Identification of photoreceptor genes affected by PRPF31 mutations associated with autosomal dominant retinitis pigmentosa

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Several ubiquitously expressed genes encoding pre-mRNA splicing factors have been associated with autosomal dominant retinitis pigmentosa (adRP), including PRPF31, PRPF3 and PRPF8. Molecular mechanisms by which defects in pre-mRNA splicing factors cause photoreceptor degeneration are not clear. To investigate the role of pre-mRNA splicing in photoreceptor gene expression and function, we have begun to search for photoreceptor genes whose pre-mRNA splicing is affected by mutations in PRPF31. Using an immunoprecipitation-coupled-microarray method, we identified a number of transcripts associated with PRPF31-containing complexes, including peripherin/RDS, FSCN2 and other photoreceptor-expressed genes. We constructed minigenes to study the effects of PRPF31 mutations on the pre-mRNA splicing of these photoreceptor specific genes. Our experiments demonstrated that mutant PRPF31 significantly inhibited pre-mRNA splicing of RDS and FSCN2. These observations suggest a functional link between ubiquitously expressed and retina-specifically expressed adRP genes. Our results indicate that PRPF31 mutations lead to defective pre-mRNA splicing of photoreceptor-specific genes and that the ubiquitously expressed adRP gene, PRPF31, is critical for pre-mRNA splicing of a subset of photoreceptor genes. Our results provide an explanation for the photoreceptor-specific phenotype of PRPF31 mutations.

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Introduction

Retinitis pigmentosa (RP), a common cause of blindness, is a group of inherited diseases characterized by the loss of photoreceptor cells. More than a hundred genetic loci have been associated with retinal degeneration (Baehr and Chen, 2001; Swaroop and Zack, 2002; see Web sites: www.sph.uth.tmc.edu/RetNet and www.uwc.m.ac.uk/uwcm/mg). As a genetically heterogeneous disease, RP displays all three modes of Mendelian inheritance: autosomal dominant (adRP), autosomal recessive (arRP) and X-linked (xIRP). Many RP genes are expressed specifically or predominantly in the retina. Recently, four adRP genes have been identified that are ubiquitously expressed in different tissues and associated with RNA processing. Three of these non-retina-specific adRP genes encode proteins essential for pre-mRNA splicing, pre-mRNA processing factors (PRPF), including PRPF31 (or PRP31; for RP11, Vithana et al., 2001), PRPF8 (PRP8 or PRPC8; for RP18, McKie et al., 2001) and PRPF3 (or HPRP3; for RP18, Chakarova et al., 2002). Another adRP gene, PAP1 (for RP9), has also been implicated in pre-mRNA splicing (Maita et al., 2004, 2005). Among these, PRPF31 has been reported as the second most common adRP gene, only second to the rhodopsin gene (Vithana et al., 1998). An interesting question is how mutations in ubiquitously expressed pre-mRNA splicing factor genes such as PRPF31 cause photoreceptor-specific disease.

Most mammalian transcription units contain at least one intron that must be removed by a process known as pre-mRNA splicing to form functional messenger RNA (mRNA). As the most upstream step of post-transcriptional regulation, pre-mRNA splicing is critical for mammalian gene expression. Pre-mRNA splicing employs a two-step transesterification mechanism. The first step involves cleavage at the 5′ splice site and formation of a lariat intermediate. The second step is cleavage at the 3′ splice site.
with concomitant ligation of the 5’ and 3’ exons. The sites of cleavage and ligation are defined by conserved cis-elements including the 5’ splice site (5’ss), the branch point sequence, the polypyrimidine tract and the 3’ splice site (3’ss) consensus sequence. The splicing reaction occurs in spliceosomes, the large RNA–protein complexes that contain pre-mRNA, five small nuclear ribonucleoprotein (snRNP) particles, U1, U2, U4/U6 and U5, as well as a number of non-snRNP protein factors (Burge et al., 1999; Hastings and Krainer, 2001; Zhou et al., 2002; Wu et al., 2004). Following the initial recognition of splice sites by U1snRNP and U2snRNP together with early-step protein factors, the assembly and incorporation of the U4/U6.U5 tri-snRNP are crucial for the formation of the catalytically active center in the spliceosome. A number of proteins, including PRPF3, PRPF8 and PRPF31, play important roles in the formation of the tri-snRNP and assembly of the mature spliceosome. These splicing factors are highly conserved through evolution, from yeast to mammals. Originally identified in a screen for splicing defects, yeast prp31 is an essential gene encoding a 60 kDa protein. It assists in recruiting the U4/U6.U5 tri-snRNP to prespliceosome complexes and is critical for pre-mRNA splicing (Maddock et al., 1996; Weidnhammer et al., 1996, 1997). Mammalian PRPF3, PRPF8 and PRPF31 proteins likely play similar roles in pre-mRNA splicing as their yeast counterparts. However, it is not clear how mutations in these splicing factors lead to photoreceptor cell death and retinal degeneration.

