PRPF31 alternative splicing and expression in human retina

Goranka Tanackovic and Carlo Rivolta

Department of Medical Genetics, University of Lausanne, Lausanne, Switzerland

Correspondence to:
Carlo Rivolta
Department of Medical Genetics
University of Lausanne
Rue du Bugnon 27
1005 Lausanne
Switzerland
Phone: +41(21) 692-5451
FAX: +41(21) 692-5455
email: carlo.rivolta@unil.ch

Short Title: Pattern of *PRPF31* splicing and expression

ABSTRACT

PURPOSE: To investigate the existence of retina-specific PRPF31 isoforms and to study *PRPF31* expression in this and in other human tissues, with the aim of providing a mechanistic link between mutations in such an essential and ubiquitously-expressed gene and retinitis pigmentosa, a disorder restricted to the eye.

METHODS: Alternatively spliced PRPF31 transcripts from human retina and other tissues, as well as from cultured human cell lines, were investigated by RT-PCR, quantitative PCR, cloning and sequencing.

RESULTS: Database searching revealed the presence of a retina-specific PRPF31 isoform in mouse, which could not, however, be experimentally identified in transcripts from human retina or from a human whole eye. Nevertheless, four different PRPF31 isoforms, that were common to all analyzed tissues and cell lines, were isolated. Three of these harbored the full-length PRPF31 coding sequence, whereas the fourth was very short and probably non-coding. The amount of PRPF31 mRNA was previously found to be lower in patients with mutations in this gene than in healthy individuals, making it likely that retinal cells are more sensitive to variation in *PRPF31* expression. However, real-time PCR experiments revealed that PRPF31 mRNA levels in human retina were comparable to those detected in other tissues.

CONCLUSIONS: Our results show that the retina-restricted phenotype caused by *PRPF31* mutations cannot be explained by the presence of tissue-specific isoforms, or by differential expression of *PRPF31* in the retina. As a consequence, the etiology of

PRPF31-associated retinitis pigmentosa likely relies on other, probably more subtle molecular mechanisms.

INTRODUCTION

Retinitis pigmentosa (RP, OMIM 268000) is the most common form of hereditary retinal degeneration, affecting 1 in 4,000 individuals worldwide. Clinically, it is characterized by constant loss of vision that can lead to complete blindness, as a consequence of the progressive death of the photoreceptors, the light-sensing cells of the retina.[1]

Genetically, RP is highly heterogeneous, with ~ 50 genes or loci found so far to be involved in the disease. Most of these genes are retina-specific or have a well-defined role in the physiology of photoreceptors [RetNet, http://www.sph.uth.tmc.edu/retnet/]. The remainders are expressed in many tissues or are ubiquitous and yet cause a phenotype that is retina-specific. Among the latter are four genes associated with autosomal dominant RP and encoding pre-mRNA splicing factors: *PAP-1 (*RP9), *PRPF31 (*RP11), *PRPF8 (*RP13), and *PRPF3* (RP18).[2-6] These genes are extremely conserved in all eukaryotes and essential for survival, as shown by experiments in yeast.[7-9]

Pre-mRNA splicing is the process by which intervening sequences (IVS, or introns) are removed from nascent transcripts and exonic sequences are joined together, to produce mature messenger RNA. It occurs in a large macromolecular complex, the spliceosome, and it can either give rise to a single type of mRNA molecule (constitutive splicing) or to different mRNA populations, through the process of alternative splicing. The importance of alternative splicing has become particularly evident after the

conclusion of the human genome sequencing project, which has revealed that only ~20,000-25,000 genes exist, while the conceptual translation of EST libraries shows that there are at least four times as many proteins in human cells. This discrepancy can indeed be explained by taking into consideration alternative splicing that, according to current estimates, occurs in more than 50% of human genes.[10] In addition to providing different biochemical/biological functions, alternative splicing events can also be used to produce tissue-specific transcripts of the same gene, similarly to what is done by post-translational modifications such as phosphorylation, acylation, glycosylation, sumoylation etc.[11]

Mechanisms linking mutations in the above-mentioned splicing factors and retina-specific cell death are currently unknown; however, some theoretical models exist. The most accepted one is based on the hypothesis of increased vulnerability of photoreceptors to a generally-reduced splicing activity. According to this model, mutations in these splicing factors would cause splicing deficiency in all tissues, however only photoreceptor cells would be affected because of their high demand for mRNA production (such as mRNA encoding rhodopsin and other phototransduction proteins). This model presumes that mRNA amounts produced by the retina greatly exceed mRNA levels produced by other tissues.[12] An alternative model proposes that these mutations do not affect general splicing, but rather impair the splicing patterns of retinal genes. A third model postulates the existence of a retina-specific isoform of any of these RP-associated splicing factors. This isoform would be translated into a protein having a function specific to the retinal tissue, perhaps through interaction with the other RP-associated splicing factors. As support for this hypothesis, retina-specific or retina-prevalent

isoforms of other RP-causing genes have been described for *RPGR*[13] (X-linked RP), *USH2A*[14] (Usher syndrome and recessive RP) and, notably, *IMPDH1*[15] (dominant RP).

PRPF31 (also called *RP11* or *hPRP31*) encodes an essential pre-mRNA splicing factor required for the assembly and recycling of the U4/U6.U5 tri-snRNP complex, which is in turn crucial for splicing.[4,16] Depletion of PRPF31 by RNAi in mammalian cells has been shown to block spliceosome assembly and therefore splicing, which leads to cell death by apoptosis.[17] At the molecular level, lymphoblast cell lines derived from patients with *PRPF31* mutations yield a decreased level of functional PRPF31 mRNA, as a consequence of a rapid degradation of the mutant mRNA by nonsense mediated decay (NMD).[18-20] Reduced mRNA leads to a decrease in the level of PRPF31 protein in the cell and therefore likely causes RP via a haploinsufficiency mechanism.[20] Recent reports of patients carrying heterozygous deletions within the *PRPF31* region[21,22] also support this model. Microarray analyses of mature mRNA from these same lymphoblast cell lines do not show any bias in the production of mRNA from spliced vs. unspliced housekeeping genes, possibly indicating that these mutations do not impair splicing as a general process,[19] against the predictions of the mainstream model (increased vulnerability of photoreceptors with respect to other tissues).

In this study, we investigate the existence of possible *PRPF31* retina-specific isoforms and analyze the expression of *PRPF31* in the retina in comparison to other human tissues, in order to provide support to any of the theories linking mutations in splicing factor genes and dominant RP.

