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Disrupted alternative splicing for genes implicated in splicing and ciliogenesis causes PRPF31 retinitis pigmentosa

Adriana Buskin et al.#

Mutations in pre-mRNA processing factors (PRPFs) cause autosomal-dominant retinitis pigmentosa (RP), but it is unclear why mutations in ubiquitously expressed genes cause non-syndromic retinal disease. Here, we generate transcriptome profiles from RP11 (PRPF31-mutated) patient-derived retinal organoids and retinal pigment epithelium (RPE), as well as Prpf31+/− mouse tissues, which revealed that disrupted alternative splicing occurred for specific splicing programmes. Mis-splicing of genes encoding pre-mRNA splicing proteins was limited to patient-specific retinal cells and Prpf31+/− mouse retinae and RPE. Mis-splicing of genes implicated in ciliogenesis and cellular adhesion was associated with severe RPE defects that include disrupted apical–basal polarity, reduced trans-epithelial resistance and phagocytic capacity, and decreased cilia length and incidence. Disrupted cilia morphology also occurred in patient-derived photoreceptors, associated with progressive degeneration and cellular stress. In situ gene editing of a pathogenic mutation rescued protein expression and key cellular phenotypes in RPE and photoreceptors, providing proof of concept for future therapeutic strategies.
Retinitis pigmentosa (RP) is one of the most common inherited forms of retinal blindness with a prevalence of about 1 in 2500 births and more than 1 million people affected worldwide. RP is characterised by progressive degeneration of the mid-peripheral retina, leading to night blindness, visual field constriction and eventual loss of visual acuity. To date, there are no effective treatments for RP and it remains a medically challenging disease. About 15% of RP are autosomal-dominant forms caused by mutations in the pre-mRNA processing factors (PRPFs). PRPF8, PRPF31, PRPF3, PRPF4, PRPF6 and SNRP200-11. The PRPFs are components of the U4/U6.U5 tri-snRNPs (small nuclear ribonucleoprotein) subunit of the spliceosome, the large RNP complex that catalyses pre-mRNA splicing. Alternative pre-mRNA splicing expands the coding capacity of eukaryotic genomes by differential inclusion of exons or retention of introns in mRNA that enables a relatively small number of genes to encode a diverse proteome. High levels of splicing diversity occur in the vertebrate nervous system where it is required for neuronal development and function. Mouse rod and cone photoreceptors, for example, have a specific splicing programme that is initiated prior to the development of outer segments. This specific splicing programme primarily affects transcripts encoding components of photoreceptor primary cilia and outer segments, both of which are essential for phototransduction. Functional primary cilia are also required for neuronal development.

Mutations in PRPFs affect the stoichiometry and kinetics of spliceosome assembly, resulting in either transcriptional dysregulation of genes required for retinal function or mis-folding and aggregation of mutant PRPF proteins that trigger apoptosis in photoreceptors. However, the disease mechanisms for PRPFs-related RP remain unclear and it is uncertain whether RPE or photoreceptors are the primary affected tissue. Paradoxically, PRPFs are ubiquitously expressed, but mutations in these genes for PRPFs-related RP remain unclear and it is uncertain whether RPE or photoreceptors are the primary affected tissue. Furthermore, PRPF animal models either do not show retinal degeneration, raising the question of why retinal cells are more susceptible to degeneration than RPE or photoreceptors, in addition to its role in vasculogenesis, and shown to be important for the survival of Müller cells and photoreceptors, in addition to its role in vasculogenesis.

Results Derivation and characterisation of RP11-iPSCs. We ascertained three related RP type 11 patients with a PRPF31 c.522_527+10del heterozygous mutation (Supplementary Data 1). Disease severity was determined according to fundus examination, visual field and visual acuity, and took account of the age at the time of examination (Supplementary Data 1). Hereafter, all patients and derived cells are referred to as RP11 accompanied by M (moderate), S (severe) and VS (very severe). Three unaffected controls are referred to as WT1 (wild type), WT2 and WT3 (Supplementary Data 1). Dermal skin fibroblasts were reprogrammed to iPSCs using a non-integrative RNA-based Sendai virus (Supplementary Figure 1A). All RP11-iPSCs harboured the mutation identified in fibroblast samples (Supplementary Figure 1B-E), expressed pluripotency markers (Supplementary Figure 2A-B), were free of transgenes (Supplementary Figure 2C), and were genetically identical to parent fibroblasts (Supplementary Figure 2D) and clear of any genomic abnormalities (Supplementary Figure 2E). Both patient-specific and control iPSCs were able to differentiate into cells belonging to all three germ layers in vitro (Supplementary Figure 3A) and in vivo (Supplementary Figure 3B).

RP11-iPSCs of severe clinical severity were used to generate RPE and three-dimensional (3D) retinal organoids from iPSCs derived from four RP11 patients with variable clinical severity caused by two different PRPF31 deletion mutations. Large-scale transcriptome analyses identified mis-splicing of cell type and patient-specific target genes affected by PRPF31 mutations, providing unprecedented molecular characterisation of splice-factor RP clinical phenotypes. CRISPR/Cas9 correction of a PRPF31 mutation in cells derived from an RP11 patient with very severe RP, resulted in the rescue of molecular and cellular phenotypes, providing proof-of-concept evidence for the effectiveness of in situ gene correction.

RP11-photoreceptors have progressive degenerative features. We differentiated control and RP11-iPSCs into 3D retinal organoids (Fig. 2a), using an established method. Bright-phase neuroepithelium developed on the apical side of retinal organoids derived from RP11 patients and controls (Fig. 2b and Supplementary Figure 5A). By week 21, retinal organoids derived from RP11 patients and controls had a well-developed apical layer.
**Fig. 1** Characterisation of RP11 - RPE cells revealed polarity and functional defects. a Schematic of RPE differentiation timeline; b Bright-field images of iPSC-derived RPE: representative examples from at least ten independent experiments, scale bar 100 μm; c Immunostaining for basolateral markers BEST1 and Na⁺/K⁺-ATPase: representative images from three independent experiments, scale bar 50 μm; d Correct basolateral distribution of collagen IV (C-IV) and apical MERTK in unaffected control (WT3) but not RP11 RPE cells: representative images from three independent experiments, scale bar 50 μm; e, f ELISA assays for apical and basal secretion of PEDF and VEGF, respectively, in control and RP11 - RPE cells; g Trans-epithelial resistance measurements revealed a significant difference between patient and RP11 - RPE cells; h Reduced phagocytic capacity in RP11 - RPE cells. Statistical significance is calculated for MFI (mean fluorescence intensity) values. e–h Data shown as mean ± SEM, n = 3. Statistical significance of pairwise comparisons is indicated by n.s.: not significant; ***p < 0.001; ****p < 0.0001 (Student’s paired t test). b–h Data obtained from RPE at week 21 of differentiation.
**Fig. 2** Generation of retinal organoids following long-term suspension culture. 