Here we describe our efforts to identify downstream “target” genes for PRPF31 using a combined molecular and biochemical approach. Immunoprecipitation of PRPF31-containing ribonucleoprotein complexes followed by microarray led to the identification of 146 RNA transcripts, including several known adRP genes. We focused on RDS and FSCN2, two photoreceptor-specific genes linked to adRP, to further test effects of PRPF31 on splicing. Cotransfection of adRP mutants of PRPF31 with a minigene of RDS or FSCN2 indicated that mutant PRPF31 proteins inhibit the pre-mRNA splicing of RDS and FSCN2 genes. Expression of the mutant PRPF31 proteins led to a significant reduction in RDS expression in cultured retinal cells. These experiments show that mutations in PRPF31 inhibit pre-mRNA splicing of certain genes expressed in photoreceptor cells. Our study reveals a functional relationship between the general splicing factor, PRPF31, and expression of photoreceptor-specific genes, RDS and FSCN2. Taken together, these observations demonstrate that PRPF31 plays an important role in the pre-mRNA splicing of a subset of photoreceptor-specific genes.

Experimental procedures

Plasmid construction and antibody preparation

The mammalian plasmids expressing either wild-type or mutant PRPF31 proteins were constructed by inserting the corresponding cDNA fragments into pCS2 vector downstream of the cytomegalovirus (CMV) promoter. Anti-PRPF31 polyclonal antibodies were prepared in chicks using synthetic peptides (corresponding to amino acid residue 416–432) (Aves Labs, Inc). Antibodies used in this study include anti-peripherin/RDS monoclonal antibody Per SH2 (Connell et al., 1991), anti-rhodopsin (Chemicon), anti-synapsin (Chemicon), anti-GFP (Roche) and rabbit anti-chicken immunoglobulin (Ig) (Jackson Immunoresearch Laboratories, Inc).

Immunoprecipitation-microarray analysis

Mouse retinae were prepared from 8-week-old mice (Charles Rivers Laboratories). Retinal cells were dissociated by gentle treatment with trypsin and lysed in cell lysis buffer (20 mM HEPES, pH 7.4, 10 mM NaCl, 5 mM MgCl2, 0.1% NP-40). After 10 min incubation on ice, cell nuclei were collected by centrifugation at 2000 rpm for 10 min and resuspended in cell lysis buffer in the presence of RNase inhibitor and protease inhibitors (Roche). The cell nuclei were pelleted by centrifugation as above and resuspended in dilution buffer (20 mM HEPES, pH 7.4, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 0.1% Triton X-100, 1 mM DTT, 1× protease inhibitor (Roche), 40 unit/ml RNAsin). The nuclear lysate was sonicated for 10×30 s, and insoluble particles were removed by centrifugation at 13000 rpm for 10 min at 4°C. Protein A/G-Sepharose beads (Sigma) were added to the supernatant to pre-clear the nuclear lysate and then incubated with affinity purified anti-PRPF31 antibody and rabbit anti-chicken Ig for 4 hrs at 4°C. The affinity purified polyclonal anti-PRPF31 antibody was prepared by Aves Labs (Oregon). The pre-immune Ig preparation was used as a control. Pelleted beads were then sequentially washed with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM HEPES pH 7.5, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM HEPES pH 7.5, 500 mM NaCl) and DOC Buffer. DNA contaminants were removed by DNasel treatment on beads. The enriched protein–RNA complex was eluted twice using freshly prepared Elution Buffer (1% SDS, 0.1 M NaHCO3) and centrifuged. Proteins were digested by proteinase K treatment on beads. The enriched protein–RNA complex was eluted twice using freshly prepared Elution Buffer (1% SDS, 0.1 M NaHCO3) and centrifuged. Proteins were digested by proteinase K treatment for 30 min at 45°C. Six volumes of Trizol (Invitrogen) was used for the RNA extraction, and RNA was precipitated. The RNA samples immunoprecipitated with anti-PRPF31 or control pre-immune Ig were amplified by RT-PCR for 25 cycles using the SMART amplification kit according to the manufacturer’s instructions (Clontech). The amplified samples (approximately 5 μg) were labeled, then fragmented and hybridized to Affymetrix mouse genome chips (430 array, version 2.0) at Vanderbilt microarray core facility.