MATERIALS AND METHODS

Cell lines and tissue samples. The following human cell lines were used: HeLa (epithelial cells), A431 (fibroblasts), Y79 (retinoblastoma) and ARPE19 (retinal pigmented epithelia-derived cells). The cells were propagated at 37° C in the following growth media: HeLa and A431 cells were grown in DMEM+GlutaMAX-I (Gibco), Y79 cells in RPMI 1640+GlutaMAX-I (Gibco/Invitrogen, Carlsbad, CA) and AREP19 cells in N1 growth medium [DMEM-F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 0.6% glucose (Sigma-Aldrich), 0.11% $NaHCO_3$ (Sigma-Aldrich), 5 mM Hepes (Gibco/Invitrogen), 1x N1 Supplement (Sigma-Aldrich) and 1 mM glutamine (Sigma-Aldrich), as final concentrations]. All media were supplemented with 10% heat-inactivated Fetal Calf Serum (Sigma-Aldrich).

Total RNA samples from human retina, brain, heart, testis and skeletal muscle were purchased from Clontech (Mountain View, CA). All tissue samples were a pool from different individuals displaying different gender and age, who died of sudden death or trauma. Specifically, retinal RNA used in our investigations was obtained by pooling total RNA preparations from 29 Caucasian individuals (males and females) who died from trauma or sudden death. These donors, aged 20 to 60, had all morphologically normal retinas. cDNA from a whole human eye was also acquired from commercial sources (BioChain, Hayward, CA).

RT-PCR and cloning. Total RNA samples from cell lines were prepared with the RNeasy Mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions for the isolation of total RNA from animal cells, and subsequently treated with 10 U of RNase-free DNase (Roche, Basel, Switzerland) for 30 min at 37° C, phenol/chloroform extracted and precipitated with ethanol. Reverse transcription reactions were performed by using 2 μg of total RNA as template and oligo-dT as primer, with the ProtoScript cDNA Synthesis kit (New England Biolabs, Ipswich, MA). The reaction was carried out for 2 h at 42° C. Following the cDNA synthesis, the remaining RNA was eliminated by treatment with RNase H for 20 min at 37° C.

Semi-quantitative PCR reactions were performed with the Expand High-Fidelity PCR System (Roche) for 30 cycles. The following primers were used: GAPDH-E7: 5'-tgcctcctgcaccaccaact-3', GAPDH-E8: 5'-gccatccacagtcttctggg-3', RHO-E3: 5'-atccccgagggcctgcagtgc-3', RHO-E5: 5'-gaggcctcatcgtcacccagtg-3', PRPF31-E1: 5'-acagtggtgcgcggagag-3', PRPF31-E2: 5'-cgccaagctatgggatagtaaga-3', PRPF31-E4: 5'-cggcctccactggtccc-3', PRPF31-E8: 5'-cacgatgtcactgtggtagatgt-3' and PRPF31-E14: 5'-cttggacacacgcagtcatt-3', where "E" indicates the exon in which the primer lies.

The obtained PCR products were assessed by agarose electrophoresis, cut from the gels, purified using the PeqGold Gel Extraction kit (Peq Lab, Erlangen, Germany), and cloned into the pCRII-TOPO cloning vector (Invitrogen). The cloning reactions were used to transform competent TOP-10 *E. coli* cells (Invitrogen) and individual clones were sequenced with either insert-specific or vector-specific primers. To analyze PRPF31 isoforms ten clones were sequenced for each PCR band. To identify PRPF31 isoforms in

human tissues, 30 clones of E1-E14 PRPF31 PCR product were sequenced for every tissue.

RT-PCRs performed with primers PRPF31-E1 (forward) and PRPF31-E4 (reverse) were further analyzed by polyacrilamide gel electrophoresis with the eGene HDA-GT12 Multi-Channel Genetic Analyzer (eGene Inc., Irvine, CA), to maximize separation of the obtained bands, ranging from 306 to 336 bp.

Quantitative-PCR (Q-PCR). Real-time PCR was performed to quantify PRPF31 mRNA in HeLa cells and in different human tissues by using the 18S ribosomal RNA, as well as GAPDH and ACTB (Actin β) mRNA as endogenous controls.

Real-time PCRs were performed by using the ABI Prism 7500 Sequence Detector in a final volume of 20 µl containing Power SybrGreen (Applied Biosystems, Foster City, CA) and 18S ribosomal RNA specific primers or PRPF31 specific primers in exons 3 and 5.[20] For the GAPDH and ACTB reactions, commercial master mixes were used (Applied Biosystems). As amplification template, 4 µl of cDNA corresponding to 20 ng of retrotranscribed RNA was used.

Relative quantification of the PRPF31 mRNA amount was done by using the "standard curve" method. Standard curves for PRPF31, GAPDH, Actin β mRNA and 18S RNA were generated via 5 serial dilutions over a 250-fold range using a pool of cDNA samples. Individual Ct values from each gene were interpolated from the relevant standard curve, and the amount of PRPF31 mRNA for each time point was quantified

relative to each endogenous control. Each quantification was the average of two independent experiments, and each PCR amplification was performed in triplicate.

Western blots. HeLa cell nuclear extracts were prepared as described previously.[23] Twenty micrograms of such extracts were loaded on 8% SDS-PAGE to be analyzed by western blot. The western blotting procedure is essentially as described in one of our previous communications.[24] The primary antibodies used are: N-terminal anti-PRPF31 antibody[20] and C-terminal anti-PRPF31 antibody.[4] The secondary antibody used to reveal the primary antibodies was polyclonal swine anti-rabbit-HRP antibody (Dako, Glostrup, Denmark).

RESULTS

In silico analysis of PRPF31 isoforms: The *PRPF31* gene is highly conserved in all eukaryotes. Using comparative genomics, we first analyzed its conservation between human and mouse. At the DNA level, the two coding sequences show 89.7% identity; in addition, size and structure of all coding exons are conserved. The constitutively-expressed form of PRPF31 encodes in both human and mouse proteins that are 499-amino acid (aa) long. Their alignment reveals 99.0% amino acid identity between the two species and 99.6% conservation, by ClustalW alignment.

This extremely high conservation between human and mouse, at the DNA as well as the protein level, suggests also conservation of splicing events, including those originating putative retina-specific variants. Therefore, we investigated both human and mouse EST databases for PRPF31 alternative splicing isoforms. Analyses of the FastDB[25,26] and Ensembl[27] databases showed that, in addition to the constitutively-expressed PRPF31 isoform that yields full-length PRPF31 protein, at least 4 other human and 3 other mouse PRPF31 isoforms exist (data not shown). Among all these, only one is specific to the retina, and is detected in mouse. This isoform partly shares its 5' region with full-length PRPF31 (with the exception of the first 59 codons) and has a distinct 3' sequence, because it retains intron 7 and uses an alternative poly-adenylation site in intron 9 (cDNA clone AK044457; Figure 1). Conceptual translation of this mRNA yields a protein of 195 aa with distinct N- and C-termini in comparison to the constitutive PRPF31 protein.

