitin,[30] and Prp8p,[10,11,28] which is ubiquitylated in the U4/U6-U5 snRNP.[30] As for hBrr2, three of the splicing factors linked to adRP are components of the U4/U6-U5 triple snRNPs, including the U4/U6 components *PRPF31* and *PRPF3* and the U5 component *PRPF8*,[15,31] and the fourth factor *PAP1* interacts with *PRPF3*.[32] adRP, associated with mutations in splicing genes, is presently regarded as resulting from defective triple-snRNP assembly.[33,34] However, adRP mutations in *PRPF8* affect not only triple snRNP assembly but also U4/U6 unwinding,[28] suggesting the involvement of complementary or alternative disease mechanisms.

Given the association of hBrr2 with adRP factors in the context of the triple snRNP, we selected *SNRNP200* as a candidate for *RP33*. In this study, we report that a missense alteration p.S1087L in the first Sec63 domain of hBrr2 cosegregates with adRP in the *RP33*-linked family. A recent crystal structure of budding yeast Brr2p localizes the equivalent of S1087 (N1104) to the nucleic-acid-interacting face of a "ratchet" helix implicated in the directionality and processivity of duplex nucleic acid unwinding.[10,11,35] Indeed, the *brr2-N1104L* mutation in yeast permitted assembly of U4/U6-U5 snRNPs but compromised unwinding of U4/U6. Thus, our results support identification of

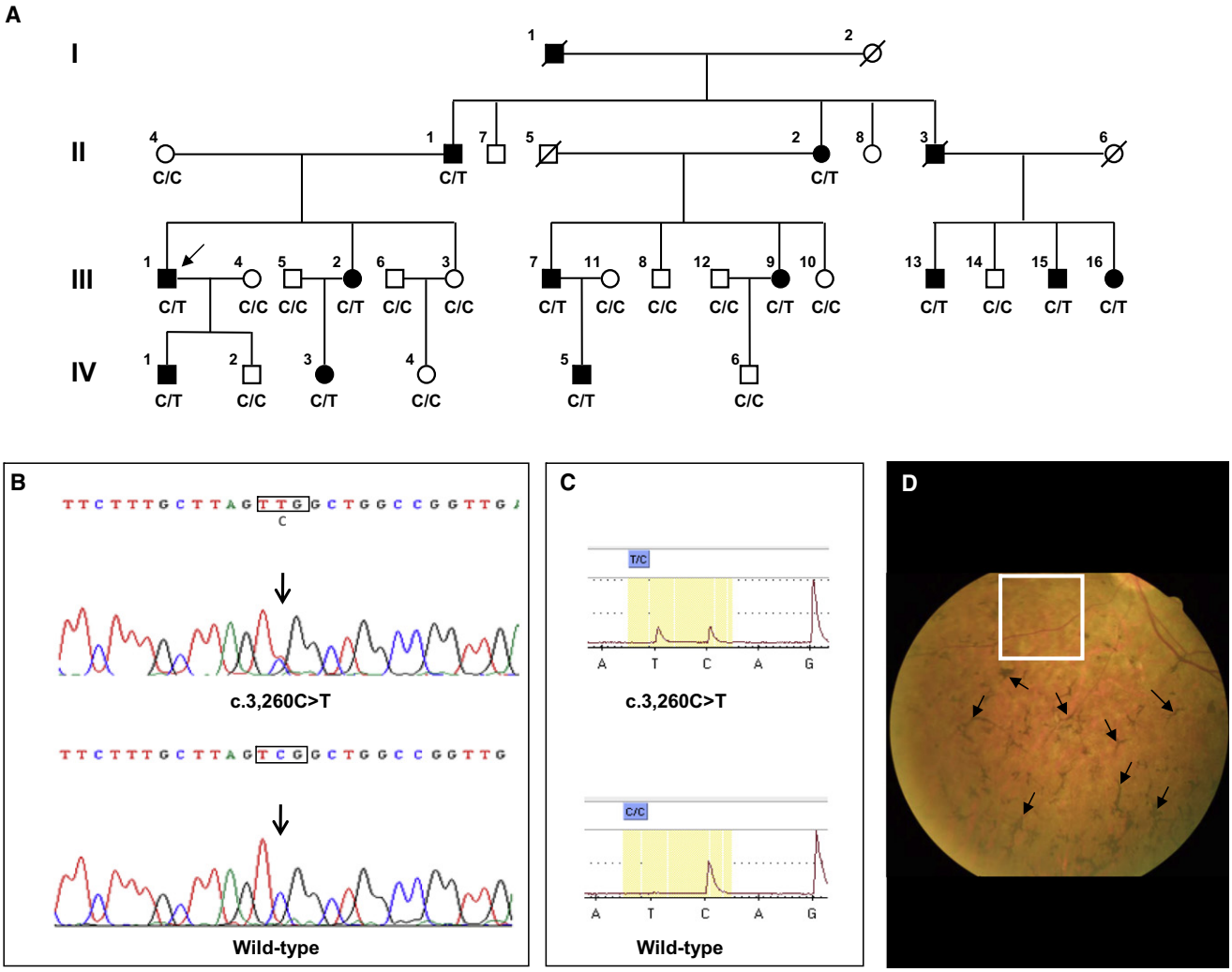

Figure 2. Identification of Missense Mutation c.3260C>T in Exon 25 of the *SNRNP200* Gene Segregating Completely with the Affected Phenotype in a Four-Generation adRP Family

(A) Pedigree of the kindred in which the *RP33* locus was initially assigned.[7] The genotypes C/T (heterozygous mutation) and C/C (wild-type) are given below the pedigree symbols.