Primary retinal neuronal culture, transfection and immunostaining assays

Primary retinal cell culture was established using retinae from P0–P5 interspecific consomic strain (ICS) mice (Charles Rivers Laboratories) following the protocol described by (Luo et al., 2001). Immediately after euthanasia, enucleation procedure and retinal dissection were carried out in Ca2+/Mg2+-free Hank’s medium (Life Technology) containing 20% glucose. The retinal tissue was treated with 0.25% trypsin for 15 min at 37°C, with the digestion terminated by adding heat-inactivated fetal bovine serum (HiFBS) to a final concentration of 20%. Following DNase (10 mg/ml, Sigma) treatment, and trituration using polished pasteur pipets, the retinal cell suspension was washed with culture medium three times. Dissociated cells were counted and plated on pre-coated coverslips (poly-l-lysine and laminin, PD/LN) at 4 × 10^5 cells per coverslip in 6-well TC dishes. Cells were allowed to attach and were cultured in DMEM/F-12 media containing 10% HiFBS, and then were used for transfection and immunostaining.

Transfection was carried out using a modified calcium precipitation method as described previously (Jiang et al., 1998) 24–48 h after the retinal cultures were established. The transfection
efficiency was approximately 5% as determined by the expression of GFP using fluorescent microscopy. Cell morphology and viability were monitored by microscopic examination following staining with bis-benzimide (Sigma). Immunofluorescent staining was performed to examine the expression of peripherin/RDS and synapsin in cultured retinal cells using specific primary antibodies followed by secondary antibodies conjugated with either Cy-3 or Cy-2. Microscopic images were taken under an inverted microscope using an AxioCAM digital camera (Zeiss).

**Transfection and pre-mRNA splicing assay**

HEK cells were transfected with splicing substrate genes together with wild-type or mutant PRPF31 expression plasmids using a modified calcium phosphate method as described (Cote et al., 2001). Thirty-six hours following transfection, cells were harvested and total RNA was extracted. RT-PCR reaction was carried out using specific primers corresponding to different exonic regions to detect pre-mRNA and splicing products as described (Jiang et al., 1998).

**Results**

**Identification of RNA transcripts associated with PRPF31-containing splicing complexes using an immunoprecipitation-coupled microarray approach**

PRPF31 mutations cause photoreceptor loss and retinal degeneration. This finding prompted us to test the hypothesis that PRPF31 may play a critical role for pre-mRNA splicing of retina-specific genes. To begin to search for potential target genes whose pre-mRNA splicing is affected by PRPF31 mutations, we designed an immunoprecipitation-coupled microarray approach to identify RNA transcripts associated with PRPF31-containing splicing complex. Using an affinity-purified polyclonal anti-PRPF31 antibody with the pre-immune Ig preparation as a negative control for immunoprecipitation, we performed immunoprecipitation experiments using cell lysates prepared from mouse retinae (see Experimental procedures). Following immunoprecipitation, RNAs were extracted from the immunoprecipitated RNA–protein complexes. Microarray analysis was carried out to identify the RNA species associated with the PRPF31-containing splicing complexes. One hundred forty-six genes were detected in the samples immunoprecipitated using PRPF31 antibody, but not in those prepared using the control pre-immune antibodies (Supplemental Table 1). Among the potential target genes identified by this approach are a number of retina-specific genes involved in phototransduction, the visual cycle, photoreceptor structure, transcription factors and other genes important for photoreceptor survival and function (Table 1). It is worth noting that among these, several genes have been implicated in retinal degeneration. Such genes include those involved in phototransduction: rhodopsin (RHO) and the β-subunit of cGMP phosphodiesterases (PDE6B), genes important for visual cycles: photoreceptor ATP-binding cassette transporter (ABCA4), cellular retinaldehyde binding protein (Rbp), and retinal G-protein coupled receptor. Also detected among these PRPF31 associated transcripts were those encoding photoreceptor cell structural proteins, including peripherin/RDS (also called peripherin-2), rod cell outer membrane protein 1 (ROM1), retinal actin-binding protein fascin (FSCN2) and photoreceptor transcription factors such as neural retina leucine zipper (NRL) and cone-rod homeobox (CRX). In addition, a number of non-retina-specific genes were also found associated with PRPF31 complex, including those implicated in cell proliferation and cell death, synaptic transmission and neuronal function, transcription and RNA processing, cell metabolism and stress response as well as signal transduction. The remaining transcripts are ESTs or genes with unknown function. The association of these RNA transcripts with PRPF31-containing splicing complexes suggests that PRPF31 may be involved in the pre-mRNA splicing of these genes.