PRPF31 isoforms in human: To experimentally investigate PRPF31 alternative splicing, we first analyzed PRPF31 transcripts by PCR on cDNA derived from human retina from 29 control donors, a whole eye (positive control), and HeLa cells (negative control) with primers located within the first and the last exon. The obtained amplification products always separated into two distinct DNA bands of 333 bp and 1550 bp (Figure 2A), which were cloned and sequenced.

The 333 bp band resulted in all samples from the joining of exon 1 to exon 12 of PRFP31, using the constitutive splice sites, followed by exons 13 and 14. Conceptual translation of this isoform yields three possible open reading frames (ORF). The first one would be a target for degradation by NMD, since it contains a premature stop codon. The second results in a hypothetical protein that would use an AUG codon within exon 13 and it would stop in exon 14, producing a 28-aa long peptide with a predicted molecular weight of 3.1 kDa. Neither of the two antibodies that were available to us would recognize this protein, since it is devoid of both the N- and C-terminal epitopes used to raise them. The third ORF could also be translated into a hypothetical protein that would have both the start and stop codons within exon 14; it would be only 17-aa long and have a predicted molecular weight of 1.9 kDa. If expressed, this peptide should be recognized by the C-terminal anti-PRPF31 antibody; however, we could not detect it by western blot, possibly because of its small size (data not shown). In addition, the same mRNA isoform can be found as the human EST clone BM772272 and as one of the Vega transcripts in Ensembl; this latter database proposes no protein product for this mRNA isoform.

Sequences obtained from the cloning of the 1550-bp band revealed in all samples three distinct PRFP31 isoforms, which were further confirmed by a second round of PCR amplification of PRPF31 exons 1 to 4, followed by agarose gel separation and excision, as well as additional cloning and sequencing (Figure 2B). These three isoforms resulted from the use of the constitutive 5' splice site (ss) in intron 1 (IVS1) in combination with the constitutive 3' ss in the same IVS or with either of two alternative 3' ss (Figure 2C). The first alternative ss is located 23 nt and the second one 30 nt upstream of the constitutive 3' ss. All three isoforms should produce full-length PRPF31 protein, since the translation start codon is within exon 2 (Figure 2C).

We then performed western analyses with two different antibodies raised against peptides derived from either the N- or the C-terminus of the PRPF31 protein,[4,20] to test for the presence of possible isoforms at the protein level. For this analysis we used HeLa nuclear extracts, since protein extracts from the human tissues assayed in expression experiments were unavailable, and all identified mRNA isoforms were shown to be invariantly expressed in all tested cell lines and human tissues (see below). Both antibodies identified a single protein band of 61 kDa, which corresponds to full-length PRPF31; no additional bands were detected (Figure 3).

Analysis of PRPF31 expression: We next investigated whether the retina-restricted pathological effect of PRPF31 mutations could be due to differential expression or to a differential splicing pattern of this gene in retina vs. other tissues. First, we compared *PRPF31* expression in the eye and the retina vs. standard laboratory cell lines of ocular origin or not (HeLa, A431, Y79 and ARPE19) by semi-quantitative RT-PCR. PRPF31

PCR products, obtained with primers that allowed the amplification of various parts of the mRNA (exons 1-14, exons 2-14, exons 2-8 and exons 1-4), revealed that in all instances *PRPF31* expression was much lower in the human eye or retina than in any of the cell lines (Figure 4A). However, this difference in expression disappeared when ocular or retinal PRPF31 mRNA was compared with transcripts from various normal human tissues (Figure 4B), indicating that the increased levels of *PRPF31* expression displayed by human cell lines were likely due to the high growth rate, and hence splicing demand, typical of cultured cells. Indeed, when directly compared with HeLa cells, all normal tissues displayed markedly reduced levels of PRPF31 transcripts (Figure 4B). RT-PCR products obtained with primers located in exons 1 and 4 were apparently less abundant when cDNAs from eye and retina, as well as form skeletal muscle, were used as template material. However, the semi-quantitative nature of these amplifications did not allow a precise assessment of these cDNA amounts, which were then further measured by Q-PCR (see below). Furthermore, the relative ratio of the 3 isoforms producing the 1550 bp band was always roughly the same in all tissues analyzed (70% constitutive PRPF31 isoform, i.e. exons 1-14; 20% exon 1+30 nt from IVS1+ exons 2-14; 10% exon 1+23 nt from IVS1+exons 2-14, not shown).

In addition, amplification of cDNA from the different human cell lines (Figure 4A) and tissues (Figure 4B), with primers located in the first and the last exon of *PRPF31*, invariably produced only the two DNA bands corresponding to the PRPF31 isoforms described above (at 1550 and 333 bp), as confirmed by cloning and sequencing. Since these isoforms were present in all cell lines and tissues tested, we concluded that none of them is specific to or is specifically absent from the retina. Use of other combinations of

primers in the coding sequence of *PRPF31*, to exclude the possibility of preferential amplification of one isoform by a given set of primers, as well as to test the presence of the human equivalent of the murine AK044457 isoform, did not result in the appearance of additional isoforms (Figure 4). We also investigated the presence of putative alternative transcripts extending before the first or after the last canonical exons by performing 5' and 3' RACE on RNA from human retina, brain, heart, testis and skeletal muscle. No additional or retina-specific isoforms could be detected by such analyses (data not shown).

To precisely assess the amounts of PRPF31 mRNA in different normal tissues and refine the data obtained by semi-quantitative methods, we measured *PRPF31* expression by Q-PCR. We initially normalized PRPF31 mRNA levels to the expression of 3 housekeeping genes: the 18S ribosomal RNA, *ACTB* and *GAPDH*. Subsequent analysis of these reference genes by the geNORM software[28] revealed that the only reliable gene, expressed at a constant rate across all analyzed tissues, was the 18S ribosomal RNA, which was therefore the only one retained for quantification of *PRPF31* expression. The Q-PCR results were consistent with semi-quantitative RT-PCR amplification and indicated that PRPF31 mRNA expression in the retina was similar to that in the brain or heart and inferior only to high-proliferating samples such as testis and HeLa (Figure 5).