(B) Sequencing chromatograms from affected (top) and unaffected (bottom) family members, showing the c.3260C>T substitution in exon 25 of *SNRNP200* and corresponding wild-type sequence.

(C) Confirmation of the c.3260C>T mutation by pyrosequencing. Genotypes are highlighted by blue color, as C/T in the presence of mutation and as C/C in wild-type sequence.

(D) Fundus photography of the right eye of affected individual IV:5. Notably, bone-spicule-like pigmentary deposits (denoted by arrows) were found in the peripheral retina, whereas the posterior retina is less affected (denoted by white box).

p.S1087L as a pathogenic mutation and implicate the pre-mRNA splicing gene *SNRP200* in adRP. In a parallel study, *SNRP200* was similarly implicated in adRP by the nearby mutation p.R1090L,[36] which we show also permits assembly of U4/U6-U5 snRNPs but compromises U4/U6 unwinding in yeast. Importantly, our work suggests that a defect in Brr2p-dependent duplex unwinding contributes to, if not accounts for, the association of splicing genes with adRP.

Subjects and Methods

Retinitis Pigmentosa Patients and Controls

The adRP family studied was previously used for assignment of the *RP33* locus to the long arm of chromosome 2. This *RP33*-linked family was clinically examined in Tianjin Eye Hospital as described in 2006.[7] At a recent follow-up, individual IV:5, who was previously unaffected, presented symptoms and features in agreement with affected status. In total, 12 affected and seven unaffected family members, as well as six spouses, were included in the genetic analyses of the present study (Figure 2). Furthermore, 137 affected cases were studied, representing 87 probands in previously reported adRP families collected at the Human Genetics Center of the University of Texas Health Science Center[37] and 50 Chinese sporadic and unrelated RP cases collected in the Hong Kong Eye Hospital and the Prince of Wales Hospital in Hong Kong.[38,39] Informed consent was obtained from all case individuals for sample collection and molecular analysis, and human studies were approved by local ethical review boards in accordance with the Declaration of Helsinki. Genomic DNA isolated from peripheral leukocytes was used for direct sequencing and pyrosequencing analyses.

Normal references constituted DNA samples from 400 unrelated individuals with no personal or family history of RP, including 200 Chinese individuals and 200 individuals of different ethnic backgrounds.

Genomic Sequencing of *SNRNP200* and *ADRA2B*

SNRNP200 and *ADRA2B* were sequenced in one unaffected and two affected family members (III:1, III:2, and III:8; Figure 2), with the use of primer sequences and annealing conditions detailed in Table S1 (available online). Primers were designed so that they allowed amplification by polymerase chain reaction (PCR) of predicted exons and exon-intron boundaries of the entire coding regions. PCR amplicons were directly sequenced in both directions via methods previously described[7] and were analyzed in an automated ABI 3730 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA) available at the KIGene facility of the Karolinska Institute (KI) in Stockholm, Sweden. After the detection of a c.3260C>T substitution, exon 25 was sequenced in all members of the *RP33*-linked family, 137 adRP patients, and 400 unaffected controls.

Pyrosequencing

Pyrosequencing was performed for the specific verficiation of the presence or absence of the c.3260C>T substitution in the *RP33*-linked family. The three primers that were used included 5′-AACG TTCTTCTGCAAGCCTTCATC-3′ (forward primer), 5′-ACCTCGGT TCAGGACAATTTCAAA-3′ (reverse primer), and 5′-ATGGTGTATG TCACACAGT-3′ (sequencing primer). The pyrosequencing assay was carried out via the protocol recommended by the manufacturer, with the use of PSQ 96 (Pyrosequencing AB, Uppsala, Sweden) available at the KIGene facility of KI in Stockholm, Sweden.

Analyses of HeLa-Cell-Derived Samples

HeLa S3 cells (human cervical cancer cells) were kindly provided by Dr. K-L Wallin of KI and were used for DNA extraction followed by genomic sequencing of exon 25. HeLa S3-cell-line-derived RNA was purchased from Ambion (Ambion, Applied Biosystems) and used for cDNA synthesis and subsequent sequencing of exon 24 to exon 26 with the use of primers detailed in Table S1.

Expression of *SNRNP200* and *Snrnp200*

PCR was performed for evaluation of the expression of *SNRNP200* in a commercial cDNA panel derived from multiple normal human tissues (Clontech, Mountain View, CA, USA), including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Similarly, expression of *Snrnp200* was also determined in different murine tissues via reverse-transcriptase PCR (RT-PCR) analysis. C57BL/6 mice were sacrificed at 2 mo of age and different murine tissues were dissected, including lung, kidney, brain, spleen, heart, liver, retina, and RPE-choroid-sclera eye cup. Total RNA was isolated with the TRIzol Plus RNA purification system and cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Primers are detailed in Table S1 for human and murine *SNRNP200* and *Snrnp200* and the housekeeping genes *GAPDH* and *Gapdh* that were analyzed in parallel as a positive control. Animal experiments were approved by the local ethical review board and conformed to the Guide for the Care and Use of Laboratory Animals (see Web Resources).