**Effects of PRPF31 mutants on pre-mRNA splicing of photoreceptor genes RDS and FSCN2**

We chose four known adRP genes, RDS, FSCN2, RHO and ROM1, to further examine their relationship to PRPF31. In this report, we focus on RDS and FSCN2, whereas characterization of RHO and ROM1 splicing was described in a separate paper (Yuan et al., 2005). The fact that RDS and FSCN2 RNA transcripts were associated with splicing complex(es) containing PRPF31 suggests that PRPF31 may be important for their pre-mRNA splicing. We set out to test this hypothesis using a minigene cotransfection approach. Several factors were taken into consideration when we selected the regions for preparing minigene constructs for testing pre-mRNA splicing. We focused on the genomic regions highly conserved between mouse and human and on the exonic regions, in particular those encoding functionally important domains. The terminal exons were not included in our minigenes to avoid potential compounding effects related to polyadenylation or other regulatory processes in the formation 3’ end of pre-mRNA. We designed a RDS minigene containing a shortened intron 1 with flanking exon 1 and exon 2 (Fig. 1), because this is a functionally important region in which a number of mutations associated with retinal degeneration have been identified. Intron 1 was shortened to facilitate minigene construction and detection. In the case of FSCN2, we chose the genomic region containing exon 3 to exon 4, a highly conserved region. We constructed a FSCN2 minigene containing its genomic fragment with exon 3–intron3–exon 4 (Fig. 1).

Two PRPF31 mutants were constructed to mimic two frameshift mutations found in either sporadic (SP117) or familial (AD5) forms of adRP (Fig. 2). As a result of either a single base insertion (SP117) or an 11-bp deletion (AD5), these mutations lead to truncations of the PRPF31 peptide after the PRPF31 amino acid residue 256 or 371, respectively. The wild-type or mutant PRPF31 proteins were expressed as GFP-tagged proteins.

To test effects of wild-type or mutant PRPF31 on splicing, the RDS minigene was transfected into HEK cells together with either wild-type or mutant PRPF31 (AD5 or SP117). Following the transfection, RDS pre-mRNA splicing was examined using RT-PCR with primers specific for RDS exon 1 and exon 2. When cotransfected with the AD5 or SP117 mutant PRPF31 (Fig. 3B, lanes 3 and 4, respectively), RDS splicing was significantly inhibited as compared with that in cells transfected with wild-type PRPF31 or vector control (Fig. 3B, lanes 2 and 1, respectively; compare lanes 3, 4, with 1 and 2). Quantification of splicing efficiency from data obtained from three independent experiments is shown in Fig. 3C.

The effects of PRPF31 mutations on FSCN2 pre-mRNA splicing were also examined using such a minigene cotransfection assay. FSCN2 minigene was introduced into HEK cells and
Table 1
A list of genes whose transcripts are associated with PRPF31-containing complex as detected by immunoprecipitation-coupled microarray experiment