**a** Schematic representation of iPSC differentiation to retinal organoids; 

**b** Bright-field images showing development of retinal neuroepithelium over time, scale bar 50 μm; 

**c** Immunostaining of retinal organoids showing the expression of cell-specific markers; 

**d**–**e** Representative examples from iPSC-derived retinal organoids from RP11S2 patient are shown, scale bar 25 μm apart from ARL13B, where scale bar is 10 μm; 

**d** TEM revealed the presence of outer limiting-like membrane (white arrows), inner segments (IS), connecting cilia (CC) and developing outer segments (OS) in retinal organoids after 21 weeks in culture, top panel scale bars: 10 μm, 500 nm, 500 nm, 10 μm, bottom panel scale bars: 5 μm, 2 μm, 500 nm, 500 nm; 

**e** At 43 weeks in culture, TEM showed that patient photoreceptors contained apoptotic nuclei with electron dense structures of condensed chromatin (white arrow) and stress vacuoles (black stars). 

**d**, **e** Representative examples of three independent experiments.
packed with photoreceptors (expressing RECOVERIN) with connecting cilia (expressing ARL13B; Fig. 2c), some of which also expressed NRL indicating a rod precursor phenotype. Müller glia, ganglion and bipolar cells were also present, indicated by CRALBP, HuC/D and PKCa expression (Fig. 2c). TEM of retinal organoids at week 21 revealed the presence of outer limiting-like membrane, inner segments, connecting cilia and developing outer segments in photoreceptors residing in the apical layer of retinal organoids (Fig. 2d). There were striking morphological differences between control and patient-derived cells. RP11 - retinal organoids had a 150% increase in cells with apoptotic nuclei compared to the controls (a total of 50 TEM sections analysed). In addition, unlike WT cells, RP11 cells had ’stress vacuoles’ (17% compared to the controls (a total of 50 TEM sections analysed). 

Organoids had a 150% increase in cells with apoptotic nuclei and stress vacuoles in RP11 patient-derived photoreceptors residing in the apical layer of retinal organoids (Fig. 2d). At week 43, TEM revealed the continuing presence of apoptotic nuclei and stress vacuoles in RP11 patient-derived photoreceptors, suggesting ’adaptive survival’ in response to environmental or oxidative stress (Fig. 2e).

At week 21 of differentiation, the 3D retinae were flattened down on multi-electrode arrays (MEAs) with the presumed ganglion cell layer facing down on the electrodes, in order to record action potentials generated by these cells. Measuring activity in retinal ganglion cells (RGCs) reflects the global network function of the organoid, similar to the retina, since RGCs carry the output signal to central visual areas in the form of spike trains. Control and RP11-retinal organoids had no difference in response to 8-Br-GMP (a membrane permeable analogue of cGMP), indicating that phototransduction responses, specifically Na+ influx similar to the inward dark current, were intact in photoreceptors (Supplementary Figure 5B, C & F). Control retinal organoids responded to the addition of the neurotransmitter GABA with an increased firing rate, but this response was significantly reduced for those derived from the very severe RP11 patient (Supplementary Figure 5D, E & G). GABA signalling emerges during very early development, and at that time it is depolarising and can induce spiking. Reduced responses to GABA therefore indicate the impairment of emerging functional neural networks in RP11 patients.

Impaired pre-mRNA splicing in RP11 RPE and retinal organoids. To better understand the impact of PRPF31 mutations, we performed semi-quantitative RT-PCR and western blot analysis of PRPF31 expression in primary fibroblasts, iPSCs and iPSC-derived RPE and retinal organoids. We observed and confirmed by Sanger sequencing the presence of nonsense-mediated decay (NMD)-insensitive long mutant (LM) and NMD-sensitive short mutant (SM) transcripts only in cells derived from RP11 patients with the c.1115_1125del11 mutation, but not in the control cells (Fig. 3a). PRPF31 expression levels were decreased more significantly in RP11-RPE cells (Fig. 3b), and this was further confirmed by western blot analysis (using an anti-PRPF31 C terminus antibody) (Fig. 3c, d). Interestingly, the PRPF31 LM isoform (detected by using an anti-PRPF31 N terminus antibody) was expressed only in RP11-RPE (Fig. 3c). Furthermore, RP11-RPE showed a substantial downregulation of SART1, a U5 snRNP protein important for the formation of the pre-catalytic spliceosomal B complex, but no changes in the expression of the U5 protein PRPF8 or the U4/U6 protein PRPF4 were observed (Fig. 3c).

To test if splicing efficiency was altered in patient-specific cells, we performed splicing assays following lentiviral transduction of an E1A minigene reporter with multiple 5’-splice sites. This can be alternatively spliced into at least five mRNAs (sizes 13S, 12S, 11S, 10S and 9S; Fig. 3e). Both RP11-RPE and retinal organoids had impaired alternative splicing of the E1A reporter, indicated by the accumulation of pre-mRNA and the decrease in the 9S and 10S isoforms in RPE and the 12S isoform in retinal organoids (Fig. 3f) compared to unaffected control and patient-specific RP11 fibroblasts and RP11-iPSCs. There were no differences in tri-snRNP stability for RP11 and control iPSCs, as determined by sedimentation of nuclear extracts on density gradients followed by detection of snRNAs (Supplementary Figure 6). However, RP11 retinal organoids had decreased expression of U4 snRNA (Fig. 3g) compared to controls, suggesting a likely reduced function of the U2-dependent spliceosome.

Disrupted splicing in cellular adhesion and cilia genes. To identify differences in transcription and splicing profiles between RP11 patients and unaffected control cells, we next performed large-scale transcriptome analyses in primary dermal fibroblasts, iPSCs, RPE and retinal organoids as biological triplicates from all subjects (Supplementary Data 2). We identified differentially expressed transcripts by using DESeq2 (Supplementary Data 2; threshold value \( p_{\text{adj}} < 0.05 \)). Of these differentially expressed genes were identified in iPSCs (\( n = 163 \)) and RPE (\( n = 59 \)) group comparisons, in contrast to fibroblasts (\( n = 1395 \)) and retinal organoids (\( n = 1367 \)). The most significant differentially expressed genes in RP11 retinal organoids (Supplementary Data 2) were enriched for Gene Ontology (GO) categories related to actin cytoskeleton, ciliary membrane, primary cilium, photoreceptor inner and outer segments, axon terminals and phototransduction (Supplementary Data 3). In RP11 fibroblasts, significant differentially expressed genes were enriched for lysosome and endosomal processes, focal adhesion, cell-substrate junctions and extracellular matrix organisation. There were no notable-enriched pathways in RP11-iPSCs and RP11-RPE.