S. cerevisiae Strains and Plasmids

Triple snRNPs were purified from yJPS1115 (*BRR2*), yJPS1378 (*brr2-N1104A*), yJPS1379 (*brr2-N1104L*), yJPS1380 (*brr2-N1104S*), and yJPS1383 (*brr2-R1107L*). These strains were generated by transformation of yJPS1114 (*MAT*a *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ *brr2::LEU2 PRP28-TAP::KanMX* pSN123)[26] with the *HIS3*-marked plasmids pPR130[25] (*BRR2*), bJPS2383 (*brr2-N1104A*), bJPS2410 (*brr2-N1104L*), bJPS2411 (*brr2-N1104S*), and bJPS2433 (*brr2-R1107L*) and subsequent selection against the endogenous *URA3*-marked *BRR2* plasmid by the streaking of colonies onto 5-fluroorotic acid.[40] The mutated plasmids were generated by Quikchange mutagenesis (Stratagene) of pPR130 and confirmed by sequencing.

Triple-snRNP Purification

Triple snRNPs were purified with the use of TAP-tagged-Prp28p essentially as described previously,[26] with one exception: the snRNPs were not eluted from the IgG-Sepharose beads but were instead stored in an equal volume of Buffer D (20 mM HEPES [pH7.9], 0.2 mM EDTA, 50 mM KCl, and 20% [v/v] glycerol).

U4/U6 Unwinding Assay

U4/U6 unwinding assays were performed and analyzed as described previously.[30] In brief, purified triple snRNPs were incubated in standard splicing buffer[41] at 4°C or 20°C, and unwinding was initiated by the addition of 2 mM ATP.

Results

Identification of a Missense *SNRNP200* Mutation in adRP

The selected candidate genes *SNRNP200* and *ADRA2B* were sequenced in one unaffected and two affected members of the *RP33*-linked family. The analyses involved the entire coding regions and exon-intron splice junctions. Disease-associated nucleotide alterations were not revealed in *ADRA2B*. However, for *SNRNP200* a single base variation, C>T, was observed at c.3260 in exon 25 (Figure 2). This c.3260C>T substitution was predicted to give an amino acid alteration from serine to leucine at amino acid residue 1087 (p.S1087L) of the encoded hBrr2 protein (Figure 3). Extended sequencing of exon 25 in all available members of the *RP33*-linked family revealed that c.3260C>T (p.S1087L) cosegregated with the RP phenotype in all affected members but was excluded from all unaffected members (Figure 2A). The mutation status was subsequently verified by direct analysis of the c.3260C>T substitution by pyrosequencing, which confirmed the presence of c.3260C>T in all affected members (Figure 2C).

Complete penetrance for the c.3260C>T (p.S1087L) Mutation

As previously reported, the *RP33*-linked family demonstrated variable expression of RP phenotypes with relatively late onset.[7] In 2006, all affected family members, as well as one unaffected family member (IV:5), were found to carry the disease-associated haplotype of the *RP33* interval. This observation was in complete agreement with the segregation of the *SNRNP200* mutation observed here. At a recent follow-up, it was observed that individual IV:5 had developed decreased nighttime visual acuity at 17 years of age, which is similar to the onset ages of most