DISCUSSION

Proteomic and functional diversity in metazoans is largely generated through alternative splicing,[29] resulting in isoforms that may have distinct cellular functions, as well as different temporal and spatial expression patterns.[30] In the case of RP, relevance of alternative splicing has already been demonstrated by the retina-specific or retina-prevalent isoforms of IMPDH1, RPGR and USH2A.[13-15]

In this study, we examined the presence of retina-specific isoforms of *PRPF31*, which could explain the retina-restricted pathological phenotype caused by mutations in this ubiquitous gene. We selected this gene following the identification in expression databases of a PRPF31 retina-specific isoform in the mouse, while the 3 other RP genes encoding splicing factors produce only one major transcript in mouse and human tissues.[5,31-32] However, we could not detect this murine retina-specific PRPF31 transcript in a human eye or retina, nor from freshly-dissected retinas and other tissues or organs from mouse (data not shown), indicating that this particular mRNA form may represent a very rare alternative splicing event with no major biological functions, or perhaps a database artifact. Indeed, this specific variant seems to be present only in the single entry AK044457, among more than 300 available PRPF31 expressed sequences deposited in public databases.

Nevertheless, we identified four different PRPF31 transcripts, ubiquitously expressed in all different cell lines and normal human tissues tested. Three of these contained the same coding sequence, since alternative splicing affects the 5'UTR, but not

the reading frame (as confirmed by western blot analyses, showing the presence of a single band, corresponding to the full-length protein). These transcripts could have variable translation efficiencies, because of their different 5'UTRs, and therefore lead to variable amounts of PRPF31 protein in different tissues. However, our data suggest that these isoforms invariably contribute in constant proportions to the total *PRPF31* expression in all tissues. The fourth identified *PRPF31* transcript (exon 1 + exons 12-14) is also expressed in a ubiquitous fashion and can potentially encode only short peptides, if it is translated at all. Altogether, our results show that none of the identified variants could *per se* explain the retina-restricted phenotype associated with PRPF31 mutations, since all cells and tissues analyzed display the same pattern of PRPF31 transcripts. Therefore, it is likely that mechanisms other than alternative splicing of PRPF31 mRNA are involved in the molecular etiology of the disease.

Since retina-specific isoforms could be ruled out, we next investigated whether differential *PRPF31* expression in retina vs. other human tissues could explain the disorder. It has been previously shown that *PRPF31*-associated RP occurs when an insufficient amount of the PRPF31 protein is made, either because of large genomic heterozygous deletions[21,22] or of mutations that are sensitive to NMD, which destroys all PRPF31 mRNA produced by mutant alleles.[20] Furthermore, semi-quantitative RT-PCR analysis of different human tissues (including retina) has suggested that another RP gene, *PRPF8*, might be over-expressed in the retina with respect to other tissues.[5] Our Q-PCR results show that *PRPF31* expression is comparable across all tissues analyzed and is in particular very similar in retina, brain and heart, making it unlikely that retina could have a higher demand for this splicing factor than these other organs. As concerns spatial

distribution, it has been recently shown by *in situ* hybridization that *PRPF31* is expressed in all retinal layers in human and mouse, with a gradient from the ganglion cell layer (strong expression) to photoreceptors (very low expression).[33] However, both these *in situ* analyses and our Q-PCR measurements were limited to mRNA levels only, and therefore we cannot exclude that there may be a difference in the amount of PRPF31 among tissues at the protein level, which could be caused by different translational yields,[34] tissue-specific codon-mediated translation control,[35] or different stability of the translated product. This issue remains then open, and should be addressed by careful assessment of PRPF31 protein distribution and of possible post-translational modifications within the human retina.

Altogether, our results seem to indicate that the etiological mechanisms for retinitis pigmentosa caused by mutations in splicing factor genes may rely on subtle alterations of the pre-mRNA splicing process, possibly via the reduced processing of transcripts that are important for retinal maintenance and function. Very recently, the molecular mechanisms of another neurodegenerative disease, spinal muscular atrophy (SMA, OMIM 600354) have been elucidated.[36] SMA is characterized by the degeneration of motor neurons and occurs when the splicing protein SMN is mutated. More specifically, it has been shown that absence of SMN in mouse causes ubiquitous and generalized splicing defects that are nonetheless pathogenic for specific cell types and are more pronounced on pre-mRNA carrying particular sequences.[36] An intriguing possibility is therefore that a similar situation could occur when RP-associated splicing factors are mutated and photoreceptors, instead of motor neurons, become the primary targets of splicing impairment.

ACKNOWLEDGMENTS

We would like to thank Drs. Jacques S. Beckmann, Thomas Rio Frio and Angela Krämer for discussions, and Dr. Nicholas M. Wade for the critical reading of the manuscript. We are also indebted to Drs. Reinhard Lührmann and Cindy Will for the C-terminal anti-PRPF31 antibody and to Drs. Estelle Arnaud and Karim Nadra for samples of mouse retina and other murine tissues. Finally, we are grateful to Drs. Corinne Kostic and Yvan Arsenijevic for providing the Y79 and ARPE19 cell lines. This work was supported by the Marie Heim-Vögtlin Programme - Promotion of women by the Swiss National Science Foundation, grant PMPDA--114446.

FIGURE LEGENDS

FIG. 1. Schematic representation of PRPF31 isoforms in human and mouse, as detected in silico.

Constitutive human and mouse PRPF31 isoforms are shown, as deduced from publicly-available databases. The representation of the mouse exons and introns are drawn to scale, as well as the exons of the human isoform. PRPF31 introns from the human sequence, which have different sizes with respect to the murine sequence, are rescaled to allow the alignment of the exons between the two species. Lines joining different exons indicate the constitutive splicing pattern (above the exons) or alternative splicing pattern (below the exons). Asterisks indicate the position of the translation initiation codons.

FIG. 2. Experimentally determined PRPF31 isoforms.

A) cDNA derived from 29 control human retinas, a whole eye, and HeLa cells was amplified with primers located in exon 1 (E1) and exon 14 (E14, the last exon) of PRPF31. Two PCR bands were obtained. The band at 1550 bp represents a mix of three PRPF31 isoforms, detailed in panels B and C. The 333 bp band is a single isoform of PRPF31 (E1 spliced to E12+E13+E14), as indicated. M, DNA ladder (sizes in bp).

B) Analysis of the 3 PRPF31 isoforms producing the 1550 bp band shown in panel A, obtained by RT-PCR with primers complementary to exons 1 and 4. The image shows normalized electropherograms from capillary polyacrylamide gel electrophoresis (left), their corresponding reconstructed images (middle), as well as the size and the structure of

these isoforms (right), as deduced after further agarose gel separation, band excision, cloning and sequencing.

C) Representation of the sequences spanning the variable region from the 3 PRPF31 isoforms from panel B. The end of the exon 1 (E1), part of the intron 1 (IVS1) and the beginning of the exon 2 (E2) of PRPF31 are depicted. Constitutive 5' and 3' splice sites are shown in green and orange, respectively, the two alternative 3' splice sites in red, and the translation start codon in blue.

FIG. 3. Western blots of PRPF31 protein.