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Genbank number</th>
<th>Gene name</th>
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<tbody>
<tr>
<td>A. Photoreceptor structure and phototransduction or visual cycle</td>
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<tr>
<td>BC013125.1</td>
<td>Rhodopsin</td>
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<td>NM_007538.1</td>
<td>Blue opsin</td>
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<td>NM_008938.1</td>
<td>Peripherin 2</td>
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<tr>
<td>NM_009073.1</td>
<td>Rod outer segment membrane protein 1 (Rom1)</td>
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<tr>
<td>NM_172802</td>
<td>Fascin homolog 2</td>
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<td>NM_012065.1</td>
<td>Phosphodiesterase 6G, cGMP-specific, rod, gamma (Pde6g)</td>
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<tr>
<td>NM_008806.1</td>
<td>Phosphodiesterase 6B (PDE6B)</td>
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<td>NM_008801.1</td>
<td>Phosphodiesterase 6D, cGMP-specific, rod, delta (Pde6d)</td>
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<td>NM_020599.1</td>
<td>Retinaldehyde-binding protein 1 (Rlbp1)</td>
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<td>BC017610.1</td>
<td>Similar to retinol-binding protein 3, interstitial</td>
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<td>NM_007378.1</td>
<td>ATP-binding cassette, sub-family A, member 4 (Abca4)</td>
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<td>NM_024458.1</td>
<td>Rod photoreceptor 1 (Rpr1)</td>
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<td>AF155141.2</td>
<td>Oxygen-regulated photoreceptor protein 1 (Rp1)</td>
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<td>NM_009038.1</td>
<td>Recoverin (Rcvrn)</td>
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<tr>
<td>B. Cell proliferation and cell death genes</td>
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<td>NM_008015.1</td>
<td>Fibroblast growth factor inducible 14</td>
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<td>NM_007610.1</td>
<td>Caspase 2</td>
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<td>NM_007611.1</td>
<td>Caspase 7</td>
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<td>Caspase 8 associated protein 2</td>
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<td>NM_011810.1</td>
<td>Fas apoptotic inhibitory molecule (Faim)</td>
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<td>NM_009628.1</td>
<td>Activity-dependent neuroprotective protein</td>
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<td>Programmed cell death 6 (Pdcd6)</td>
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<tr>
<td>AF100927.1</td>
<td>Programmed cell death 8 (Pdc8d, apoptosis inducing factor)</td>
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<td>C. Synaptic transmission and neuronal functions</td>
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<td>NM_009306.1</td>
<td>Synaptotagmin 1 (Syt1), mRNA</td>
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<td>Synaptosomal-associated protein 91 kDa (Snap91)</td>
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<td>Gamma-aminobutyric acid (GABA-A) transporter 1 (Gabt1)</td>
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<td>GABA-A receptor-associated protein-like protein1</td>
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<td>AV075715</td>
<td>Clusterin</td>
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<td>D. Transcription or RNA processing or translation</td>
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<td>NM_007770.1</td>
<td>Cone-rod homeobox containing gene (Crx)</td>
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<td>NM_008736.1</td>
<td>Neural retinal leucine zipper gene (Nrl)</td>
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<td>TSC22-related inducible leucine zipper 1b</td>
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<td>Nuclear receptor subfamily 2, group E, member 3 (Nr2e3)</td>
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<td>AF443223.1</td>
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<td>BB722680</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
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<td>NM_011732.1</td>
<td>Y box protein 1</td>
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<td>Cold inducible RNA binding protein (Cirbp)</td>
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<td>DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide 5 (Ddx5)</td>
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<td>Splicing factor, arginineserine-rich 5 (SRp40, HRS)</td>
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<td>Small nuclear ribonucleoprotein N (Snurpn)</td>
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<td>NM_020007.1</td>
<td>Muscleblind-like (Mbnl)</td>
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<td>Karyopherin (importin) alpha 2</td>
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<td>BC018223.1</td>
<td>Eukaryotic translation elongation factor 1 alpha1</td>
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<td>E. Cell metabolism and stress responses</td>
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<tr>
<td>NM_011829.1</td>
<td>Inosine 5-phosphate dehydrogenase 1 (IMPDH1)</td>
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<td>ATP synthase, alpha subunit</td>
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<td>AF354051.1</td>
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<td>ATPase, H+ transporting, lysosomal 16 kDa, V0 subunit C</td>
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<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4</td>
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<td>BG967663</td>
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<td>Pyruvate kinase 3 (Pkp3)</td>
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<td>Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (Hspa5)</td>
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<td>Heat shock protein, 86 kDa 1 (Hspa8-1)</td>
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<td>F. Signal transduction and protein modification</td>
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<td>NM_013636.1</td>
<td>Protein phosphatase 1, catalytic subunit, gamma isoform (Ppp1cc)</td>
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<td>Guanine nucleotide binding protein, alaphatransducing 1</td>
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<tr>
<td>NM_008142.1</td>
<td>Guanine nucleotide-binding protein, beta-1 subunit (Gnb1)</td>
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cotransfected with either wild-type or mutant PRPF31 expression plasmids. As shown in Fig. 4, the expression of AD5 mutant PRPF31 significantly inhibited the splicing of FSCN2 intron 3 (compare lane 3 with lanes 1 and 2). In this case, AD5 mutant strongly inhibited pre-mRNA splicing of FSCN2 whereas SP117 mutant showed little effect, as compared to cells transfected with wild-type PRPF31 or vector control (Fig. 4).

The expression of PRPF31 mutants reduces peripherin/RDS expression in primary retinal cells