Since RP11-RPE and retinal organoids had impaired pre-mRNA splicing (Fig. 3f, g), we next analysed transcripts in all four cell types for differential exon usage (skipped exons, retained introns, alternative 5’ and 3’ splice sites, and mutually exclusive exons; Supplementary Data 4) using rMATS software (threshold values \( p_{\text{adj}} < 0.05 \) and inclusion difference >5%). Differential exon usage analyses revealed that RP11-RPE had the highest level of transcripts with retained introns and alternative 3’ splice sites (Fig. 4a and Supplementary Data 4). GO enrichment analysis of biological processes for each cell type (Fig. 4b) showed that RP11 fibroblasts had significant differential exon usage for transcripts implicated in cilium formation categories (cilium assembly, cilium organisation, microtubule organising centre and centrosome; Supplementary Data 5). This suggests that PRPF31 has a role in fibroblast ciliogenesis and corroborates our previously published data on decreased cilia length and incidence in RP11 fibroblasts.34 In iPSCs, enriched GO biological processes included DNA recombination and DNA double-strand break repair, whereas enriched cellular components identified the centrosome, cilio and microtubule organising centre (Fig. 4b). RP11-RPE were enriched in genes implicated in cells-to-substrate adherens junctions and focal adhesions, and mitochondrial inner membrane, whereas RP11 retinal organoids were enriched for cilio and microtubule organisation (Fig. 4b and Supplementary Data 5).

Importantly, in both RP11-RPE and retinal organoids, the most significantly enriched GO biological process was pre-mRNA and alternative mRNA splicing via the spliceosome (Supplementary Data 5 and Fig. 4b), consistent with our observations (Fig. 3f, g) that PRPF31 mutations lead to impaired pre-mRNA splicing of key components involved in the splicing process itself. Specific human transcripts included those implicated in spliceosome assembly (e.g., SF1, SART1/Snu66 and DDX5), formation of the U4/U6 snRNP (LSM2), 3’-end processing of pre-mRNAs (CPSF1...
and U2AF1L4), association of U2 snRNP with pre-mRNA (DDX39B and PTBP1) and 5'-splice site selection (LUCL7). To validate some of these findings, we performed RT-PCR experiments in RPE and retinal organoids derived from RP11 and control iPSC samples (Fig. 4c). For this validation we selected key genes involved in cilia formation and/or outer segments of photoreceptors (RPGR, RPRGIP1L and CNOT3), intraflagellar transport (IFT122), actin filament organisation, centrosome and focal adhesion (SORBS1) and pre-mRNA 3'-end processing (CPSF1). As predicted by the rMATS analysis, the RP11 RPE
**Fig. 3** PRPF31 expression in patient-specific cells and effects on pre-mRNA splicing. a Gel electrophoresis showing the presence of a long mutant transcript (LM) isoform for the exon 11 deletion in patient-specific cells. The short mutant (SM) isoform is present only upon inhibition of NMD with puromycin (indicated by + ). b The bar graph shows wild-type PRPF31 mRNA in patient cells relative to controls from a, b. Data are representative of at least three independent repeats, RO retinal organoids; c Wild-type PRPF31 is significantly reduced in patient RPE cells and less notably in retinal organoids. The LM form and reduced SART1 is observed only in the patient RPE cells; d The bar graph shows wild-type PRPF31 levels in patient cells relative to normal cells quantified from c, n = 3; e, f Patient RPE cells and retinal organoids exhibit a notable defect in the alternative splicing of EIA minigene reporter. Schematic representation of alternative splice variants of the EIA reporter (e) and denaturing PAGE and autoradiography using a phosphorimager (f). n = 3, g Northern blot analysis showing the level of snRNAs in various normal and patient cells. Total RNA was isolated from each sample and snRNA levels were analysed by denaturing PAGE followed by Northern blotting using probes against U1, U2, U4, U5, U6 and 5S rRNA (top). The levels of snRNAs were quantified and normalised to the amount of 5S rRNA (bottom), n = 2. All error bars represent SEM.

**Fig. 4** RNA-seq analysis of alternative splicing in fibroblasts, iPSC, RPE, retinal organoids and Prpf31+/− retina. a nMATS analysis showing that RP11 - RPE have the highest percentage of transcripts containing retained introns (RI) and alternative 3′ splice sites (A3SS); b Gene Ontology enrichment analysis showing biological and cellular processes affected by alternative splicing, respectively, in human cells; c Gel electrophoresis of RT-PCR for the indicated genes in RPE and retinal organoids derived from patient RP11VS and unaffected control WT3. Sizes (in bp) for major and minor isoforms (arrowheads), and percentage-spliced-in (PSI) values, are indicated; d Sashimi plots for the indicated genes for validation of alternative splicing events in RPE and retinal organoids derived from RP11 patients (blue) and unaffected controls (red). Data are representative of at least three independent experiments. Green highlights in Sashimi plots indicate alternative splicing events with the number of junction reads indicated for each event; e, f Gene Ontology enrichment analysis showing biological and cellular processes affected by alternative splicing, respectively, in mouse Prpf31+/− retinæ and RPE. Data are representative of at least three independent experiments.
showed a significant change in alternative splicing of CPSF1 and CNOT3, while RP11 retinal organoids showed alternative splicing for RPRGR, RPRGRIP1L, IFT122 and SORB51 (Fig. 4d). These in vitro data were strongly corroborated by differential exon usage analyses of Prprf31+/− mouse retinae and RPE.20,21 The most significantly enriched GO processes and categories in Prprf31+/− mouse mutant compared to wild-type control retinae were for RNA splicing, mRNA processing and ribonucleoprotein complex biogenesis, as well as microtubules, cilium and centrosomes (Fig. 4e). Similarly, Prprf31+/− mouse mutant compared to wild-type control RPE were enriched for genes involved in mRNA processing and microtubules (Fig. 4f). These data suggest that disrupted alternative splicing programmes in RP11 result in exacerbation of splicing deficiencies, in turn disrupting specific biological processes that cause the unique cellular phenotypes observed in RP.

**RP11 - RPE and photoreceptors show ciliary abnormalities.** To analyse the role of PRPF31 in cilia formation and function, we first determined the extent of co-localisation of PRPF31 with an snRNP-specific marker (Y12, Supplementary Figure 7A) and a cilia-specific marker (ARL13b, Supplementary Figure 7B) in fibroblasts, iPSCs, RPE and photoreceptors. PRPF31 co-localised with both snRNPs and ARL13b for all patient cell types and controls, confirming PRPF31 localisation in both splicing complexes and cilia.

We then measured cilia length and incidence in RP11 - RPE cells using a combination of ARL13b and a basal body marker (pericentrin) that is located at the base of the cilia (Fig. 5a). Both cilia incidence and cilia length were significantly reduced in all RP11 - RPE cells when compared to controls (Fig. 5b). TEM analysis revealed the presence of long cilia with clearly aligned microtubules in control RPE cells, while RP11 - RPE cells had shorter, abnormal, bulbous cilia (Fig. 5c). Structural defects in axonemal microtubules were confirmed by serial block face scanning electron microscopy (SBFSEM, Fig. 5d). RP11 photoreceptors also had significantly reduced cilia incidence (Fig. 5e, f) and defective, bulbous cilia with misaligned microtubules (Fig. 5g) that was also confirmed by SBFSEM analysis (Fig. 5h).