A HeLa nuclear extract was analyzed by western blots with either N-terminal or C-terminal anti-PRPF31 antibodies, as indicated at the bottom of the images. Both antibodies reveal the presence of a single 61 kDa band, that corresponds to full-length PRPF31. The sizes of protein ladder (M, in kDa) are indicated on the left of the blots.

FIG. 4. PRPF31 mRNA expression in cell lines (A) and various normal human tissues (B).

cDNA from the indicated samples was used in semi-quantitative RT-PCRs, with primers for PRPF31 (exons E1 to E14, E2 to E14, E2 to E8 and E1 to E4), GAPDH (exons E7 to E8), and the rhodopsin gene (RHO; as specific marker of rod photoreceptors; exons E3 to E5), as indicated on the left of the gels. The sizes of PCR products are indicated on the right of the gels. The lane "-" shows the negative control of the RT-PCRs.

FIG. 5. Quantitative PCR analysis of PRPF31 mRNA expression in various normal human tissues.

cDNA from HeLa cells and five normal human tissues was amplified by Q-PCR with primers for PRPF31 (exon 3 to exon 5). The expression was normalized to the 18S ribosomal RNA. The result represents an average of two independent experiments done in triplicate.

REFERENCES

1. Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet* 2006;368:1795-1809.
2. Chakarova CF, Hims MM, Bolz H, Abu-Safieh L, Patel RJ, Papaioannou MG, Inglehearn CF, Keen TJ, Willis C, Moore AT, Rosenberg T, Webster AR, Bird AC, Gal A, Hunt D, Vithana EN, Bhattacharya SS. Mutations in HPRP3, a third member of pre-mRNA splicing factor genes, implicated in autosomal dominant retinitis pigmentosa. *Hum Mol Genet* 2002;11:87-92.
3. Maita H, Kitaura H, Keen TJ, Inglehearn CF, Ariga H, Iguchi-Ariga SM. PAP-1, the mutated gene underlying the RP9 form of dominant retinitis pigmentosa, is a splicing factor. *Exp Cell Res* 2004;300:283-296.
4. Makarova OV, Makarov EM, Liu S, Vornlocher HP, Lührmann R. Protein 61K, encoded by a gene (PRPF31) linked to autosomal dominant retinitis pigmentosa, is required for U4/U6.U5 tri-snRNP formation and pre-mRNA splicing. *EMBO J.* 2002;21:1148-1157.
5. McKie AB, McHale JC, Keen TJ, Tarttelin EE, Goliath R, van Lith-Verhoeven JJ, Greenberg J, Ramesar RS, Hoyng CB, Cremers FP, Mackey DA, Bhattacharya SS, Bird AC, Markham AF, Inglehearn CF. Mutations in the pre-mRNA splicing factor gene PRPC8 in autosomal dominant retinitis pigmentosa (RP13). *Hum Mol Genet* 2001;10:1555-1562.
6. Vithana EN, Abu-Safieh L, Allen MJ, Carey A, Papaioannou M, Chakarova C, Al-Maghtheh M, Ebenezer ND, Willis C, Moore AT, Bird AC, Hunt DM,

Bhattacharya SS. A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). *Mol Cell* 2001;8:375-381.

7. Lossky M, Anderson GJ, Jackson SP, Beggs J. Identification of a yeast snRNP protein and detection of snRNP-snRNP interactions. *Cell* 1987;51:1019-1026.

8. Potashkin J, Li R, Frendewey D. Pre-mRNA splicing mutants of Schizosaccharomyces pombe. *Embo J* 1989;8:551-559.

9. Weidenhammer EM, Singh M, Ruiz Noriega M, Woolford JLJ. The PRP31 gene encodes a novel protein required for pre-mRNA splicing in Saccharomyces cerevisiae. *Nucleic Acids Res.* 1996;24:1164-1170.

10. Stetefeld J, Ruegg MA. Structural and functional diversity generated by alternative mRNA splicing. *Trends Biochem Sci* 2005;30:515-521.

11. Mann M, Jensen ON. Proteomic analysis of post-translational modifications. *Nat Biotechnol* 2003;21:255-261.

12. Baehr W, Chen CK. RP11 and RP13: unexpected gene loci. *Trends Mol Med* 2001;7:484-486.

13. Neidhardt J, Glaus E, Barthelmes D, Zeitz C, Fleischhauer J, Berger W. Identification and characterization of a novel RPGR isoform in human retina. *Hum Mutat* 2007;28:797-807.

14. van Wijk E, Pennings RJ, te Brinke H, Claassen A, Yntema HG, Hoefsloot LH, Cremers FP, Cremers CW, Kremer H. Identification of 51 novel exons of the Usher syndrome type 2A (USH2A) gene that encode multiple conserved

functional domains and that are mutated in patients with Usher syndrome type II. *Am J Hum Genet* 2004;74:738-744.

15. Bowne SJ, Liu Q, Sullivan LS, Zhu J, Spellicy CJ, Rickman CB, Pierce EA, Daiger SP. Why do mutations in the ubiquitously expressed housekeeping gene IMPDH1 cause retina-specific photoreceptor degeneration? *Invest Ophthalmol Vis Sci* 2006;47:3754-3765.

16. Nottrott S, Urlaub H, Luhrmann R. Hierarchical, clustered protein interactions with U4/U6 snRNA: a biochemical role for U4/U6 proteins. *Embo J* 2002;21:5527-5538.

17. Schaffert N, Hossbach M, Heintzmann R, Achsel T, Lührmann R. RNAi knockdown of hPrp31 leads to an accumulation of U4/U6 di-snRNPs in Cajal bodies. *EMBO J.* 2004;23:3000-3009.

18. Vithana EN, Abu-Safieh L, Pelosini L, Winchester E, Hornan D, Bird AC, Hunt DM, Bustin SA, Bhattacharya SS. Expression of PRPF31 mRNA in patients with autosomal dominant retinitis pigmentosa: a molecular clue for incomplete penetrance? *Invest Ophthalmol Vis Sci* 2003;44:4204-4209.

19. Rivolta C, McGee TL, Rio Frio T, Jensen RV, Berson EL, Dryja TP. Variation in retinitis pigmentosa-11 (PRPF31 or RP11) gene expression between symptomatic and asymptomatic patients with dominant RP11 mutations. *Hum Mutat* 2006;27:644-653.

20. Rio Frio T, Wade NM, Ransijn A, Berson EL, Beckmann JS, Rivolta C. Premature termination codons in PRPF31 cause retinitis pigmentosa via

haploinsufficiency due to nonsense-mediated mRNA decay. *J Clin Invest* 2008;118:1519-1531.

21. Abu-Safieh L, Vithana EN, Mantel I, Holder GE, Pelosini L, Bird AC, Bhattacharya SS. A large deletion in the adRP gene PRPF31: evidence that haploinsufficiency is the cause of disease. *Mol Vis* 2006;12:384-388.