To study the effects of PRPF31 mutants on retinal cells, we used primary retinal cell culture prepared from P0–P5 mice. Dissociated retinal cells were cultured on coverslips coated with laminin and poly-L-lysine. Wild-type or mutant PRPF31 expression plasmids were transfected into primary retinal cells using a modified calcium phosphate precipitation method (Jiang et al., 1998) that allowed us to achieve a moderate transfection efficiency in retinal cells without causing detectable nonspecific cytotoxicity. Peripherin/RDS expression was examined by immunofluorescent staining assay using a specific monoclonal antibody (Connell et al., 1991). Approximately 70% of the retinal cells were peripherin/RDS-positive by day 2 in culture when cells were cultured under the conditions described above. Following the transfection of the plasmid expressing the GFP vector, GFP-tagged wild-type PRPF31, AD5 mutant or SP117 mutant, we examined cells expressing these GFP-tagged proteins using fluorescent microscopy. Transfection with wild-type PRPF31 or vector control did not affect the expression of peripherin/RDS (see Fig. 5). In cells expressing mutant PRPF31, either AD5 or SP117, peripherin/ RDS immunostaining was significantly reduced as compared to cells expressing wild-type PRPF31 (Fig. 5, compare the red immunofluorescent staining signals for RDS in cells marked with arrows expressing PRPF31-GFP fusion proteins in panels A1, B1 and C1). Comparison of the peripherin/RDS-immunofluorescent staining signals in cells transfected with mutant PRPF31-GFP plasmid with those in surrounding non-transfected cells in the same tissue culture dishes also indicates that RDS expression was reduced in cells expressing either the AD5 or the SP117 mutant PRPF31 proteins (Fig. 5). Peripherin/RDS-expressing cells were quantified at different time points after transfection. By 12 h post-transfection, the percentage of peripherin/RDS-positive cells was significantly decreased in cells expressing mutant (either AD5 or SP117) PRPF31 protein as compared with cells expressing the vector or wild-type PRPF31 (Fig. 6). There was no detectable change in the expression of another neuronal marker, synapsin, at the same time period (data not shown; also Yuan et al., 2005), suggesting that the reduction in peripherin/RDS expression observed is not due to general inhibition of protein synthesis or due to secondary effects of cell death. These experiments demonstrate that mutant PRPF31 proteins inhibit the expression of RDS gene in primary retinal cells. Together with the data on RDS pre-mRNA splicing, our study shows that PRPF31 mutations lead to defective RDS splicing and reduced peripherin/RDS protein expression.

Discussion

Pre-mRNA splicing, the most upstream post-transcriptional regulatory process, is a critical step in mammalian gene expression. Most mammalian transcription units contain introns that must be precisely removed to form functional mRNAs. Defects in pre-
mRNA splicing play an important role in the pathogenesis of many human diseases. Neurodegenerative diseases have been associated with mutations affecting splicing machinery. One example is spinal muscular atrophy (SMA), a motor neuron degenerative disorder caused by mutations in Survival of Motor Neuron (SMN), a ubiquitously expressed gene important for splicing machinery assembly (Pellizzoni et al., 2002). Recent genetic studies indicate that mutations in pre-mRNA splicing factors, PRPF3, PRPF8 and PRPF31, can cause autosomal dominant retinitis pigmentosa (reviewed in Pacione et al., 2003).

Significant progress has been made in understanding normal function as well as the pathogenetic mechanisms of the retina-specific RP genes, such as rhodopsin. However, mechanisms underlying mutations in ubiquitously expressed adRP genes, such as those encoding splicing factors, remain largely unknown (see Inglehearn, 1998; Phelan and Bok, 2000; Dejneka and Bennett, 2001; Himis et al., 2003; Pacione et al., 2003; www.sph.uth.tmc.edu/RetNet and www.uwm.ac.uk/uwem/mg). Our experiments using minigene transfection and primary retinal cell culture demonstrate that PRPF31 plays an important role in the expression of a subset of photoreceptor-specific genes and that mutant PRPF31 inhibits pre-mRNA splicing of these photoreceptor-specific genes, including RDS and FSCN2.

The gene responsible for photoreceptor degeneration in the retinal degeneration slow (rds) mouse was first identified by Travis et al. (1989). The RDS gene was subsequently shown to encode a 36 kDa photoreceptor membrane protein known as peripherin/rds or peripherin-2 (Connell and Molday, 1990; Connell et al., 1991; Travis et al., 1991) that is localized along the rims of rod and cone photoreceptor discs where it plays an essential role in outer segment disc morphogenesis and structure (Molday et al., 1987; Arikawa et al., 1992). Biochemical studies indicate that peripherin/RDS forms oligomeric complexes with another photoreceptor membrane protein ROM1 (Goldberg and Molday, 1996; Loewen and Molday, 2000). These complexes interact with other photoreceptor proteins including the cGMP-gated channel to further stabilize rod outer segments (Clarke et al., 1994; Nichols et al., 1993; reviewed in Gregory-Evans and Phelan, 1998; Zack et al., 1999; Chang et al., 2002; Bhattacharya et al., 2000; Poetsch et al., 2001). RDS mutations in both human and mice lead to photoreceptor degeneration. In mice, an insertion mutation in exon 2 of RDS gene causes the loss of peripherin/RDS expression and photoreceptor degeneration associated with the inability of photoreceptors to form outer segments (Travis et al., 1989; Ma et al., 1995; Sanyal and Jansen, 1981). RDS deficiency cause photoreceptor cell death in animal models of monogenic and digenic adRP (Kedzierski et al., 2001; Loewen et al., 2003). Mutations in the RDS gene have been associated with a number of human retinal dystrophies, including cone-rod dystrophy, cone dystrophy, macular degeneration and retinitis pigmentosa (Weleber et al., 1993; Wells et al., 1993; Keen et al., 1994; Nichols et al., 1993; reviewed in Gregory-Evans and Bhattacharya, 1998; Zack et al., 1999; Chang et al., 2002; Pacione et al., 2003). The most common RDS mutations reported in retinal degeneration are missense mutations in exon 1 and exon 2. Splice site mutations in RDS gene have also been reported (Sohocki et al., 2001). Our observation that PRPF31 mutations affect RDS pre-mRNA splicing indicates that defects in upstream gene regulators such as pre-mRNA splicing factors may also affect RDS expression, providing a new mechanism for defective RDS expression.

