**Sequence-Specific Suppression of Alleles Causing Dominantly Inherited Retinal Degeneration Using the RNA-Guided Nuclease Cas9**

The Harvard community has made this article openly available. **Please share** how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:27007731">http://nrs.harvard.edu/urn-3:HUL.InstRepos:27007731</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

1 ABSTRACT .................................................................................................................................4

2 GLOSSARY ...............................................................................................................................5

3 BACKGROUND ..........................................................................................................................6

   3.1 Brief History ..........................................................................................................................6
   3.2 State of Gene Therapy Today ...............................................................................................6
   3.3 Applications in Ophthalmology ...........................................................................................7
   3.4 CRISPR-Cas9 Technology ....................................................................................................8
   3.5 Purpose of Inquiry ................................................................................................................9

4 MATERIALS AND METHODS .................................................................................................10

   4.1 Patient Selection ..................................................................................................................10
   4.2 Cell Culture ........................................................................................................................10
   4.3 pCAG-EGxxFP Plasmids with Targets and COS-7 Cell Transfection ..............................11
   4.4 Lentivirus Production and Transduction of Fibroblasts ......................................................11
   4.5 Next-Generation Sequencing and Indel Analysis ..............................................................12
   4.6 Off-target Analysis by Surveyor Assay ............................................................................12

5 RESULTS .....................................................................................................................................13

   5.1 Principles of Allele-Specific Targeting ..............................................................................13
   5.2 Cas9 Cleavage of pCAG-EGxxFP Target Plasmids in COS-7 Cells .................................14
   5.3 Validation of Sequence-Specific Cas9 Cleavage in Primary Patient-Derived
       Fibroblasts ..............................................................................................................................15
       5.3.1 Verification of Controls in the Experimental Design ....................................................15
       5.3.2 Allele-Specific Silencing in Primary Patient-Derived Fibroblasts ............................16
       5.3.3 Subanalysis of Indel Formation Rates in Known WT and Mutant Reads ..............17
   5.4 Off-target Analysis in Fibroblasts Using the Surveyor Assay ......................................17

6 DISCUSSION .............................................................................................................................18

   6.1 Allele Specific Targeting In Patient Dermal Fibroblasts ................................................18
1. ABSTRACT

**Background:** Dominant mutations account for a substantial proportion of inherited retinal degenerations (IRDs) through haploinsufficiency or gain-of-function (GOF) effects. This work explores a potential strategy to treat diseases caused by dominant GOF mutations by specifically suppressing the function of the mutant allele at the genetic level using the CRISPR/Cas9 system. After taking into account the likelihood of allele pathogenicity, we identified six targets suitable for this form of genome editing therapy, including mutations in the *RHO, BEST1, IMPDH1, SNRNP200, PRPH2,* and *PRPF8* genes.

**Methods:** Cas9 single-guide RNAs (sgRNAs) for target sequences were tested in COS-7 cells with the pEGxxFP assay. Cas9-sgRNA plasmids were co-transfected with reporter plasmids containing either the mutated allele target sequence or the wildtype sequence of the identified genes and the efficiency of Cas9 cleavage was quantified by FACS. Using this methodology we identified allele-specific guides for *BEST1, PRPF8,* and *PRPH2.* The rate of allele-specific targeting by Cas9 was then assessed for each sgRNA in patient-derived and control fibroblasts by next-generation sequencing (NGS).

**Results:** In patient fibroblasts, we saw up to 16.3% indel formation for *PRPF8,* 14% for *BEST1,* and 15% for *PRPH2.* The low rates of indel formation in wildtype alleles (<2.5%) suggest specific targeting of pathogenic mutations. In addition, 76-94% of the total indels formed were out-of-frame in nature, a promising sign that Cas9 cleavage is likely to produce a loss or insertion of bases that disrupts the coding sequence.