22. Sullivan LS, Bowne SJ, Seaman CR, Blanton SH, Lewis RA, Heckenlively JR, Birch DG, Hughbanks-Wheaton D, Daiger SP. Genomic rearrangements of the PRPF31 gene account for 2.5% of autosomal dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 2006;47:4579-4588.

23. Krämer A, Keller W. Preparation and fractionation of mammalian extracts active in pre-mRNA splicing. In: Abelson JN, ed. *Methods Enzymol.* Vol 181. New York: Academic Press, 1990;3-19.

24. Tanackovic G, Kramer A. Human splicing factor SF3a, but not SF1, is essential for pre-mRNA splicing in vivo. *Mol Biol Cell* 2005;16:1366-1377.

25. de la Grange P, Dutertre M, Martin N, Auboeuf D. FAST DB: a website resource for the study of the expression regulation of human gene products. *Nucleic Acids Res* 2005;33:4276-4284.

26. de la Grange P, Dutertre M, Correa M, Auboeuf D. A new advance in alternative splicing databases: from catalogue to detailed analysis of regulation of expression and function of human alternative splicing variants. *BMC Bioinformatics* 2007;8:180.

27. Hubbard TJ, Aken BL, Beal K, Ballester B, Caccamo M, Chen Y, Clarke L, Coates G, Cunningham F, Cutts T, Down T, Dyer SC, Fitzgerald S, Fernandez-

Banet J, Graf S, Haider S, Hammond M, Herrero J, Holland R, Howe K, Johnson N, Kahari A, Keefe D, Kokocinski F, Kulesha E, Lawson D, Longden I, Melsopp C, Megy K, Meidl P, Ouverdin B, Parker A, Prlic A, Rice S, Rios D, Schuster M, Sealy I, Severin J, Slater G, Smedley D, Spudich G, Trevanion S, Vilella A, Vogel J, White S, Wood M, Cox T, Curwen V, Durbin R, Fernandez-Suarez XM, Flicek P, Kasprzyk A, Proctor G, Searle S, Smith J, Ureta-Vidal A, Birney E. Ensembl 2007. *Nucleic Acids Res* 2007;35:D610-617.

28. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.

29. Blencowe BJ. Alternative splicing: new insights from global analyses. *Cell* 2006;126:37-47.

30. Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. *Genes Dev* 2003;17:419-437.

31. Takahashi A, Muramatsu H, Takagi S, Fujisawa H, Miyake Y, Muramatsu T. A splicing factor, Prp8: preferential localization in the testis and ovary in adult mice. *J Biochem* 2001;129:599-606.

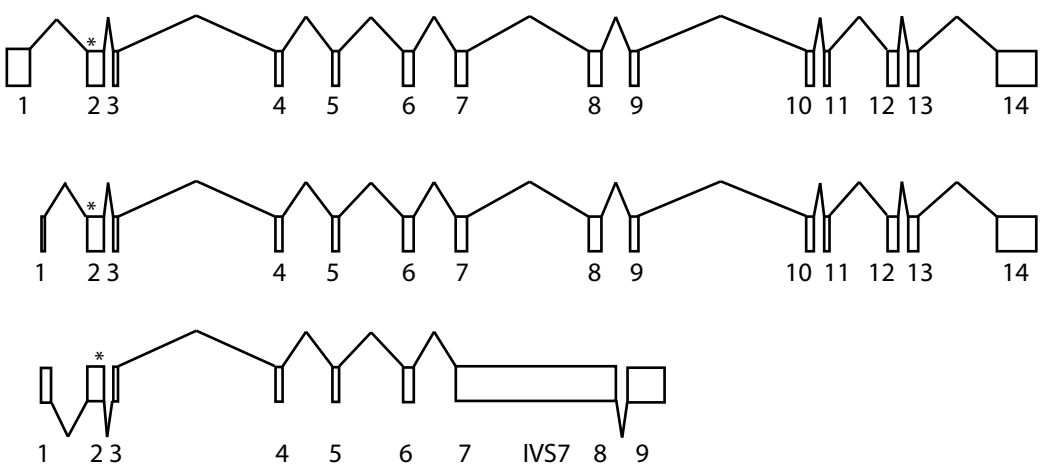
32. Gonzalez-Santos JM, Cao H, Wang A, Koehler DR, Martin B, Navab R, Hu J. A complementation method for functional analysis of mammalian genes. *Nucleic Acids Res* 2005;33:e94.

33. Trifunovic D, Karali M, Camposampiero D, Ponzin D, Banfi S, Marigo V. A high-resolution RNA expression atlas of retinitis pigmentosa genes in human and mouse retinas. *Invest Ophthalmol Vis Sci* 2008;49:2330-2336.

34. Bracey LT, Paigen K. Changes in translational yield regulate tissue-specific expression of beta-glucuronidase. *Proc Natl Acad Sci U S A* 1987;84:9020-9024.

35. Plotkin JB, Robins H, Levine AJ. Tissue-specific codon usage and the expression of human genes. *Proc Natl Acad Sci U S A* 2004;101:12588-12591.

36. Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, Dreyfuss G. SMN Deficiency Causes Tissue-Specific Perturbations in the Repertoire of snRNAs and Widespread Defects in Splicing. *Cell* 2008;133:585-600.