Retinal fascin (also named FSCN2) is a photoreceptor-specific protein of the fascin family. Fascins are a family of actin-bundling proteins that crosslink F-actin into highly ordered bundles, and they play an important role in the formation of neuronal growth cone filapodia. The human FSCN2 protein contains 492-amino acid and is coded by a gene located on chromosome 17q25 (Saishin et al., 1997; Tubb et al., 2000). A frame-shift mutation in FSCN2, 208delG, has been reported in approximately 3% of Japanese adRP patients (Wada et al., 2003).

In this study, we report the identification of gene transcripts associated with PRPF31-containing splicing complexes. A number of known adRP genes are among these genes transcripts. We have tested two such genes, RDS and FSCN2 in cells transfected with...
corresponding minigenes and shown that mutant PRPF31 inhibits pre-mRNA splicing of these genes. Our findings suggest a functional link between ubiquitously expressed PRPF31 protein and the expression of the retina-specific RDS and FSCN2 genes. This reveals a previously unknown relationship between PRPF31 and other adRP genes including RDS and FSCN2.

Fig. 4. FSCN2 pre-mRNA splicing is inhibited by mutant PRPF31. (A) Effects of PRPF31 mutant proteins on the pre-mRNA splicing of FSCN2 intron 3. Following cotransfection of plasmids expressing either vector control (lane 1), wild-type (lane 2) or mutant [AD5 (lane 3) or SP117 (lane 4)] PRPF31 proteins together with the FSCN2 intron 3 minigene into HEK cells, the corresponding pre-mRNA and splicing products are detected using RT-PCR with specific primers in the corresponding regions as shown in panel A. DNA size markers are in lane 5 with sizes indicated on the right. (B) Quantification of the splicing efficiency using a PhosphorImager is shown from data collected of 3 independent experiments. The splicing efficiency is expressed as the ratio of mRNA to pre-mRNA.

Fig. 5. The expression of mutant PRPF31 reduced RDS expression in primary retinal cells. Following the transfection of murine primary retinal neurons with plasmids expressing either wild-type or mutant PRPF31 as GFP-fusion proteins, wild-type (A) or AD5 (B) or SP117 (C) PRPF31 proteins or GFP vector control (panel D) was detected in the transfected primary retinal cells by monitoring GFP fluorescence (panels A2–D2). Peripherin/RDS expression was demonstrated by immunofluorescent staining (red fluorescence) using anti-peripherin/RDS antibody followed by the staining with the secondary antibody conjugated with Cy3 (A1–D1). Nuclear morphology was revealed by staining with bis-benzamide (panels A3–D3). In panels A4–D4 are superimposed images in columns 1, 2 and 3, and in panels A5–D5 are phase-contrast images. The cells marked with arrowheads in panels B3 and C3 showed abnormally condensed or fragmented nuclei, indicative of apoptosis. In panels B2 and C2, cells expressing mutant PRPF31 that underwent apoptosis showed mutant PRPF31 proteins outside of condensed or fragmented nuclei. As compared to cells expressing wild-type PRPF31 or vector control (panels A or D respectively), cells expressing AD5 or SP117 mutant PRPF31 (panels B1 and C1 respectively, marked by arrows) showed a reduction in RDS staining.
Our previous study showed that PRPF31 mutations did not affect ROM1 intron 2 splicing. Interestingly, in cells transfected with SP117 mutant PRPF31, RDS intron 1 splicing was significantly inhibited (Fig. 3B, lane 4) whereas FSCN2 intron 3 splicing was not severely affected (Fig. 4, lane 4). It remains to be determined what molecular features in pre-mRNAs determine their sensitivity to PRPF31 mutations.