To further confirm that loss of human PRPF31 negatively regulates ciliogenesis, we performed siRNA knockdown in the human ciliated retinal pigment epithelial hTERT-RPE1 cell line. Knockdown of PRPF31 protein levels caused a significant decrease in cilia incidence (Fig. 6a). Since SHH activity is known to require functional cilia, we confirmed that PRPF31 siRNA knockdown caused a dysregulated response to Smoothened agonist (SAG; Fig. 6b). To investigate possible defects in ciliary morphogenesis and structural organisation as a consequence of ciliary gene mis-splicing, we used structured illumination microscopy (SIM) to resolve the detailed localisation of proteins along the ciliary axoneme and at the transition zone (TZ). PRPF31 knockdown caused significant mislocalisation of IFT88 to the ciliary tip (Fig. 6c), and the TZ proteins CC2D2A and RPRGRIP1L were either entirely excluded from the TZ (Fig. 6d) or mislocalised from the TZ into the ciliary axoneme (Fig. 6e). Similar mislocalisation was also evident in RP11 - RPE (Fig. 6f, g).

**Correction of PRPF31 mutation restores molecular and cellular defects.** To further validate the function of PRPF31 in retinal cells, CRISPR/Cas9 genome editing was used to correct the PRPF31 c.11115_1125del11 genetic mutation in cells from the patient with the most severe clinical phenotype (RP11VS). For in situ gene correction, an ssODN template with wild-type PRPF31 sequences was designed with 91 bp homology arms on each side of the mutation region (Supplementary Data 6). Two hundred iPSC clones were selected and tested (Fig. 7a), and candidates identified by PCR were sequenced to confirm gene editing of PRPF31 (Fig. 7b). Quantitative RT-PCR analysis confirmed the increased expression of PRPF31 in the CRISPR/Cas9-corrected clone when compared to uncorrected iPSCs (Fig. 7c). We also excluded potential off-target effects (Supplementary Data 7) and CytoSNP analysis confirmed the identity to the parental cell line and lack of genomic abnormalities (Supplementary Figure 8). The CRISPR/Cas9 iPSC clone expressed pluripotency-associated markers Nanog and TRA-1-60, and gave rise to cells belonging to all three germ layers (Supplementary Figure 9).

The CRISPR/Cas9-corrected iPSC clone was differentiated to RPE and retinal organoids, in parallel with uncorrected RP11-iPSCs using our established protocols. Cilia length and incidence was significantly increased in both corrected RPE (Fig. 7d, e) and corrected photoreceptors (Fig. 7f, i). TEM analysis also revealed cilia with well-aligned axonemal microtubules in corrected cells that did not display the aberrant morphology observed in the RP11-derived retinal cells (Fig. 7f, m). Importantly, flow cytometry confirmed the rescue of photoreceptor capacity (Fig. 7g), suggesting an improvement of functional characteristics in corrected RPE. Immunostaining and cytokine secretion assays also revealed the restoration of cytokine secretion and basal collagen IV and apical MERTK expression, suggesting that corrected RPE apical – basal polarity was restored (Fig. 7b–j). These data indicate that in situ gene editing restored key cellular and functional phenotypes associated with RP type 11.

To further assess the impact of alternative splicing on protein abundance, quantitative proteomic analysis was carried out in RP11VS and CRISPR/Cas9-corrected RPE and retinal organoids using TMT labelling and mass spectrometry (Supplementary Figure 10A-F and Supplementary Data 8, 9). GO enrichment analysis for biological processes indicated that RNA metabolic processes, mRNA processing, RNA splicing, and both RNA and DNA metabolic processes, to be the most affected pathways in RPE and retinal organoids, respectively (Supplementary Figure 10A, D). Several components of the mRNA surveillance pathway (MSI2 and RNPS1), the PRP19 complex (PLRG1 and CTNNB1), the SF3a/SF3b complex (SF3A1 and SF3B4), spliceosomal tri-snRNP proteins (PRPF3, USP39, PRPF6 and DDX23) and SR proteins (SRSF1, 2, 5 and 6) were downregulated in either RP11 patient-specific RPE, retinal organoid cells or both (Supplementary Data 8, 9). The endoplasmic reticulum, nucleoplasm, ribonucleoprotein and spliceosomal complex were the most affected cellular components (Supplementary Figure 10B, D), corroborating the splicing deficiency highlighted by our RNA-Seq and splicing assays. Ten per cent of the differentially expressed genes and 10% of differentially spliced transcripts showed differential protein expression in RPE cells (Supplementary Figure 10F). Of the 49 differentially spliced transcripts and differentially expressed proteins, nine were associated with the ribonucleoprotein complexes and shown to be involved in pre-mRNA splicing, RNA binding and translation initiation, further corroborating the impact of PRPF31 mutations on the spliceosome complex. The impact of differential gene expression and exon usage was less pronounced in retinal organoids than in RPE cells (1.6% and 0.74%, respectively) (Supplementary Figure 10C). Of the 14 differentially spliced and expressed proteins, PRPF31 itself was identified, in addition to superoxide dismutase mitochondrial protein (SOD2) for which reduced expression has been linked to retinopathies. The latter was also significantly downregulated in mutant RPE cells. Collectively, the proteomic data suggest that differential splicing may play a more significant role in protein isoform generation in RPE when compared to retinal organoids. This data highlights key candidate genes and
Fig. 5 RP11 - RPE cells and photoreceptors have defective ciliogenesis and cilia morphology. a Immunostaining of RPE with cilia markers ARL13B (green) and pericentrin (red), with representative images shown from n = 3 independent experiments, scale bar 10 μm; b Quantification of cilia length and incidence showing significant reduction across both parameters in RP11 patients compared to the controls; c, d 2D TEM and 3D SBFSEM images showing shorter cilia in RP11 - RPE cells, with abnormal bulbous morphology, with representative images shown from n = 3 independent experiments, scale bar 500 nm (c), 1 μm (d); e Immunostaining of photoreceptors with cilia marker ARL13B (red), with representative images shown from n = 3 independent experiments; f Quantification of cilia length and frequency in photoreceptors showing significant reduction in RP11 patients compared to the controls; g, h 2D TEM and 3D SBFSEM images showing shorter cilia in patient-derived photoreceptors, with abnormal bulbous morphology, with representative images shown from n = 3 independent experiments, scale bar 500 nm (g), 1 μm (h). Data shown as mean ± SEM, n = 3. Statistical significance of the indicated comparisons is indicated by n.s. not significant; **p < 0.01; ***p < 0.001; ****p < 0.0001 (one-way ANOVA test with Dunnett’s post hoc test correction for multiple testing).
proteins that are affected by alternative splicing in RP11 retinal cells and deserve further investigation.