**Conclusions:** This study demonstrates that CRISPR genome editing has single base specificity and can be used to selectively knock down mutant alleles even when the mutation does not create a new protospacer adjacent motif (PAM) sequence. The RNA-guided Cas9 nuclease is a potentially useful tool for targeted therapy in IRDs. This work is a proof of concept that genome editing tools could be used to provide long-term suppression of GOF alleles to treat dominant genetic diseases.
2. GLOSSARY

ADA – Adenosine deaminase
Cas9 – CRISPR associated 9
COS-7 – African green monkey kidney fibroblast-like cell line
CRISPR – Clustered regularly interspaced short palindromic repeats
DSB – Double-stranded break
FDA – U.S. Food and Drug Administration
Indels – Small insertion or deletion
IRD – Inherited retinal degenerations
GOF – Gain-of-function
HDR – Homology-directed repair
HEK – Human embryonic kidney cell line
LCA – Leber congenital amaurosis
MEEI – Massachusetts Eye and Ear Infirmary
NGS – Next generation sequencing
NHEJ – Nonhomologous end joining
PAM – Protospacer adjacent motif
pCAG-EGxxFP – Reporter plasmid used in this study; 500-600 basepairs of target genomic DNA can be inserted into the middle of the EGFP expression cassette
SCID – Severe combined immunodeficiency
sgRNA – Single-guide RNA
SNP – Single nucleotide polymorphisms
TALENs – Transcription activator-like effector nucleases
ZFNs – Zinc finger nucleases
3. BACKGROUND

3.1 Brief History

Gene therapy holds tremendous potential for genetic diseases for which no effective treatment exists. The FDA defines gene therapy as an intervention aimed at modifying genetic material of living cells to produce therapeutic effects. This is achieved by replacing or suppressing the mutated gene in question within the affected cells. While the concept seems simple, researchers have faced many obstacles to producing effective gene therapy treatments for patients, including cellular and tissue barriers, efficient delivery mechanisms, and safety and ethical aspects. Nevertheless, on September 14, 1990, the FDA approved the first gene therapy clinical trial in the US aimed at achieving a therapeutic outcome in patients, and two children with SCID caused by adenosine deaminase (ADA) deficiency became the first recipients of gene therapy.\(^1\) White blood cells were extracted from the two patients and modified ex vivo to correct the mutation in the nonfunctioning ADA gene. These cells with the modified genes were implanted back into patients. Although the therapeutic effects of these early trials were equivocal, the field of gene therapy garnered much international attention. However, the tragic death of 18-year-old Jesse Gelsinger in 1999 from a high dose of adenovirus administration\(^2\) led a halt in progress for many years.

3.2 State of Gene Therapy Today

Since then, the field of gene therapy has made slow but important advances. Several phase I and II clinical trials have reported clear positive results, showing efficacious and safe treatment of a number of different inherited diseases, including β-thalassemia, hemophilia B, B-cell lymphoma, and Type 2 Leber congenital amaurosis (LCA).\(^3\)

Therapeutic effects in gene therapy clinical trials are often achieved by exposing affected cells or tissues to viral vectors that stably express a transgene to replace a non-functional gene product. For gene therapy treatment to be successful, the genetically modified cells must be present in large enough quantity to prevent or reverse the disease in question. The necessity of obtaining a large number of stably gene-corrected cells has limited gene therapy successes, mainly to diseases where 1) a selective advantage exists for gene corrected cells or 2) the
affected tissue can be efficiently transduced with safe vectors. This requirement has limited the success of gene therapy mainly to eye, liver and hematopoietic disorders. However, with the improved efficacy and safety of new vectors, a wide range of indications is now being tested for gene therapy. Currently, nearly 2,000 gene therapy clinical trials exist worldwide targeting an extensive range of diseases.  

3.3 Applications in Ophthalmology

Gene therapy for ophthalmologic disorders is of tremendous interest due to the availability of a number of vectors that effectively transduce cell types affected by genetic eye disorders. Furthermore, the immune privilege property of the eye also keeps the delivery vector or transgene product from disseminating systemically and the body from mounting an immune response, thus reducing the risk of adverse systemic effects.