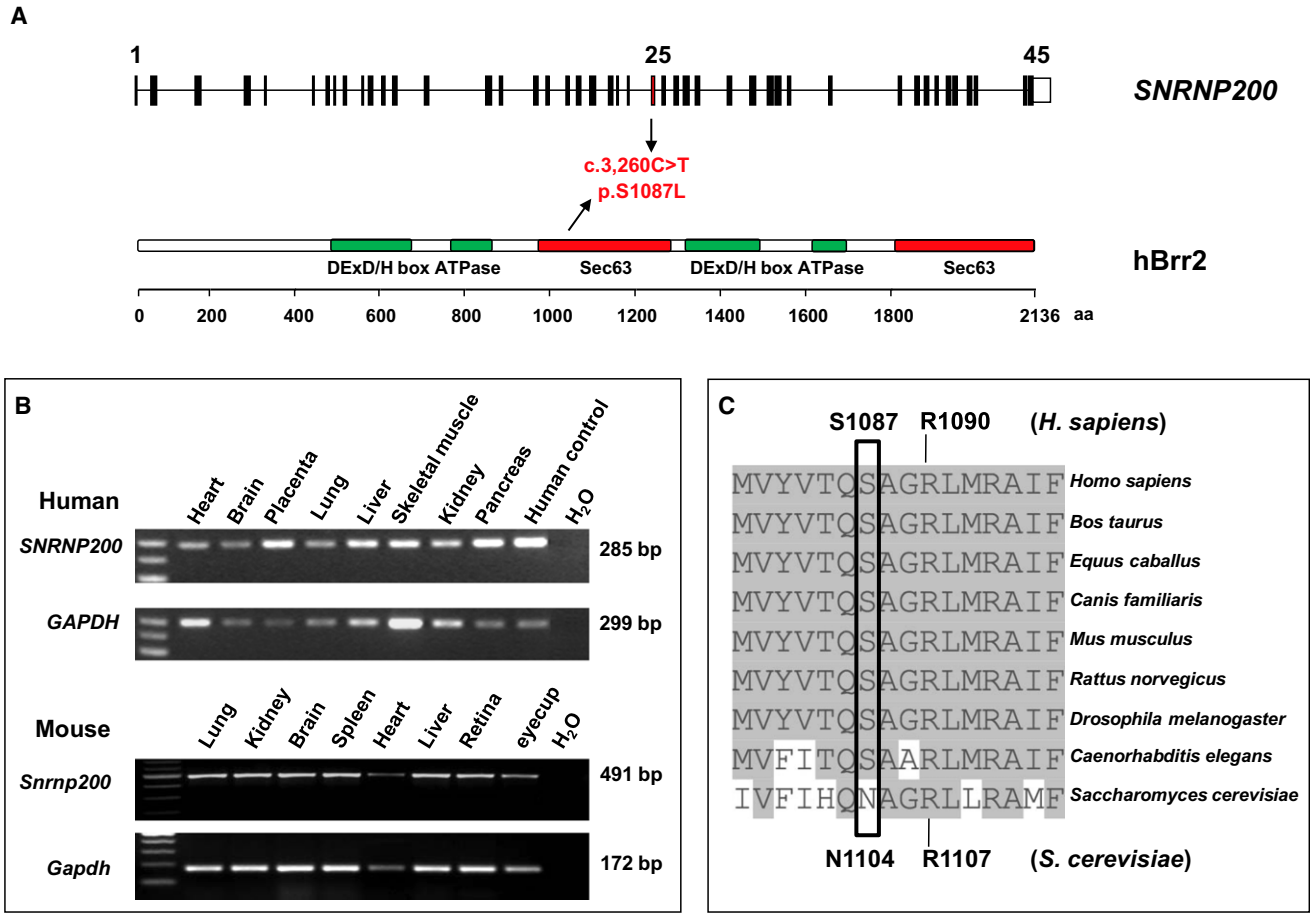

Figure 3. Analysis of the *SNRNP200* Gene and the c.3260C>T (p.S1087L) Mutation
(A) The *SNRNP200* gene, including 45 exons, is illustrated at the top, and the mutated exon 25 is highlighted in red. The encoded hBrr2 protein includes 2136 amino acids and contains two DExD/H box ATPase domains (green) and two Sec63 domains (red). The p.S1087L mutation resides in the first Sec63 domain.
(B) Ubiquitous expression of human *SNRNP200* and murine *Snrnp200* demonstrated by PCR of cDNA samples. The expected product of 285 bp for human (top panel) and 491 bp for mouse (third panel) was found in all tested tissues. Human *GAPDH* and murine *Gapdh* transcripts were analyzed in parallel as a positive control (second and bottom panels).
(C) Sequence alignment of hBrr2 from human (*Hs*), cow (*Bt*), horse (*Ec*), dog (*Cf*), mouse (*Mm*), rat (*Rn*), fruit fly (*Dm*), worm (*Ce*), and budding yeast (*Sc*). Conserved residues are shaded, the position of the mutated human S1087 residue in the *RP33* family is boxed, and the R1107 mutation[36] is indicated by a line.

affected members in the family. Consistently, bone-spicule-like pigmentary deposits were observed in the local peripheral retina of both eyes during funduscopy (Figure 2D). Altogether, these findings suggest that the p.S1087L mutation is associated with complete penetrance of the adRP phenotype in this family.

Screening for c.3260C>T (p.S1087L) in adRP Probands and Controls

According to the Human SNP databases (Ensembl and HapMap), the c.3260C>T alteration has not been previously reported in any population. However, c.3,260C>T (p.S1087L) was reported as a conflict variation in one HeLa-cell-derived cDNA clone (GenBank ID: gi|45861371)[42] but not in others.[8] Sequencing of exon 25 in HeLa S3-derived samples showed the presence of the C/T genotype in genomic DNA and of the c.3260C>T (p.S1087L) alteration in cDNA samples (data not shown). For further assessment of whether c.3260C>T (p.S1087L) is a rare polymorphism or a disease-associated mutation, direct sequencing of exon 25 in 400 unaffected controls and 137 unrelated adRP probands was carried out. This revealed the wild-type sequence c.3260C in all cases, suggesting that c.3260C>T (p.S1087L) is a disease-associated mutation in the *RP33*-linked family.

Expression of the *SNRNP200* Gene

SNRNP200 expression was previously detected in one retinal-derived cDNA library (NbLib0013) deposited in the NEIbank database. We further assessed *SNRNP200* gene expression in other human tissues by routine PCR analyses of a cDNA panel, using primers flanking exons 4 to 6. The housekeeping gene *GAPDH* was amplified in parallel as a positive control. As illustrated in Figure 3B (upper two panels), the PCR reaction generated single products of the expected sizes of 285 bp for *SNRNP200* and of 299 bp for *GAPDH* in all tissues. These results demonstrate that