Discussion
Retinitis pigmentosa (RP) is one of the most common forms of hereditary progressive sight loss. Autosomal-dominant inheritance accounts for about 40% of RP, with an estimated 15% of cases of this RP inheritance type caused by mutations in pre-mRNA processing factors (PRPFs). PRPFs are ubiquitously expressed and involved in the formation of stable U4/U6.U5 tri-snRNPs and the spliceosomal B complex leading to spliceosome activation, yet human PRPF mutations result in retinal-specific phenotypes. Despite a large body of work in immortalised cell lines and animal models, there are no described cellular phenotypes for PRPF31-related RP type 11 that define the primary affected cell type or provide clear insights into the pathomechanisms that can explain the retinal specificity of phenotypes.
To gain insights into RP pathomechanisms, we characterised the cellular phenotypes and splicing programmes of RPE and retinal organs in comparison to fibroblasts and iPSCs derived from RP11 patients with PRPF31 mutations. Through large-scale transcriptome and biochemical analyses, we provide evidence that impaired in vivo splicing is restricted to patient-derived retinal cells only, and that impaired pre-mRNA splicing appears to be limited to splicing programmes that affect RNA processing itself (Fig. 4). These splicing defects appeared to be correlated with ultrastructural, cellular and functional deficiencies that are characteristic of RPE in the RP disease state. These include shorter microvilli and primary cilia, loss of polarity, reduced barrier characteristic of RPE in the RP disease state. These include shorter microvilli and primary cilia, loss of polarity, reduced barrier characteristic of RPE in the RP disease state. 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Fig. 7 Gene correction of PRPF31 mutation results in reversal of cellular and functional phenotypes in RPE and photoreceptors. a–c CRISPR/Cas9 correction of the PRPF31 deletion in exon 11; d, e Quantification of cilia length and incidence in PRPF31- and WT-RPE; f TEM analysis of PRPF31-edited RPE cilia showing morphologically normal cilia, scale bar: 500 nm; g increased phagocytosis in PRPF31-edited RPE; h, j Restoration of apical–basal polarity in PRPF31-edited RPE, scale bar: 50 μm. k, l Quantification of cilia length and frequency in PRPF31- and WT-photoreceptors; m TEM analysis of PRPF31-edited photoreceptor cilia showing morphologically normal cilia, scale bar: 500 nm. c–e, g–i, k, l Data shown as mean ± SEM, n = 3. Statistical significance of pairwise comparisons is indicated by n.s.: not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Student’s paired t test).
with previous studies. Nevertheless, the presence of PRPF31 can be ascribed to PRPF31 haploinsufficiency, which is consistent with previous studies. Nevertheless, the presence of PRPF31 mutant proteins in RPE suggests that the use of allele-specific antisense or morpholino oligonucleotides will be required as an additional treatment strategy to modify PRPF31 gene expression in order to more fully rescue all retinal phenotypes in splicing factor RP. However, there are important caveats to extrapolating disease modelling in iPSCs and derivatives to future preclinical studies of RP. In particular, cellular phenotypes of RP are detectable in the iPSC-derived retinal cell types weeks after differentiation, whereas the clinical phenotype in RP patients manifests as a late-onset condition. Part of this disparity may be explained by the observation of rapid premature senescence in RPE differentiated from iPSCs.

In conclusion, our data provide a detailed mechanistic explanation of retinal-specific phenotypes in PRPF31-mutated RP type 11 (summarised in Fig. 8) and, more generally, the characterisation of potential pathomechanisms during retinal degeneration. Our transcriptome data sets comprise a comprehensive catalogue of target genes affected by PRPF31 mutations. These delineate retinal-specific splicing programmes in the RP disease state, providing new insights into the contribution of mRNA processing to human disease.

**Methods**

**Human subjects.** All samples used in this study were obtained with informed consent according to the protocols approved by Yorkshire and the Humber Research Ethics Committee (REC ref. no. 03/362). Further information on the patients and controls is provided in Methods and in Supplementary Data 1.

**Animals.** The in vivo experiments using mice were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary. All procedures were performed to minimise suffering in accordance with the animal care rules in the institution in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**iPSC generation.** Three age-matched unaffected controls (WT1, WT2 and WT3) and four R11 dermal skin fibroblasts (RP11M, RP11S1, RP11S2 and RP11VS) were transduced at a density of 30,000 cells/cm² using the CytoTune®-iPS 2.0 Reprogramming Kit (Life Technologies, A16517) following the manufacturer’s instructions. iPSC colonies were established on inactivated primary mouse embryonic fibroblasts feeder layer and then adapted to the feeder-free system described below.

**iPSC culture.** Human iPSCs were cultured on six-well plates on Matrigel™ GFR (Corning, 354230)-coated wells with mTeSR1 (StemCell Technologies, 05850) medium supplemented with penicillin/streptomycin (Gibco, 15140). Cell culture medium was replaced on a daily basis. Cells were allowed to grow for 4–5 days prior to passaging or induction of differentiation. Passaging was carried out using Versene (EDTA 0.02%) (Lonza, BE17–771E) solution at 37 °C for 3–5 min and
cells were transferred to fresh matrigel plates in a 1:3–1:6 ratio. All cultures were maintained at 37 °C, in a humidified environment, with 5% CO₂. Cells were cryopreserved with freezing media containing 90% fetal bovine serum (Gibco, 10270) and 10% dimethyl sulfoxide (Sigma, D2650).

Detection of pluripotency markers by immunocytochemistry. iPSC colonies were fixed in 4% paraformaldehyde (Sigma, 47608) for 15 min at room temperature and permeabilised with 0.2% Triton X-100 (Sigma, T8787) for 40 min. Blocking solution was applied (10% FBS + 1% bovine serum albumin—Sigma, A33111) for 45 min at room temperature before proceeding with the addition of anti-human SSEA4 conjugated with Alexa Fluor® 555 (BD Biosciences, 560218, 1:200) and anti-human OCT4 primary antibody (R&D, AF1167, 1:200). Secondary staining was performed with the antibody anti-goat IgG with FITC (Jackson Immuno Research, 705-096-147, 1:500) diluted in blocking solution, followed by nuclear counterstaining with DAPI (Partec, 05-0050). Colonies were imaged using a Bioptechs Axiovert 200 microscope in combination with the associated Carl Zeiss software, AxioVision. All antibody details are shown in Supplementary Data 6.

Detection of pluripotency markers by flow cytometry. iPSCs were treated with 0.02% EDTA (Lonza, BE17-711E) for 3 min at 37 °C to dissociate the colonies. The suspension was collected in phosphate buffer saline (PBS) and centrifuged for 3 min at 300 g. Supernatant was removed and replaced with PBS with 0.1% BSA containing TRA-1-60-conjugated FITC (Merck Millipore, FCMAB115F, 1:60) and NANOG conjugated with Alexa Fluor® 647 (Cell Signaling Technology, 5448S, 1:150). Samples were incubated in the dark at room temperature for 60 min on a shaker. Cells were washed with PBS and resuspended in FACS buffer (PBS with 2% FBS). At least 10,000 events were analysed using a FACS Canto II flow cytometer. Results were analysed using the FACS Diva software.