Constitutive human isoform
NCBI entry: AY040822

Constitutive mouse isoform
NCBI entry: BC018376

Retinal mouse isoform
NCBI entry: AK044457

Figure 1

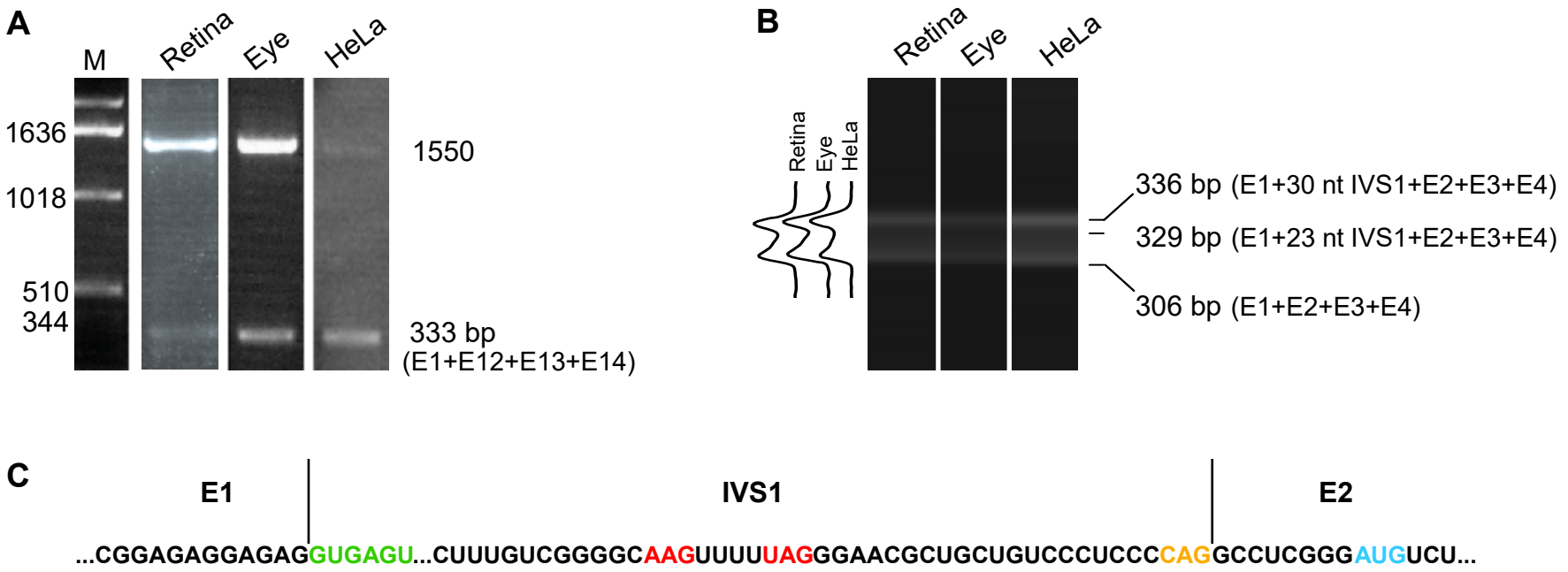

A
M
Retina
Eye
HeLa
1636
1018
510
344
1550
333 bp
(E1+E12+E13+E14)
B
Retina
Eye
HeLa
Retina
Eye
HeLa
336 bp (E1+30 nt IVS1+E2+E3+E4)
329 bp (E1+23 nt IVS1+E2+E3+E4)
306 bp (E1+E2+E3+E4)
C
E1
IVS1
E2
...CGGAGAGGAGAGGUGAGU...CUUUGUCGGGGCAAGUUUUUAGGGAACGCUGCUGUCCCUCCCCAGGCCUCGGGAUGUCU...

Figure 2

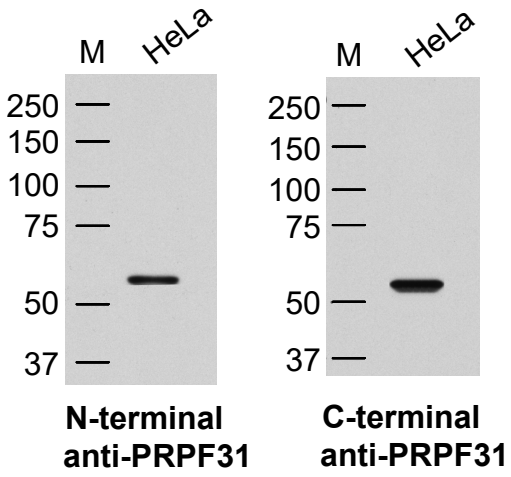
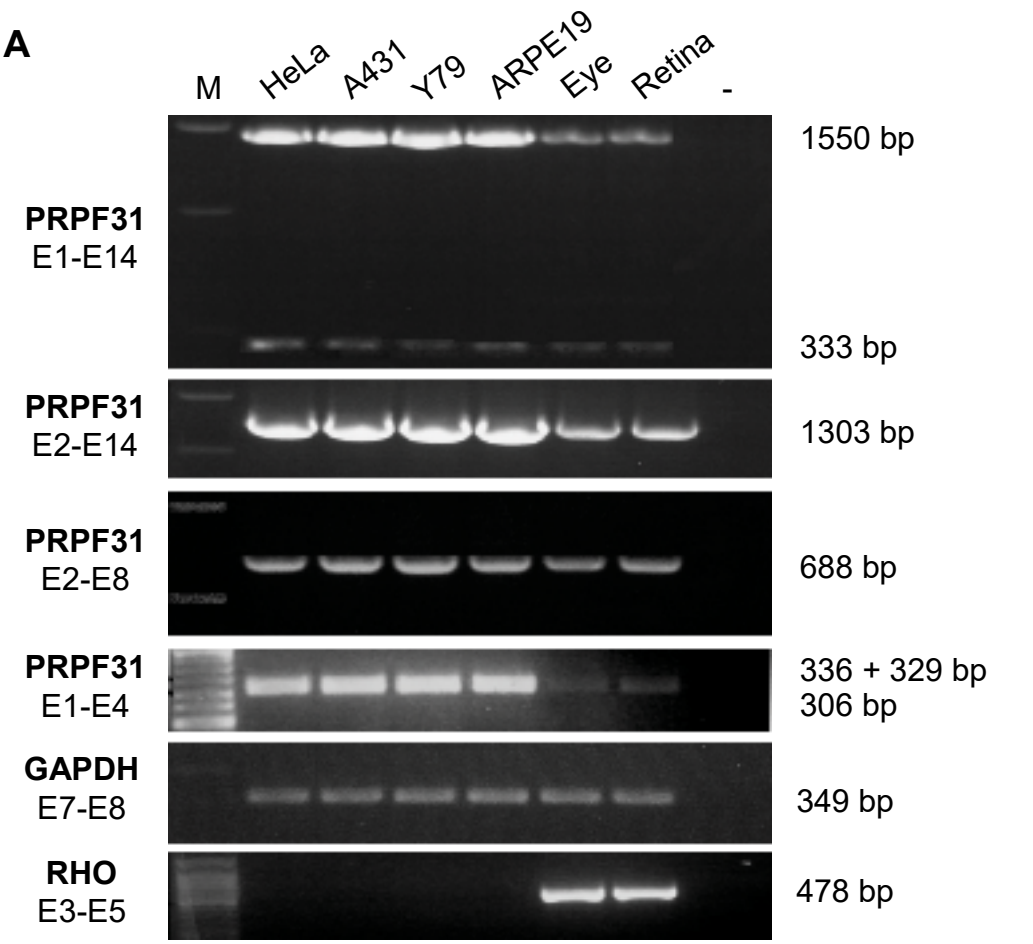

M
HeLa
250
150
100
75
50
37
N-terminal
anti-PRPF31
M
HeLa
250
150
100
75
50
37
C-terminal
anti-PRPF31

Figure 3


A
M
HeLa
A431
Y79
ARPE19
Eye
Retina
-
PRPF31
E1-E14
1550 bp
333 bp
PRPF31
E2-E14
1303 bp
PRPF31
E2-E8
688 bp
PRPF31
E1-E4
336 + 329 bp
306 bp
GAPDH
E7-E8
349 bp
RHO
E3-E5
478 bp