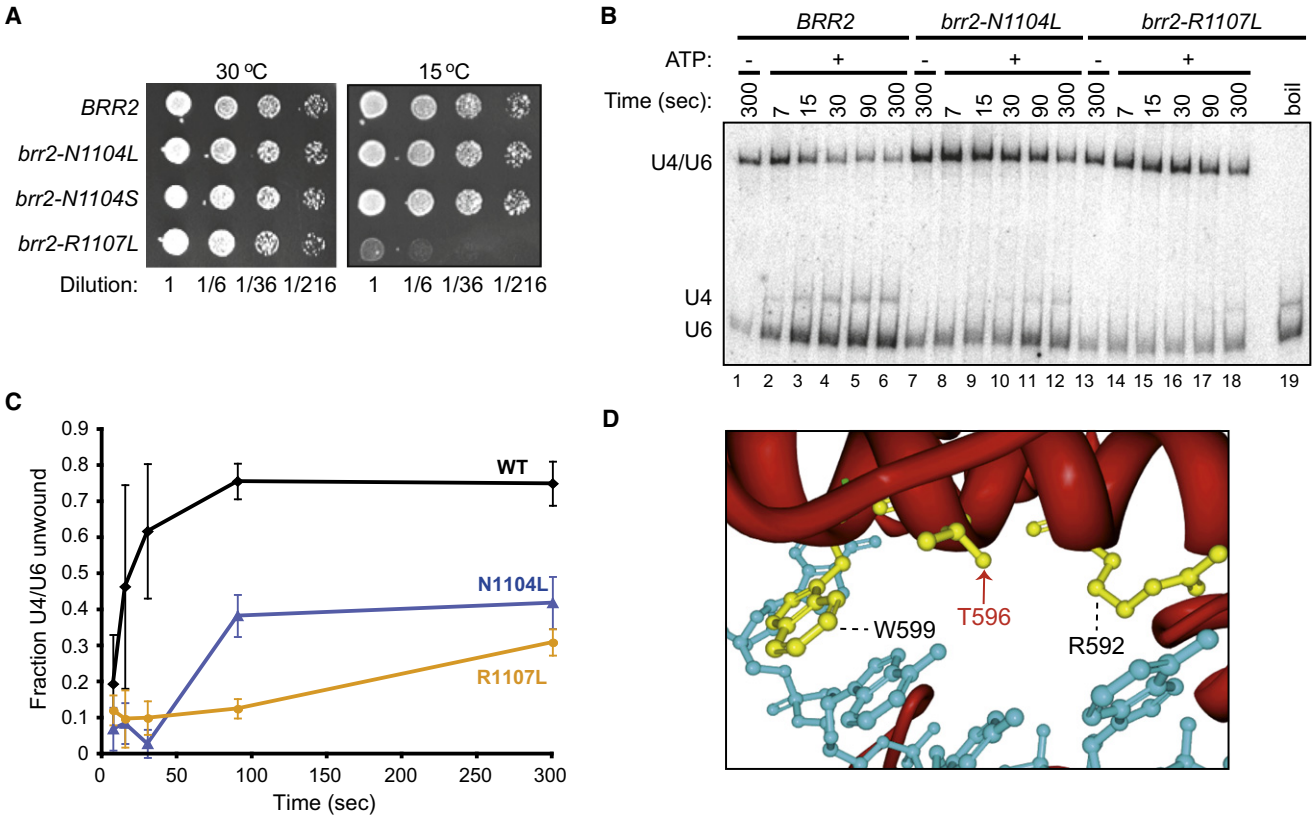

Figure 4. Two adRP Mutations in Budding Yeast Brr2p Impair Unwinding of U4/U6 snRNAs
(A) The mutant yeast strains *brr2-N1104L*, *brr2-N1104S*, and *brr2-R1107L* were assayed for growth relative to wild-type. Strains were grown to log phase, serially diluted, spotted onto rich media, and grown at 30°C (1 day) or 15°C (7 days).
(B) Triple snRNPs purified from wild-type *BRR2* (lanes 1–6), mutant *brr2-N1104L* (lanes 7–12), or mutant *brr2-R1107L* (lanes 13–18) were assayed for their ability to unwind U4/U6 snRNAs in the presence of ATP at 20°C. Quenched samples were cold-phenol extracted, RNA was resolved by native gel electrophoresis, and the U4 and U6 snRNAs were detected by RNA blotting analysis. For comparison, lane 19 shows a sample from *brr2-R1107L* that was hot-phenol extracted.
(C) Quantification of U4/U6 unwinding at 20°C by triple snRNPs purified from *BRR2* (black closed diamonds), the *RP33* mutant *brr2-N1104L* (blue closed triangles), and the other adRP mutant *brr2-R1107L* (orange closed circles). Data from (B) and data not shown were quantified and plotted. Error bars represent the standard deviation from three or more experiments.
(D) The *SNRNP200* adRP mutations reside in a "ratchet" helix of the Sec63 domain of Brr2p implicated in promoting the directionality and processivity of unwinding. A close-up of the "ratchet" helix of the corresponding domain in Hel308 (pdb 2P6R[35]) (red) is shown with the "ratchet" helix at the top. Residues of the ratchet helix that face and/or interact with nucleic acid (cyan) are highlighted (yellow). T596 corresponds to N1104 in yeast and to the *RP33* residue S1087 in humans. W599 corresponds to R1107 in yeast and to the adRP residue R1090 in humans.[36] R592 corresponds to F1100 in yeast and to Y1083 in humans.

SNRNP200 is expressed in a wide range of human tissues. A similar expression pattern of the *Snrnp200* gene was detected in murine tissues, as shown in Figure 3B (lower two panels). A 491 bp band representing an RT-PCR amplicon flanking exons 24 to 26 of *Snrnp200* was detected in all tissues, including, among others, the retina.