Initially reported by Al-Maghtheh et al. (1994), RP11 represents the second most common adRP locus. Since 2001, a number of mutations have been identified in the PRPF31 gene in RP11 patients, including missense substitutions, deletions and insertions (Vithana et al., 2001; Wang et al., 2003; Abu-Saifeh et al., 2006; Fig. 2). In this study, we used the immunoprecipitation-coupled-microarray approach to identify candidate target genes for the PRPF31 protein. These experiments revealed more than 100 RNA transcripts associated with PRPF31-containing splicing complex(es). Our results show that multiple RNA species associated with PRPF31-containing splicing complexes in retinal cells. Cotransfection experiments using RDS and FSCN2 minigenes with wild-type or RP-mutant forms of PRPF31 expression constructs in HEK cells indicated mutant PRPF31 inhibited pre-mRNA splicing of RDS and FSCN2 minigene in vivo. These results show that RDS and FSCN2 transcripts are among pre-mRNA splicing substrates for PRPF31 protein-containing splicing complexes. Our study suggests that PRPF31 may act upstream of a number of retina-specific genes and control their pre-mRNA splicing.

We also examined effects of PRPF31 mutations in primary retinal cultures. Retinal cells expressing mutant PRPF31 protein showed significantly reduced expression of peripherin/RDS protein as compared with those expressing wild-type PRPF31, supporting the theory that mutant PRPF31 acts in a dominant-negative manner to inhibit RDS pre-mRNA splicing. Consistent with the observation in cotransfected HEK cells, peripherin/RDS protein expression was markedly decreased in retinal cells transfected with the RP-mutant forms of PRPF31. Thus, mutations in PRPF31 affect pre-mRNA splicing of a subset of photoreceptor-specific genes, including rhodopsin (Yuan et al., 2005), peripherin/RDS and FSCN2 (this study). As shown in our previous study, the expression of mutant PRPF31 proteins led to apoptosis of retinal cells (Yuan et al., 2005). Although the effect of mutant PRPF31 on global gene expression profiles remains to be determined, it is likely that the defective or aberrant pre-mRNA splicing of photoreceptor-specific genes, including vital photoreceptor structural genes, caused by PRPF31 mutations contributes to photoreceptor loss and retinal degeneration in patients carrying PRPF31 mutations.

Defective pre-mRNA splicing has been associated with the pathogenesis of neurodegenerative disorders including amyotrophic lateral sclerosis, spinal muscular atrophy and dementia (Grabowski and Black, 2001; Faustino and Cooper, 2003; Wu et al., 2004). Both cis-acting and trans-acting mutations that cause defective pre-mRNA splicing have been identified in patients with retinal degeneration. For example, splice site mutations in rhodopsin and RDS genes have been found in adRP patients (Kim et al., 1993; Jacobson et al., 1994; Sohocki et al., 2001; Greenberg et al., 2003). These mutations affect pre-mRNA splicing of the corresponding genes by a cis-acting mechanism. In addition to such cis-acting splicing mutations, trans-acting mutations also affect pre-mRNA splicing. These mutations, including adRP associated ones in PRPF3, PRPF8 and PRPF31, cause the formation of defective splicing machinery, which in turn leads to defective pre-mRNA splicing of target genes involved. Our results on PRPF31 provide an explanation why genetic defects in the ubiquitously expressed general splicing factors cause photoreceptor-specific disease.

It is interesting to note that RP11 shows incomplete penetrance. Individuals carrying PRPF31 mutations can be either completely asymptomatic or fully symptomatic (Al-Maghtheh et al., 1994, 1996, McGee et al., 1997; Wang et al., 2003). It was reported that the expression levels of PRPF31 in lymphoblastoid cell lines derived from patients and asymptomatic mutation carriers correlate with phenotypes, suggesting that modifier genes may exist that influence the level of PRPF31 expression (Vithana et al., 2003). In this study, the expression of the mutant PRPF31 protein was not examined. Therefore, this study, although consistent with the mechanism of haploinsufficiency, does not exclude the possibility of gain-of-function toxicity of the mutant PRPF31. It is conceivable that modifier genes that functionally antagonize or promote the removal of mutant PRPF31 proteins could affect the penetrance or expressivity of PRPF31 mutations. Elucidation of molecular mechanisms underlying this incomplete penetrance awaits further investigation.

Currently, no effective treatment exists for any form of retinitis pigmentosa. Given the genetic basis of the disease, the most promising prospect may be gene transfer therapy. In an adRP mouse model that was homozygous null for rds, the application of an adeno-associated virus encoding rds resulted in significant structural and electrophysiological improvement in photoreceptor cells (Ali et al., 2000). Specific knockdown ribozymes that target adRP mutant rhodopsin mRNA have also been shown to delay photoreceptor degeneration in mice (Lewin et al., 1998). With regard to the RP11 form of adRP, gene transfer therapy that enhances the expression of wild-type PRPF31 or reduces the level of mutant PRPF31 may provide benefits by restoring the normal splicing of photoreceptor-expressed genes and preventing the loss of photoreceptor cells.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2006.08.026.

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