In vitro and in vivo three germ-layer differentiation. iPSCs were detached from six-well plates (20–30 colonies per well) using 1 ml of 1 µg/ml Collagenase type IV (Gibco 17104–019) and 0.5 µg/ml Dispase II (Gibco, 17105–041) solutions. The colonies were dissociated, washed, resuspended in 0.1% BSA containing 20% fetal bovine serum (FBS) (Gibco, 10270), 1% penicillin/streptomycin (Gibco, 15140), 1% essential amino acids (Gibco, 11141), and added to a 10 cm Petri dish and media was changed every day. After 1 week, the embryoid bodies (EBs) were transferred to an embryoid body differentiation media, containing DMEM-F12 (Gibco, 11330), 20% FBS (Gibco, 17100), 10% N2 supplement (Gibco, 17200-041), 0.02% EDTA (Lonza, BE17-711E) for 3 min at 37 °C to dissociate the colonies. The suspension was collected in phosphate-buffered saline (PBS) and centrifuged at 400 g. Supernatant was removed and replaced with PBS with 0.1% BSA containing TRA-1-60-conjugated FITC (Merck Millipore, FCMAB115F, 1:60) and NANOG conjugated with Alexa Fluor® 647 (Cell Signaling Technology, 5448S, 1:150). Samples were incubated in the dark at room temperature for 60 min on a shaker. Cells were washed with PBS and resuspended in FACS buffer (PBS with 2% FBS). At least 10,000 events were analysed using a FACS Canto II flow cytometer. Results were analysed using the FACS Diva software.

Genomic DNA extraction. Genomic DNA was extracted from the pelleted cultures of the iPSC and corresponding parental fibroblast cell lines using the QIAGen DNA Mini Kit (Qiagen, 56304) following the manufacturer’s instructions.

Mutation screening. An aliquot of 10 ng of DNA from control and patients’ fibroblasts and iPSCs was amplified by standard PCR (40 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s) using primers described by Dong et al. for the specific mutations being looked for. The products were purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and quantified using the Quibit® 2.0 Fluorometer. The sequencing files were analysed in the Seqscape v.2.5 software and forward and reverse sequences from both fibroblasts and iPSCs were aligned and compared with the PRPF31 reference sequence (NC_009759.1) from GenBank to identify the PRPF31 mutations. The consensus sequences from the forward and reverse sequences were then extracted from the software and pairwise aligned against the coding PRPF31 sequence. Here the nucleotide designated as 1 commences at position 36 of GenBank accession number AL505369. All primer details are shown in Supplementary Data 6.

SNP array. DNA samples from the iPSCs and corresponding parental fibroblasts were analysed using the Illumina HumanCytoSNP-12 (Illumina, WG-320-2101) SNP array following the manufacturer’s instructions. The results were analysed using the BioNumerics Multi 4.3 software (Illumina, San Diego, USA).

iPSC differentiation to retinal pigment epithelium. iPSC colonies were grown to 80–95% confluence and all differentiation areas were removed. mTeSR®1 media was replaced with 2 ml of differentiation medium [Advanced RPMI 1640, 12633, GlutaMAX-1 (Gibco, 35050), Penicillin/Streptomycin (Gibco, 15140) and B-27 (Gibco, 17200)] supplemented with 10 µg/ml BSA (Sigma, A9641) and 10 ng/ml Noggin (R&D Systems, 6057-NG-025) from days 0 to 5. From days 6 to 9, only 10 ng/ml Noggin (R&D Systems, 6057-NG-025) was added to the medium. From days 10 to 15, the medium was supplemented with 5 ng/ml Activin A (PeproTech, 71-14-A) and from days 16 to 21, Activin A was replaced with 3 µl CMHR9004 (Sigma, SML10406). The cells were then fed every 2 days until the first RPE patches appeared, normally by week 4 of differentiation. RPE patches were mechanically picked and placed in TrypLE® (Invitrogen) for 30 min to dissociate the cells, agitated by gentle pipetting at 10, 20 and 30 min. Cells were collected and grown on 24-well plates or 0.33 cm² PET hanging cell culture inserts (Merck Millipore; Billerica, USA) coated with PLO/laminin (50 µg/ml) (Sigma-Aldrich, USA).

iPSC differentiation to retinal ganglion cells. The method for generating retinal ganglion cells from iPSC was based on a previously described protocol® with minor modifications. Briefly, iPSCs were dissociated into single cells using Accutase (Gibco, A110501). iPSCs were re-aggregated using low-cell adhesion 96-well plates with U-bottomed conical well (Lipidure® COAT Plates, NOF Corp.) at a density of 12,000 cells/well in mTeSR1 media supplemented with ROCK inhibitor (Y-27632, Chemdade, CD0141, 20 µM). After 48 h, the media was changed to

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mutation site. The online design tool (http://tools.genome-engineering.org) was used to design the sgRNA for PRPF31 mutation in the RP11VS iPSCs was achieved by using the CRISPR/Cas9 vector (pSpCas9(BB)-2A-Puro) following the protocol described previously46. Broad white light pulses (200 ms, 217 µW/cm²) were administered at 1 Hz in the dark. This process was repeated for a total of 5 min to achieve the desired effect. After each light pulse, the organoids were allowed to recover for 5 min in the dark.

**Phagocytosis assay.** Bovine rod photoreceptor outer segments (POS) (Invitrogen Biosciences, 98740) were centrifuged at 2600 x g for 4 min and the pellet was resuspended in 100 µl of advanced RPMI (AdRPMI) 1640 medium (12633, Gibco). The POS were incubated with 0.4 mg/ml FITC (Sigma, F7250) for 1 h at room temperature and agitated in the dark. The POS were centrifuged at 2600 x g for 4 min and washed three times with PBS (Gibco, 14190). Then, they were resuspended in AdRPMI 1640 (12633, Gibco) + B-27 Supplement (Gibco, 17504) + 10% fetal bovine serum (FBS) (Gibco, 10270) and the staining was confirmed under a Bioscience Axiovert microscope. RPE cells were treated with 1 x 10⁶ POS-FITC per cm² and incubated for 4 h at 37°C. For the control experiments, RPE cells were treated with the same number of non-stained POS and incubated for the same time.