Evolutionary Conservation of p.S1087L

Screening of *SNRNP200* orthologs with the use of the Ensembl protein blast database revealed that S1087 is extremely well conserved between all mammalian species, *D. melanogaster*, and *C. elegans* (Figure 3C). Application of the polymorphism-phenotyping program PolyPhen for prediction of functional consequences resulted in a position-specific independent counts (PSIC) profile score of 2.45 for the p.S1087L amino acid change. This value indicates that p.S1087L is likely to functionally impair the hBrr2 protein,[43] thus suggesting that p.S1087L is a functional mutation.

ad*RP* Mutations Impair U4/U6 snRNA Unwinding in Budding Yeast

To investigate whether the *RP33* mutation might compromise the catalytic activity of hBrr2, we utilized an assay for U4/U6 unwinding from budding yeast.[25] With this assay, we tested the impact of three mutations—N1104L, corresponding to the *RP33* mutation; N1104S, corresponding to the natural human residue at this position; and N1104A. None of these mutations compromised growth (Figure 4A, data not shown), except subtly at low temperature on minimal media (15°C; data not shown). For the unwinding assay, we first fractioned yeast extract on a glycerol gradient and then purified U4/U6-U5 triple-snRNP particles from peak fractions by using TAP-tagged Prp28p.[26] None of the mutations altered the migration

of snRNAs on the glycerol gradient, and none altered the ratio or reduced the levels of snRNAs copurified with Prp28p (data not shown). Thus, at least at 30°C, the mutations do not compromise triple-snRNP assembly, which has been suggested as a causative defect of other adRP mutations.[33,34]

Although aphenotypic in the aforementioned assays, the N1104L mutation conferred a striking defect in ATP-dependent U4/U6 unwinding (Figures 4B and 4C; Figure S1). For reference, at 20°C, the wild-type triple snRNPs unwound U4/U6 rapidly, achieving 50% unwinding at 20 s (Figures 4B and 4C). In contrast, the *RP33* mutation, N1104L, slowed unwinding significantly, failing to achieve even 50% unwinding at 300 s (Figures 4B and 4C). At 4°C, the wild-type triple snRNPs similarly unwound U4/U6 rapidly, achieving 50% unwinding at 50 s (Figures S1A and S1B), and the mutation to the human residue, N1104S, slowed unwinding only slightly, permitting 50% unwinding by 75 s (Figures S1A and S1B). In contrast, the *RP33* mutation, N1104L, slowed unwinding significantly, preventing even 50% unwinding after 300 s (Figures S1A and S1B), and the N1104A mutation also slowed unwinding but permitted 50% unwinding at 260 s (Figures S1A and S1B). Thus, the N1104L RP mutation in budding yeast permits triple-snRNP assembly but impairs RNA unwinding.

While this manuscript was under revision, a separate study was published, reporting a second Chinese family in which adRP cosegregated with a mutation in *SNRNP200*.[36] In this family, the average age of onset was 7–8 years,[36] rather than the 16–18 years observed for *RP33*.[7] Notably, this mutation (p.R1090L) is also found in the first Sec63 domain and resides on the same face of the putative ratchet helix as p.S1087L, and the equivalent residue in Hel308 (W599, Figure 4D) has been proposed to contribute to ratcheting.[10,11,35] R1090 is strictly conserved from humans to budding yeast (Figure 3C), and a mutation of the equivalent residue in yeast (R1107A) impairs ATPase and U4/U6 unwinding activities of purified Brr2p and U4/U6 unwinding within U4/U6-U5 snRNPs.[10,26] To investigate the possible consequences of the p.R1090L mutation in hBrr2p, we investigated the equivalent R1107L mutation in yeast. In contrast to the N1104L mutation, the R1107L mutation conferred a strong cold-sensitive defect on rich media (Figure 4A). Like the N1104L mutation, the R1107L mutation did not compromise U4/U6-U5 snRNP assembly in vivo at 30°C (data not shown), in agreement with its normal growth phenotype at that temperature (Figure 4A). At both 4°C and 20°C, the R1107L mutation permitted even less unwinding than did the N1104L mutation (Figures 4B and 4C; Figure S1C). Thus, both adRP mutations in *SNRNP200* confer defects in U4/U6 unwinding, proportional to the severity of the disease resulting from these mutations.

Discussion

In this study, we report the identification of a single amino acid substitution (p.S1087L) of the *SNRNP200* gene in four generations of a Chinese family, previously linked to the critical region for the adRP locus *RP33* (Figure 1). The p.S1087L mutation cosegregates with the RP phenotype in all affected members in the family (Figure 2) and was not identified in a panel of 400 normal controls. p.S1087 is absolutely conserved among all mammals, as well as fruit flies and worms, suggesting that it has an important function in the encoded hBrr2 protein. The mutation falls within the first Sec63 domain of hBrr2, implicating a defect in RNA unwinding. Indeed, in native U4/U6-U5 snRNP particles from budding yeast, the analogous mutation (N1104L) impaired U4/U6 unwinding (Figure 4; Figure S1), a critical step in the catalytic activation of the spliceosome. Additionally, a newly identified RP locus similarly links to a mutation (p.R1090L) in *SNRNP200*,[36] and we show that the equivalent mutation in budding yeast also compromises U4/U6 unwinding (Figure 4; Figure S1). These data implicate a defect in RNA unwinding as the basis of *SNRNP200*-associated adRP and implicate the contribution of a similar mechanism for adRP mutations in other splicing genes.