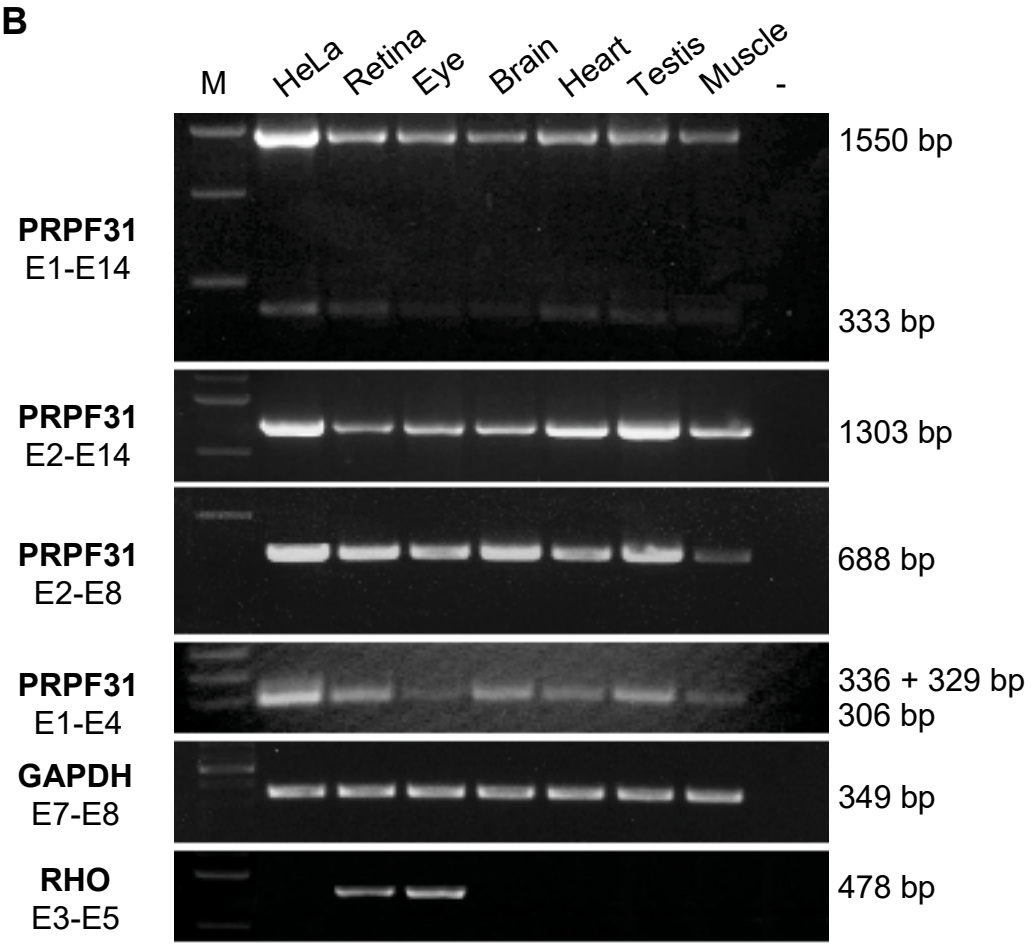

B
M
HeLa
Retina
Eye
Brain
Heart
Testis
Muscle
-
PRPF31
E1-E14
1550 bp
333 bp
PRPF31
E2-E14
1303 bp
PRPF31
E2-E8
688 bp
PRPF31
E1-E4
336 + 329 bp
306 bp
GAPDH
E7-E8
349 bp
RHO
E3-E5
478 bp

Figure 4

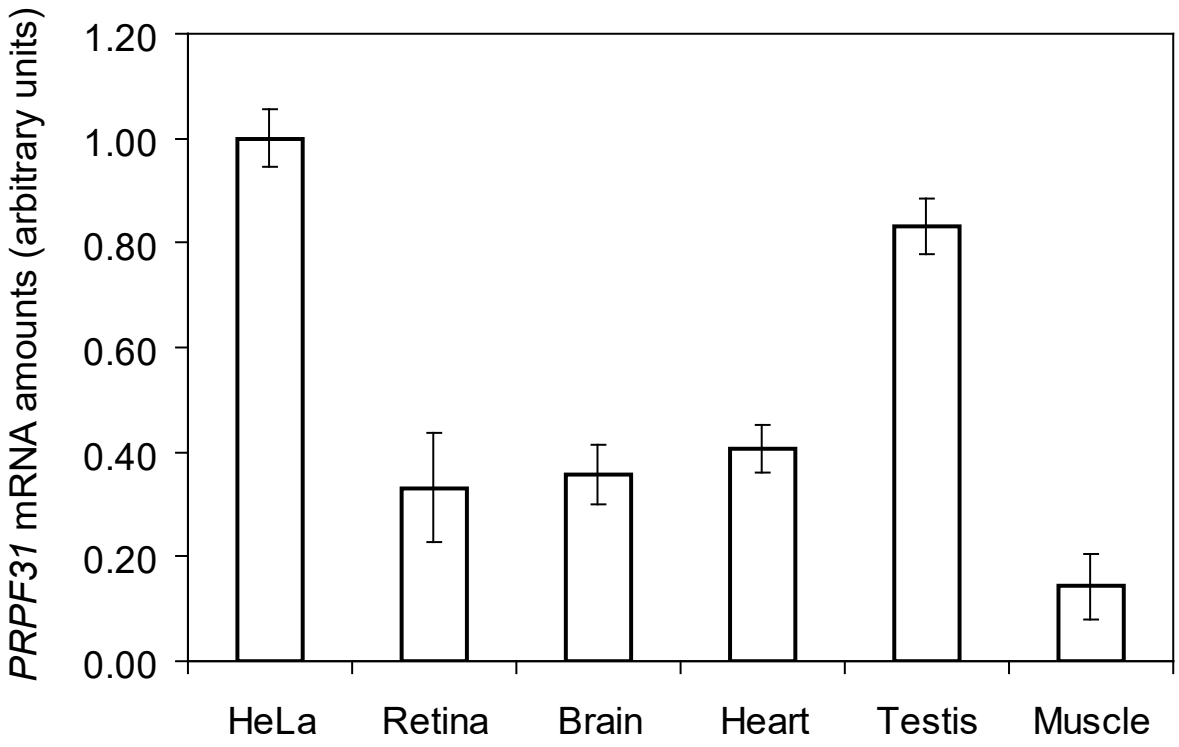

PRPF31 mRNA amounts (arbitrary units)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
HeLa
Retina
Brain
Heart
Testis
Muscle

Figure 5