In particular, this thesis focuses on inherited retinal degenerations (IRDs), a group of disorders usually caused by single gene defects. IRDs affect more that 2 million people worldwide and display broad genetic and phenotypic heterogeneity. This makes IRDs particularly difficult to treat with conventional therapies. Mutations in over 200 genes have been linked to IRDs, with dominant mutations accounting for approximately one-third of cases (RetNet: http://www.sph.uth.tmc.edu/RetNet/), some of which are SNPs resulting in toxic gain-of-function (GOF) effects. Today, clinical trials aimed at treating RPE65-associated LCA (or Type 2 LCA), Usher syndrome Type 1B, Stargardt’s disease, and choroideremia are already underway. Results from the RPE65-associated LCA clinical trials have shown stable improvement in visual sensitivity in patients for up to three years after the initial treatment.  

Despite exciting progress for treating recessive loss-of-function mutations with gene therapy, diseases caused by dominant gain-of-function mutations has eluded treatment with genetic therapies. Dominantly inherited genetic diseases are caused by either GOF mutations that result in a toxic gene product or loss of function mutations that result in haploinsufficiency. GOF mutant alleles produce gene products with dominant negative or toxic functions. In these cases, ablating the mutant gene’s expression is necessary for treatment, and gene augmentation will not produce a therapeutic benefit. In such cases, it would be ideal to selectively ablate the function of the mutant gene while preserving the function of the wildtype
allele to ensure proper gene function following treatment. For instance, Huntington’s disease is an autosomal dominant disorder caused by an abnormal CAG expansion within the HTT gene. Studies have shown that heterozygous polymorphisms in cis with the mutation can be used to target the mutant mRNAs through RNA interference (RNAi), thus selectively knocking down the function of the mutant allele while sparing wildtype expression.20

Allele specific silencing at the protein level has already proven to be effective. Small molecule therapies—such as vemurafenib for melanomas with V600E mutations in BRAF21–23 and imatinib for the BCR-ABL fusion protein in chronic myeloid leukemia24,25—have been successful in treating disease by selectively suppressing the function of the pathogenic allele at the protein level. However, identifying small molecules with specific and potent activity against pathogenic proteins are difficult to create, often requiring years of drug development. An alternative therapeutic strategy is suppression of mutant gene expression at the RNA or DNA level. RNAi is the primary method of suppression at the RNA level, but this approach is temporary and often lacks specificity and potency.15,26 Suppression of pathogenic alleles at the DNA level would be permanent and with the advent of genome editing technologies with simple design rules, applicable to a wide range of GOF mutations.

3.4 CRISPR/Cas9 Technology

The field of genome editing technology has seen rapid progress in the recent years, especially through programmable site-specific nucleases such as zinc-finger nucleases (ZFNs),27–29 transcription activator-like effector nucleases (TALENs),30,31 and the Cas9 nuclease from the microbial CRISPR (clustered regularly interspaced short palindromic repeats) system.32–34 The discovery of the CRISPR/Cas9 bacterial immunity system has had a particularly profound impact on many fields engaged in scientific research, stimulating new approaches in gene therapy and the development of new disease models. This system has been shown to be a powerful new technology for editing mammalian genomes.33,34 Cas9 is targeted to specific locations in the genome using a single-guide RNA (sgRNA) that binds and cuts a complementary DNA target sequence that is adjacent to a sequence motif called the protospacer adjacent motif (PAM). Cas9 targeting occurs via Cas9 protein-DNA interactions with the PAM and Watson-Crick base-pairing of 20 nucleotides (nt) of the sgRNA with complementary DNA. Thus, re-
targeting of Cas9 to new target sequences can be achieved through changing the 20-bp sgRNA sequence to match a new target, provided that the new target is also adjacent to a PAM sequence. Compared with the previous genome editing technologies, ZFNs and TALENs, CRISPR boasts the advantages of ease of customization, a predictable cleavage pattern, and high editing efficiency.\textsuperscript{35}