The substitution c.3260C>T (p.S1087L) was not revealed in 137 adRP probands, suggesting that mutation of exon 25 is a relatively uncommon cause of adRP. However, for full evaluation of the prevalence of *SNRNP200* mutations in adRP, mutation screening by sequencing of the entire coding region and splice junctions of *SNRNP200* is required in adRP families. Furthermore, because a direct-sequencing-based technique is limited in the detection of large deletions and/or insertions, which are relatively common for other adRP genes,[44] complementary techniques, such as multiplex ligation-dependent probe amplification (MLPA), may also be required. Indeed, the discovery of a second adRP mutation (R1090L) in *SNRNP200* suggests that more will be found.[36]

Unexpectedly, c.3260C>T (p.S1087L) has been described in a HeLa-cell-derived cDNA clone[42] and was also observed here in the heterozygous state in DNA and cDNA samples of HeLa S3 cells. The HeLa cells were established more than 50 years ago from an ovarian carcinoma of a woman who died at 31 years of age. It is presently not possible to determine whether she carried c.3260C>T (p.S1087L) at the constitutional level, whether it was acquired during ovarian cancer development, or whether it occurred during long-term cell culturing as an artifact or growth-promoting aberration.

To investigate the mechanism of pathogenesis for *RP33*, we tested whether S1087L impairs RNA unwinding, given that hBrr2 and its yeast ortholog directly unwind duplex RNA[10,11,27,28] and yeast Brr2p unwinds U4/U6 snRNAs within the U4/U6-U5 snRNP.[25,26,30] hBrr2 is composed of a tandem repeat of a DExD/H box domain followed by a Sec63 domain (Figure 3). In yeast Brr2p, only the first repeat performs a catalytic role;[10,29] the second repeat functions in protein binding and regulation by Prp8.[10,45] The p.S1087L mutation resides in the first Sec63 domain, which has been modeled in yeast on the basis of the

structure of the second Sec63 domain, which shows striking similarity with a subdomain of Hel308.[10,11,35] On the basis of this Sec63 domain model, the yeast equivalent of p.S1087 (N1104) falls within the "ratchet" helix on a face that interacts with nucleic acid. Furthermore, N1104 is predicted to be sandwiched between F1100 and R1107, and the equivalent residues in Hel308, R592 and W599, respectively (Figure 4D), were hypothesized to perform a ratchet function to promote directional and processive unwinding.[35] In agreement with this hypothesis, the mutation R1107A in yeast Brr2p dramatically reduces the ATPase, U4/U6 unwinding, and spliceosome-disassembly activities of Brr2p.[10,26] Moreover, U4/U6 unwinding was also decreased by the R1107L mutation, corresponding to the adRP mutation p.R1090L in hBrr2,[36] and by the neighboring N1104L mutation, corresponding to the adRP mutation p.S1087L in hBrr2 (Figure 4; Figure S1). Therefore, our data emphasize the importance of the ratchet helix in U4/U6 unwinding and implicate a defect in U4/U6 unwinding in adRP.

The p.S1087L and p.R1090L ratchet-helix mutations could compromise U4/U6 unwinding by either compromising the inherent unwinding activity of hBrr2 or compromising the regulation of hBrr2. Because the equivalent of residues S1087 and R1090 in Hel308 contact nucleic acid from a domain essential for duplex unwinding,[10,11,35] mutations in these residues of hBrr2 may very well compromise its inherent unwinding activity. Indeed, the Sec63 domain harboring these residues is associated with the catalytically active DExD/H box domain of yeast Brr2p,[10,11,29] and the R1107A mutation in yeast Brr2p (at the equivalent of position R1090 in hBrr2) compromises U4/U6 unwinding by purified Brr2p, suggesting that the adRP mutations in this ratchet helix cripple the inherent unwinding activity of Brr2p.[10] However, given that Brr2p is regulated by the guanine nucleotide state of the GTPase Snu114p,[26] by the ubiquitylation state of the U4/U6-U5 snRNP,[30] and by Prp8p,[28] the adRP mutations could also or alternatively compromise the regulation of hBrr2. Indeed, regulation of Brr2p by Prp8p is mediated by the C-terminal Sec63 domain,[10,45] thereby implicating additional regulatory interactions within Brr2p between the C-terminal Hel308 module, which includes the C-terminal Sec63 domain, and the active N-terminal Hel308 module; such interactions may be compromised by the adRP mutations. Resolving whether adRP mutations affect inherent unwinding activity or regulation of hBrr2 requires further study.

The p.S1087L and p.R1090L mutations could compromise a cell in several ways. First, they could decrease the expression of spliced genes overall or for certain genes by disrupting the splicing cycle, either by impeding U4/U6 unwinding and, consequently, spliceosome activation or by impeding spliceosome disassembly and, consequently, spliceosome recycling, although the adRP genes *PRPF31* and *PRPF3* are unlikely to play roles in spliceosome disassembly. Additionally, as suggested for the other splicing-related adRP mutations, p.S1087L and p.R1090L could compromise assembly of the U4/U6-U5 snRNP, although we have found no evidence that corresponding mutations N1104L and R1107L compromise assembly in budding yeast. Second, the hBrr2 mutation may also impair proofreading of pre-mRNA splicing, thereby compromising the fidelity of gene expression. Three of eight DExD/H box ATPases required for splicing have been shown to function in proofreading.[46–48] It is anticipated that Brr2p will also function in proofreading by prematurely unwinding U4/U6 or prematurely disassembling the spliceosome to reject incorrect splicing substrates. Such branched pathways are in competition with productive splicing and are consequently quite sensitive to mutations. Finally, the p.S1087L and p.R1090L mutations could compromise an as-yet-undefined function that lies outside of splicing.