**Transmission electron microscopy.** RPE and 3D optic cup samples were fixed in 1% osmium tetroxide, dehydrated in gradient acetone and embedded in epoxy resin. Ultrathin sections (70 nm) were picked up on copper grids, stained with uranyl acetate overnight and finally lead aspartate solution. Between each step the samples were rinsed thoroughly in several changes of deionised water. Samples were dehydrated through a graded series of acetone and then impregnated with increasing concentrations of Taab 812 hard resin, with several changes of 100% resin. The samples were embedded in 100% resin and left to polymerise at 60°C for a minimum of 36 h. The resin blocks were trimmed to ~0.75 mm by 0.5 mm and glued onto an aluminium pin. In order to reduce sample charging within the SEM, the block was painted with silver glue and spatter coated with a 5 nm layer of gold. The pin was placed into a Zeiss Sigma SEM incorporating the Gatan 3view system, which enables the observation of the sample without illumination in the SEM and provides images in the z-direction. Multiple regions of interest were imaged at x2000 magnification, 3000 x 1500 pixel scan, which gave a pixel resolution of ~15 nm. Section thickness was 50 nm in the z-direction. In the resulting z-stacks, clara, were identified and segmented manually using Microscopy Image Browser (MIB, University of Hel- sinki). The segmentations were imported into Amira (FEI) for construction of the 3D models.

**RPE cytokine secretion studies.** Medium from basal and apical chambers of transwell inserts were collected from RPE cells of healthy controls and patients. The levels of PEDF and VEGF secretion were measured by using human PDEF-ELISA Kit (Casabio, CSB-E08818h) and human VEGF-ELISA Kit (Life technologies KH0011) according to the manufacturer’s instructions.

**RPE characterisation by immunocytochemistry.** Cells were fixed in 4% paraformaldehyde (47608) for 15 min at room temperature and permeabilised with 0.25% Triton X-100 (Sigma, T8787) for 15 min, followed by treatment with blocking solution (3% BSA in PBS, Sigma, A3311) for 30 min at room temperature. Cells were treated with primary antibodies anti-bystrophin (Abcam, ab2182, 1,200), anti-potassium channel γ2 subunit (Alpha-Tau® 488 conjugate) (Abcam, ab21844, 1,200), pericentrin (Abcam, ab28144, 1,200), MERTK (Bethyl, A300-222A, 1,200), ARAL3B (Proteintech, 17711-1-AP, 1,500), collagen IV (Abcam, ab5866, 1,200), PRPF31 (Abnova, PAR7154, 1,500) and SNRPB monoclonal antibody (Y12) (ThermoFisher, MA-13449, 1,500) overnight at 4°C, and with secondary antibodies anti-rabbit FITC (Sigma, T9887, 1,500) or anti-mouse FITC (Jackson Immuno Research, 115-165-003, 1,500) or anti-rabbit Cy3 (Jackson Immuno Research, 115-165-003, 1,500) diluted in PBS for 1 h at room temperature. Washes with PBS were carried out between and after treatments. Finally, cells were treated with the blue counterstain (DAPI (PARTech, 05-0005) 5 mg/ml) using a NIS Airconfocal microscope in combination with the associated NIS Elements software. All antibody details are shown in Supplementary Data 6.
Immunofluorescence and microscopy of hTERT-RPE1 and RPE. Immunofluorescence staining was performed as described previously. hTERT-RPE1 cells were grown on 12-well plates and centrifuged. Coverslips with 20,000 cells were plated into each well of a 96-well plate and two rows each were reverse-transfected with 25 pmole of siRNA targeting PrP, Flt1, and a scrambled control using Lipofectamine RNAiMax. Twenty-four hours later, the cells were treated with 100 nM SAG (Cayman Chemical Company, 11914) or vehicle control for 48 h. The cells were then collected in 1XPLB buffer using the Dual Luciferase Reporter Assay system (Promega, E1910). The assays were run on a Berthold LB96 plate reader with dual injector system per the manufacturer’s protocol. Assay results were expressed as a ratio of firefly: Renilla luciferase activities in arbitrary units. The data were analysed by one-way ANOVA followed by Tukey’s multiple comparison test.

RNA sequencing. Total RNA was extracted from tissue using TRIzol (ThermoFisher Scientific Inc.). RNA samples were treated with a TURBO DNA-free Kit (Ambion Inc.) using the conditions recommended by the manufacturers, and then cleaned with a RNA Clean & Concentrator—5 spin column (Zymo Research Corp.). RNA was tested for quality and yield using a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer. RNA sequencing was performed for all patients and all controls as triplicate biological repeats in all cell types: fibroblasts, iPSC, iPSC-derived RPE and iPSC-derived retinal organoids. To minimise bubble PCR artefacts, we used 100 ng of purified total RNA in library preparation, following the TruSeq Illumina protocol. In brief, RNA was fragmented and used to prepare a single DNA library per sample. Raw data aligned to the human (Homo sapiens) full genome (GRCm38, UCSC mm10) using STAR, a splice-aware aligner. GT suffix annotation files were downloaded from Ensembl. Transcripts were analysed using STAR, followed by estimates of raw gene counts using HTSeq. Differential gene expression was analysed using DESeq2 with statistical significance expressed as a p value adjusted for a false discovery rate of 0.01 using Benjamini-Hochberg correction for multiple testing. Alternate splicing analysis was then carried out using rMAT5. For each comparison being made, we used the sorted BAM files produced by STAR to run rMAT5 on default unchanged parameters. Reported splicing changes were considered significant if they had a p value <0.05 and a change in inclusion-level difference of more than 5%. GO enrichment analysis was carried out on the genes found to have significant splicing changes via clusterProfiler. Multiple testing corrections were carried out using the Benjamini–Hochberg method with an adjusted p value <0.05, denoting significantly enriched gene ontology.

Production of lentiviral particles and transduction of cells. The minigene reporter encoding the adenovirus E1A transcript was subcloned from pmTE1A plasmid into the Pincl site of the pWPI lentiviral vector (Addgene; Trono lab). Lentiviral particles were produced in HEK293T cells grown in DMEM medium with 10% FBS. The cells were transfected with pWPI-E1A and the packaging plasmids pMD2.G (Addgene) and psPAX2 (Addgene) using PElpe transfection reagent (Polyplus transfection). After 54 h, the medium containing lentiviral particles was centrifuged at 1000 × g for 5 min and cleared using a 0.45 μm filter. The lentivirus was concentrated using Amicon Ultra 100 KD MWC0 centrifugal filter units (Millipore) and aliquots were stored at −80°C. For lentiviral transduction, cells were seeded in six-well plates with 2 ml medium and infected with the concentrated lentivirus in the presence of 8 μg/ml polybrene (Sigma). After 24 h, the culture medium was replaced, and 36 h later cells were washed with PBS and harvested.

E1A alternative splicing assays. Total RNA was extracted from cells transfected with the E1A lentivirus using a RNA extraction kit (Macherey Nagel). E1A alternative splicing was analysed by RT-PCR with 1 μg of the total RNA sample using the high-capacity cDNA reverse transcription kit (Applied Biosystems) and GoTaq DNA polymerase (Promega). PCR was performed with the 5'-end labelled exon 1 forward primer (5'-TTTTTCTCCCGACGGCTCCCG) and the exon 2 reverse primer (5'-TCCAGGCTCAGGTCACAGAGG) by using the following conditions: 95°C for 2 min, 30 cycles of 95°C for 15 s, 64°C for 30 s, 72°C for 1 min, and a final step of 72°C for 5 min. PCR products were separated by denaturing PAGE, visualised by autoradiography using a Typhoon Trio plus scanner (GE Healthcare) and quantified using Quantity One software (Bio-Rad).