After Cas9 finds its target, the nuclease produces a double-stranded break (DSB) in the genomic DNA. Upon cleavage, the DNA can undergo one of two repair pathways, the error-prone NHEJ or the high-fidelity HDR. In post-mitotic cells such as photoreceptors, NHEJ is the main DNA repair mechanism. NHEJ is an error-prone repair process and frequently results in a small deletion or insertion (indels) at the site. Thus, we can take advantage of this property to selectively edit the mutant allele while leaving the wildtype allele intact, hopefully resulting in knock down of only the toxic allele.

### 3.5 Purpose of Inquiry

In this work, we validate a strategy to treat diseases caused by GOF mutations by specifically suppressing the function of the toxic allele at the genetic level. We hypothesize that this could be achieved through 2 mechanisms: 1) by creating sgRNAs that perfectly basepair with GOF alleles created by SNPs but contain mismatches with the wildtype allele, thereby favoring suppression of the mutant allele, and 2) by targeting mutations that create a new PAM, which is required for cleavage and therefore would ensure only targeting of the mutant allele.

We demonstrate that both of these mechanisms can be used to achieve allele specific targeting and highlight the broad utility of this approach for treating genetic disorders caused by GOF mutations. For this proof-of-concept study, we targeted GOF SNP mutations causing IRDs. We envision that CRISPR-Cas9 allele specific silencing could one day be used with gene therapy vectors as a treatment for this group of devastating diseases.
4. MATERIAL AND METHODS

4.1 Patient Selection

We identified a subset of mutations with a dominant inheritance pattern from our in-house IRD cohort database. The database contains detailed clinical information, DNA samples, and any pathogenic mutations identified for nearly 10,000 IRD patients and families collected over the last four decades in the Berman-Gund Laboratory for the Study of Retinal Degeneration at Massachusetts Eye and Ear Infirmary (MEEI). After taking into account the likelihood of allele pathogenicity using the SIFT/PolyPhen prediction tools and proximity of the mutation to an NGG PAM sequence of S. pyogenes Cas9, we identified six targets for genome editing therapy, including mutations in the RHO, BEST1, IMPDH1, SNRNP200, PRPH2, and PRPF8 genes. A literature search also supported the potential gain-of-function mechanisms of the identified alleles. Patients with autosomal dominant IRDs were seen at MEEI and consented to the study. Skin biopsies were collected from patients for the establishment of fibroblast cell lines (cell lines initiated by the CHGR DNA and Tissue Culture Resource, Massachusetts General Hospital, Boston, MA, USA). The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of Massachusetts Eye and Ear Infirmary and Harvard Medical School.

4.2 Cell Culture

African green monkey kidney fibroblast-like (COS-7) cell line (Life Technologies, Carlsbad, CA, USA) and human embryonic kidney (HEK) cell line 293FT (Life Technologies) were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT, USA).

Patient fibroblast cell lines were maintained in DMEM with Nutrient Mixture F-12 (DMEM/F12) (Life Technologies) supplemented with 15% FBS, 100 U/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies). Selection was achieved with 0.25 µg/ml puromycin (Life Technologies). All cells were incubated at 37°C with 5% CO₂.
4.3 pCAG-EGxxFP Plasmids with Targets and COS-7 Cell Transfection

*Strep pyogenes* Cas9 was used (pX459, Addgene 48139). Cas9 sgRNAs were tested for allele-specific targeting in COS-7 cells using pCAG-EGxxFP (Addgene 50716) containing either the mutated allele target sequence or the wildtype sequence of the candidate genes (pEGx-target-xFP). Briefly, in the middle of the EGFP, there is a multi-cloning site flanked by regions of homology. We inserted 500-600 bp of genomic sequence containing either mutant or wildtype target into the site (primers for PCR amplification of products included in Supp Table S2). If the target sequence is cleaved by the Cas9 endonuclease, homologous recombination or single strand annealing takes place to reconstitute the EGFP expression cassette. All experiments were carried out in triplicates and repeated three times each. Using this method, we hoped to quickly assess the strength of each guide in cutting and whether it can select for the mutant over wildtype allele.