Interestingly, the earlier onset of adRP in individuals with the p.R1090L mutation (7–8 years)[36] as compared to those with the p.S1087L mutation (16–18 years)[7] correlates with the stronger cold-sensitive growth defect and stronger U4/U6-unwinding defect associated with the equivalent R1107L mutation, as compared to the N1104L mutation, in budding yeast (Figure 4). This correlation supports the utility of budding yeast as a model organism for investigation of adRP mutations. Furthermore, the correlation provides additional evidence that a defect in U4/U6 unwinding contributes to, if not accounts for, adRP resulting from mutations in *SNRNP200*.

The *SNRNP200*, *PRPF8*, *PRPF3*, and *PRPF31* gene products are all triple-snRNP components,[15,31] and PAP1 binds PRPF3 to negatively regulate splicing,[32,49] suggesting a common mechanism for pathogenesis. The prevailing hypothesis has been that RP mutations in each of these genes compromise triple-snRNP assembly. For example, a previous study suggested that mutations in *PRPF8* may cause RP by disrupting the nuclear association of Brr2p with the U5 snRNP and, consequently, the assembly of the triple snRNP.[33] In agreement with this point, another recent study demonstrated that *PRPF8* mutations in yeast Prp8p compromise binding of a C-terminal fragment of Prp8p to Brr2p.[28] However, this study also demonstrated that the C-terminal fragment of Prp8p stimulates Brr2p-dependent RNA unwinding and that *PRPF8* RP mutations compromised unwinding. Although it is difficult to uncouple the unwinding defects from binding defects, this study suggested that RP may result from a defect in spliceosome activation in addition to or instead of a defect in triple-snRNP assembly. Our own data solidify this hypothesis, because the N1104L and R1107L mutations in yeast Brr2p permit triple-snRNP assembly but compromise U4/U6 unwinding (Figure 4; Figure S1; also, data not shown). Prp3p (RP11) binds U4/U6 but may also promote its unwinding, because Prp3p interacts by two-hybrid analysis with Snu66p, which itself interacts with Brr2p.[42] PAP1, as a factor that interacts with Prp3p, could modulate such an unwinding role for Prp3p.[32] Prp31p interacts with U4 but also with Prp6p, which interacts

with Brr2p, potentially implicating a role for Prp31p in unwinding. Consistent with a role in U4/U6 unwinding, both Prp3p and Prp31p dissociate upon U4/U6 unwinding.[15] Thus, in addition to or instead of compromising triple snRNP assembly, each of the RP mutations in splicing genes may compromise Brr2p-dependent U4/U6 unwinding.

Diverse RP phenotypes[50] are frequently observed in association with mutations in adRP genes involved in the pre-mRNA splicing process. Mutations of *PRPF8* and *PRPF3* generally lead to more severe phenotypes,[3,51] in agreement with type 1 expression with early disease onset and diffuse retinal involvement. By contrast, mutations of *PRPF31* and *PAP1* have been observed in type 2 expression, with late onset and regional retinal involvement, or showed variable intrafamilial expression.[4,52,53] The *RP33* family affected by the p.S1087L mutation of *SNRNP200*, reported here, presented clinical expression suggesting type 2 and intrafamilial variable phenotype,[7] whereas the p.R1090L mutation led to a more quickly developed phenotype,[36] more likely fitting with type 1. Thus, the variable severity of the retinal phenotypes in adRP may not only depend on the disease gene involved but also result from the type and consequence of the specific mutation involved.

Why do RP mutations in splicing genes cause only retinal phenotypes if they manifest as pre-mRNA splicing defects? One hypothesis rests on the fact that the retina is a highly metabolically active tissue. Large amounts of opsin are synthesized every second,[54] and the outer segments of photoreceptors are rapidly renewed. The photoreceptors may show the highest demand for pre-mRNA splicing among all cell types, and RP mutations may compromise the efficiency of splicing. Hence, the reduced splicing efficiency after a heterozygous mutation could be sufficient in other tissues but insufficient in the retina. In an alternative hypothesis, retinal cells may be particularly demanding on the fidelity mechanisms of splicing, perhaps because of errors resulting from light-induced damage or efficient synthesis at the expense of specificity. In this case, RP would stem from a defect in a putative fidelity role for Brr2. Finally, RP mutations in splicing genes may play a role outside of splicing that is specific to the retina.

Supplemental Data

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

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Protein Blast Database, http://www.ensembl.org/Homo_sapiens/Gene/Compara_Ortholog
Ensembl SNP Database, www.ensembl.org/Multi/martview
Guide for the Care and Use of Laboratory Animals, http://books.nap.edu/catalog.php?record_id=5140#toc
HapMap, www.hapmap.org
NEIbank Database, http://neibank.nei.nih.gov/cgi-bin/search.cgi
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim
Polymorphism Phenotyping Program (PolyPhen), http://genetics.bwh.harvard.edu/pph/
Protein Data Bank, http://www.pdb.org/
Retnet, http://www.sph.uth.tmc.edu/Retnet/disease.htm

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