Western blot analysis. Cells were washed with PBS and lysed in lysis buffer (40 mM HEPES pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phe- nylmethylsulfonyl fluoride, 1 mM sodium orthovandate and 0.5 mMDTT) supplemented with phosphatase and EDTA-free protease inhibitor cocktails (Roche). The concentration of total protein in cleared lysates was measured by Bradford assay and about 20 μg of each sample was analysed by
western blotting followed by immunostaining using antibodies against SART1, PRP6F, Smn1u14, PRPF31 (against its N terminus or C terminus), PRPF4 and SF3B155 using the Amersham ECL detection kit (GE Healthcare). All antibody details are shown in Supplementary Data 6. Uncropped blots are shown in Supplementary Figure 1.

Analysis of snRNP levels by glycerol gradient fractionation. Nuclear extracts (200 µg each) were diluted with an equal volume of buffer G (150 mM HEPEs pH 7.9, 150 Mm NaCl, 1.5 mM MgCl2, and 0.5 mM DTT) and sedimented on linear (30%–10%) glycerol gradients in the G150 buffer. After ultra-centrifugation in a Sorval TH-66 rotor for 14 h at 29,000 rpm (114,000 xg), the gradients were separated into 24 fractions. To analyse the relative levels of snRNPs in the nuclear extracts, proteins in the gradient fractions were digested by Proteinase K in 20 mM HEPEs pH 7.9, 150 mM CaCl2, 10 mM EDTA, 1% (v/v) SDS for 45 min at 42°C, the RNAs were extracted by phenol/chloroform/isoamylalcohol and pre-cipitated. The isolated RNAs were separated by denaturing 8% urea PAGE followed by Northern blotting using 5'-end radiolabeled DNA probes against U1, U2, U4, U6 and U5 snRNAs. To analyse the association of selected splicing proteins with the tri-snRNP, proteins were precipitated from gradient fractions and separated on NuPAGE 4–12% Bis-tris gel (SDS) followed by blotting and immuno-staining using antibodies against PRP6F, Brr2, Smn1u14, PRPF31 (against its C terminus), PRPF4 and SF3B155, and the Amersham ECL detection kit (GE Healthcare). All antibody details are shown in Supplementary Data 6.

TMT labelling for mass spectrometry. Total cell lysates were prepared from 1 million RPI1Vs retinal organoid or RPE cells and the corresponding Cas9-corrected cells according to the protocol described for Pierce Mass Spec Sample Prep Kit (Thermo Scientific). Lysates were diluted to 130 µl and sonicated using Covaris S220 ultrasonicator (Covaris). Protein concentrations were determined using the Pierce BCA protein assay kit and 100 µg of the total proteins from patient or Cas9-corrected control cells were processed for isobaric tandem mass quantification (TMT) labelling using TMTDplex Isobaric Mass Tagging Kit (Thermo Scientific) according to the manufacturer’s instructions. Briefly, samples were reduced by the addition of TCEP, alkylated with iodoacetamide and acetone precipitated. Protein pellets were resuspended in 50 mM TEAB (triethylammonium bicarbonate) buffer followed by digestion with trypsin overnight at 37°C. The patient and Cas9-corrected control samples, respectively, labelled with TMT-127 and TMT-126 reagents for 1 h at room temperature and the reactions were quenched by 5% hydroxylamine for 15 min. Next, 50 µg of TMT-labelled peptides from patient and control cells were combined and cleaned up using C18 spin columns (Harvard Apparatus). The samples were dried down by SpeedVac (Eppendorf) and reconstituted in 100 µl buffer A (10 mM NH4OH).

Fifty microliters of peptide mixtures were separated in 80 fractions on an XBridge BEH C18 HPLC column (150 mm x 1 mm ID, 3.5 µm; Waters) using a gradient of 0.1% formic acid in H2O. The peptides were eluted with buffer B (80% acetonitrile) over 90 min. The elution fractions were combined to 20 fractions, dried down by SpeedVac and resuspended in 20 µl of 0.1% trifluoroacetic acid (TFA).

LC/MS/MS analysis. Peptides in each fraction were analysed in three replicates on either an Orbitrap Fusion or a Q Exactive HF–X mass spectrometer (Thermo Fisher Scientific), both of which are equipped with a Ultimate 3000 RSLcQnano HPLC system (Thermo Fisher Scientific). First, the peptides were desalted on a reverse phase C18 pre-column ( Dionex 5 x, 0.5 mm ID) for 3 min. After 3 min, the pre-column was switched to the analytical column (1 cm long, 75 µm ID) prepared in-house using Reprofl-Pur C18 AQ 1.9 µm reversed phase resin (Dr. Maisch GmbH). Solvent A consisted of 0.1% formic acid in H2O, and B consisted of 80% acetonitrile and 0.1% formic acid in H2O. The peptides were eluted with buffer B (8–42% gradient) at a flow rate of 300 nL/min over 70 min. The pre-column and the column temperature were set to 50°C during chromatography. On the Orbitrap Fusion, a data-dependent method was applied to improve the accuracy and sensitivity of peptide identification. The false discovery rate (FDR) was set to 1% at both the peptide spectrum match (PSM) level and the protein level, respectively, using Mascot Percolator and a built-in Protein FDR Validation tool on PD. Quantitative measurement was based on relative abundance of the detected TMT reporter ions in MS3 or MS2 spectra for raw files from the Orbitrap Fusion or Q Exactive HF–X, respectively. At least two quantifiable unique peptides in each replicate were required for protein quantification. Protein ratios were log transformed and then median normalised based on the assumption that the majority of the proteins are unaffected. The reported RPI1V5/Cas9–RPI1V5 ratios are the average of at least two replicates. To identify the differentially regulated proteins, the corresponding Z scores were calculated and those proteins with Z scores less than –1.5 or greater than +1.5 were defined as regulated. 1D annotation enrichment analysis was carried out by the Perseus software version 1.6.1.3 with a Benjamini–Hochberg FDR 25%.

Quantification and statistical analysis. P values were calculated of normally distributed data sets using a two-tailed Student’s t test, or one-way ANOVA with Dunnett’s post hoc test, or two-way ANOVA with Bonferroni post hoc tests using GraphPad Prism Software Inc. (San Diego, CA, USA). Statistical analyses represent the mean of at least three independent experiments, error bars represent standard error of the mean (S.E.M.) or as otherwise indicated. The statistical significance of pairwise comparisons shown on bar graphs is indicated by n.s. not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. For cell populations, a minimum of 100 cells were counted from >10 separate fields of view.

Data availability
The trimmed FASTQ data for all human samples were uploaded to the European Nucleotide Archive under the accession number PRJEB22885 (human) and PRJNA471002 (mouse). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE38 partner repository with the dataset identifier PXD010821.

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