COS-7 cells were seeded onto 12-well plates (Corning, Corning, NY, USA) 24 h before transfection. The cells were transfected using Lipofectamine 3000 (Life Technologies) at 70-90% confluency. In each well, 2.25 µg of Cas9+sgRNA plasmid and 0.25 µg of pEGx-target-xFP sequence plasmid were used. For the negative controls, 2.25 µg of “empty” Cas9 pX459 with 0.25 µg of pEGx-target-xFP sequence plasmid were transfected. 48 h after transfection of COS-7 cells, the efficiency of Cas9 cleavage was quantified by FACS.

The experiments were carried out in triplicates and repeated three times. However, we found that the transfection of cells with the plasmid with target alone (without Cas9) resulted in background fluorescence. Therefore, in our analysis we compared the percentage of GFP+ cells from transfections with Cas9, sgRNA and pEGx-target-xFP to percentage GFP+ from transfections with only pEGx-target-xFP.

4.4 Lentiviral Production and Transduction of Fibroblasts

Dermal fibroblasts were obtained from patients carrying the mutations, *PRPF8* [p.(Arg1935His)], *BEST1* [p.(Lys30Arg)], and *PRPH2* [p.(Phe211Leu)], and from normal controls. *EMX1* was included as a control for transduction efficiency in control and patient fibroblasts.
The production of lentiviruses and transduction of fibroblasts were achieved according to a previously described protocol. Briefly, sgRNAs targeting PRPF8, BEST1, and PRPH2 alleles were cloned into the lentiCRISPR-Puro vector (Addgene 52961) for generation of viral particles carrying the Cas9-sgRNA system. 293FT cells were seeded onto 15 cm cell culture dishes (Corning) and transfected at 80-90% confluency with lentiCRISPR-Puro vector, pMD2.G, and psPAX2 plasmids (Addgene 52961, 12259, and 12260, respectively) for lentiviral packaging and production using Lipofectamine 3000 (Life Technologies). The transfection mixture was aspirated 6 hr later and replaced with fresh DMEM + 10% FBS. Viral particles were harvested 48 hr later and stored at -80°C.

Patient-derived and control fibroblasts were infected with each single sgRNA virus at multiplicity of infection (MOI) ~0.3 and collected after 5 days in culture. Selection was achieved with 0.25 μg/ml puromycin (Life Technologies). Genomic DNA was extracted using the Allele-In-One Mouse Tail Direct PCR buffer (Allele Biotechnology, San Diego, CA, USA). The rate of allele-specific targeting by Cas9 was then assessed by next-generation sequencing (NGS). PCR amplification of the target region was performed on genomic DNA using HOT FIREPol DNA polymerase (Solis Biodyne, Tartu, Estonia) and primers (Supp. Table S3).

4.5 Next-Generation Sequencing and Indel Analysis
Deep sequencing of PCR amplicons was performed by the Sequencing group of the CCIB DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). Illumina sequencing reads were mapped to reference sequences using Burrows-Wheeler Aligner with custom scripts. Insertions and deletions were called against reference according to a previously described method.

4.6 Off-Target Analysis by Surveyor Assay
Modification rates at the top 3-4 exonic off-target sites were evaluated by the Surveyor assay. The methods for identifying potential off-target sites for each allele were based on Watson-Crick base-pairing mismatch between sgRNA and target DNA, as previously described. PCR was performed using off-target primers presented in Supp. Table S3 under the following conditions: 95°C for 5 min; 35 × (95°C for 30 s, 60°C for 30 s, 72°C for 60 s); 72°C for
5 min; hold at 4°C. PCR products were then denatured to 95°C, annealed slowly at -0.1°C/s to 16°C, and treated with Surveyor nuclease (Integrated DNA Technologies, Coralville, IA, USA). DNA concentration of each band was measured on a SYBR Safe (Life Technologies) stained 3% agarose gel in Tris-acetate-EDTA (TAE) buffer and quantified using BioRad Gel Doc XR+ System. Indel formation rate was calculated from the relative band intensities using the formula, $100 \times \left(1 - \frac{1}{(a + b + c + \ldots)}\right)^{1/2}$, where $a$ represented the band intensity of the undigested PCR product, and $(b + c + \ldots)$ the intensities of cleavage products.

5. RESULTS

5.1 Principles of Allele-Specific Targeting

We speculated that for dominant genetic disorders where gain of function mutations cause disease, the selective generation of loss of function mutations in the pathogenic allele might prevent or reverse disease progression. Patients with dominant diseases are largely heterozygous for such pathogenic GOF mutations. The two alleles often differ by a small number of bases, which we hypothesized could be exploited by the specificity of the CRISPR-Cas9 system to selectively target the mutant allele for mutagenesis. In principle, there are two mechanisms by which allele-specific targeting could be achieved. If the mutation is a SNP or a small indel proximal to a PAM, one could design an sgRNA that has perfect complementarity to the mutant allele and therefore contains one or more mismatches to the wildtype sequence. If the mismatch is sufficient to prevent efficient targeting of the wildtype allele, DSBs would selectively form on the mutant allele, leading to the formation of indel mutations via repair with NHEJ that would convert the gain of function allele into a loss of function variant (Figure 1). The net result is that only the wild-type protein will be functional in target cells.

Allele-specific targeting could also be achieved if a GOF mutation creates a new PAM. This would allow selective cleavage via favorable protein-DNA interactions between the PAM-interacting (PI) domain of Cas9 and the PAM sequence present on the mutant allele.$^{32}$

We used 2 criteria to select mutations to test the feasibility of allele specific silencing of dominant GOF mutations for IRDs. First, we selected alleles from our IRD patient database.$^{36}$
that are likely to act in a dominant GOF manner to cause disease. The mechanism of allele pathogenicity was inferred from analysis with SIFT/PolyPhen and a literature review when possible. Second, we selected alleles with mutations within 10 bp 5’ of the NGG PAM of *Streptococcus pyogenes* Cas9, where studies have shown that mismatches significantly decrease targeting efficiency, to maximize selective targeting of the mutant allele. The six alleles that fit these criteria are presented in Table 1 [the mutations in *IMDPDH1* (c.962C>T) and *PRPF8* (c.5804G>A) are novel and published for the first time here]. With the exception of the *BEST1* allele, all the single nucleotide mutation falls within the first 7 bp upstream of the PAM sequence. For *BEST1* (c.89A>G), the mutation lies within the PAM sequence, transforming the weak NAG PAM to the stronger NGG PAM.

For each candidate allele, we designed three sgRNAs (Figure 2). All sgRNAs were constructed taking into account observations from Cas9 specificity studies where the effects of single and double sgRNA:DNA mismatches were systematically tested through mutation of the sgRNA. We exploited the observation that certain single-bp mismatches, especially from 2-10 bp from the 5’ end of PAM, hampered Cas9 cleavage more than others. The first guide for each of our targets has perfect complementarity to the mutant allele at the site of the mutation. For the second and third guides of each candidate target, we introduced an additional mutation in the sgRNA sequence, which would result in one mismatch between sgRNA and mutant allele and two mismatches between sgRNA and wildtype allele. The second mismatches were designed to be highly disruptive to sgRNA targeting when present with the original mismatches, but weakly disruptive by themselves.

### 5.2 Cas9 Cleavage of pCAG-EGxxFP Target Plasmids in COS-7 Cells

We chose to use the pCAG-EGxxFP plasmid system as a reporter for Cas9 cleavage efficiency (Figure 3A). We created pCAG-EGxxFP plasmids with mutant and wildtype sequences of each candidate gene and tested them with each sgRNA (see Materials and Methods above). Cleavage of the target sites within the EGxxFP plasmid by Cas9 reconstitutes EGFP through homologous recombination, allowing quantification of cleavage efficiency by flow cytometry.
For the *PRPF8*, *BEST1*, and *PRPH2* targets, we found at least one sgRNA that led to a 2-fold increase in the percent of EGFP-positive cells for plasmids containing the mutant allele compared to the wildtype allele, suggesting the potential utility of these sgRNAs for allele-specific targeting (Figure 3B). For *PRPF8* sgRNAs 2 and 3 showed high allele-specificity, with around a two-fold increase in fluorescence in cells transfected with mutant targets than cells with the wildtype sequence. For *BEST1*, sgRNA 2 showed the highest level of allele specificity, although interestingly, it did not have the highest absolute rate of cleavage. For *PRPH2*, both sgRNAs 2 and 3 showed around a two-fold increase in Cas9 targeting of mutant over wildtype sequence (Figure 3B). For *RHO*, *SNRNP200*, and *IMPDH1*, we found no sgRNAs that targeted the mutant allele specifically. Therefore, we focused on only *PRPF8*, *BEST1*, and *PRPH2* genes for the subsequent experiments.

5.3 Validation of Sequence-Specific Cas9 Cleavage in Primary Patient-Derived Fibroblasts

We obtained dermal fibroblasts from patients carrying the mutations in *PRPF8* [p.(Arg1935His)], *BEST1* [p.(Lys30Arg)], and *PRPH2* [p.(Phe211Leu)], and quantified Cas9-mediated indel formation on chromosomal loci in these cells. We transduced the cells with lentiviral vectors carrying the Cas9-sgRNA system and quantified indel formation rate by deep sequencing for target sites.

5.3.1 Validation of Controls in the Experimental Design

In this study, *EMX1* was included as a control for transduction efficiency in control and patient fibroblasts. The *EMX1* locus was selected due to prior studies which showed efficient Cas9 cleavage at this site.33,35 The similar indel frequency (33.0-37.4%) in the control and patient fibroblasts transduced with control sgRNA targeting *EMX1* indicates comparable transduction efficiency and indel formation between the different fibroblast lines.

Additionally, we also performed NGS analysis on unmanipulated patient fibroblasts to determine the baseline ratios of wildtype to mutant reads. We would expect about 50% wildtype and 50% mutant in untransduced fibroblasts of a patient with a heterozygous mutation. Indeed,
we see 49.1% WT and 50.9% mutant reads in the **PRPF8** patient, 48.5% WT and 51.5% mutant in **BEST1**, and 49.6% WT and 50.4% mutant in **PRPH2**.

**5.3.2 Allele-Specific Silencing in Primary Patient-Derived Fibroblasts**

As mentioned, patient fibroblasts contain one mutant allele and one wildtype allele. However, because Cas9-mediated mutations are mostly in the form of indels that disrupt the sequence differentiating wildtype from mutant, it is often not possible to assay directly whether an indel was created in the mutant or wildtype copy. To quantify rates of allele specificity, we instead quantified the ratio of mutant and wildtype alleles containing no indels as an estimate of targeting (Figure 4A). For example: if the reads post-Cas9 targeting mapping to wildtype and mutant are 50% and 40%, respectively, with the remaining 10% of the reads being classified as indels, this would indicate that the mutant allele has undergone indel formation at a rate of 20% and the wildtype allele is not mutated at all. As an additional control to estimate the rates of mutagenesis on wildtype alleles, we performed similar experiments on control (+/+) fibroblasts.

NGS quantification of indel formation rates in patient fibroblasts showed that for **PRPF8** [c.5804G>A, p.(Arg1935His)], we attained 8.4% indel frequency with sgRNA 1, 16.3% with sgRNA 2, and 11.6% with sgRNA 3 (Figure 4B). The high percentage of unmutated wildtype allele reads (55-59%) compared to mutant (24-36%) suggests specific targeting of the mutant allele. In control fibroblasts transduced with lentiviruses containing sgRNAs targeting the **PRPF8** locus, there were low rates of indel formation (0.1-1.5%), supporting the idea that most of the indels formed in patient fibroblasts occurred through specific targeting of the mutant allele. sgRNA 2 showed the highest indel formation rate for **PRPF8**, with 16.3% of reads modified, 59.3% unmodified wildtype, and 24.4% unmodified mutant reads.

In patient fibroblasts with a heterozygous mutation in **BEST1** [c.89A>G, p.(Lys30Arg)], we obtained 5.2%, 0.7%, and 14.1% of indel frequency with sgRNA 1, 2, and 3, respectively (Figure 4C). sgRNA 3 showed the best specificity for modification of the mutant allele, with 14.1% indel formation rate, 45% unmodified wildtype reads, and 40.9% unmodified mutant. In control fibroblasts transduced with sgRNAs targeting the **BEST1** locus, we saw similarly low rates of indel formation (0.3%).

16
We obtained 10.2%, 14.8%, and 4.7% in patient fibroblasts with PRPH2 [c.631T>C, p.(Phe211Leu)] (Figure 4D). Here, sgRNA 2 showed the highest rate of allele specificity, with 14.8% indel formation rate, 48.0% unmodified wildtype, and 37.2% unmodified mutant. There were low rates of indel formation in control fibroblasts transduced with sgRNAs targeting the PRPH2 locus (0.05-2.5%).

The rates of indel formation can be further broken down into in-frame versus out-of-frame loss or insertion of bases (Table 2). The majority of indels formed post-modification are out-of-frame, with 88% of indels for PRPF8 sgRNA 2, 94% for BEST1 sgRNA 3, and 76% for PRPH2 sgRNA 2 resulting in a frameshift mutation in the genomic DNA after Cas9 cleavage and inactivation of the coding sequence.

5.3.3 Subanalysis of Indel Formation Rates in Known Wildtype and Mutant Reads

As discussed, modification by Cas9 often obfuscates the identity of the read. However, in approximately 25% of reads, we can still differentiate wildtype from mutant because the indel formation event does not involve the genomic position of interest. Of these reads, we found that with the exception of sgRNA 2 for BEST1, over 85% of indel formation events occurred on the mutant alleles (Table 3). Specifically for the sgRNAs that demonstrated the most selective cleavage for mutant over wildtype alleles – sgRNA 2 for PRPF8, sgRNA3 for BEST1, and sgRNA2 for PRPH2 – we saw that 94.5%, 95.7%, and 86.1% of the indel formation events occurred on the mutant alleles, respectively.

5.4 Off-Target Analysis in Fibroblasts using the Surveyor Assay

Off-target mutagenesis is a large safety concern for genome editing therapies, as the genomic DNA sequence changes introduced are permanent. To assess the safety of our allele-specific silencing strategy, we assayed potential off-target editing by the Cas9-sgRNA system in transduced fibroblasts. We determined specificity by computationally predicting the top 3-4 off-target sites in coding regions of the genome using an established algorithm (CRISPR Design: crispr.mit.edu) and assessing mutagenesis rates at chosen sites by the SURVEYOR nuclease assay. We observed low off-target modification rates in patient fibroblasts with our guides (Figure 5).
6. DISCUSSION

In this work, we described the use of CRISPR to perform allele-specific silencing at the DNA level for GOF SNPs that cause dominantly inherited IRD. This proof of concept study demonstrated that CRISPR genome editing has single-base specificity and can be used to selectively knock down mutant alleles even when the mutation does not result in a new PAM sequence.

6.1 Allele Specific Targeting in Patient Dermal Fibroblasts

We showed experimental data from patient fibroblasts with dominant mutations in PRPF8 (c.5804G>A), BEST1 (c.89A>G), and PRPH2 (c.631T>C). We observed cleavage efficiencies of 16% in sgRNA 2 for PRPF8, 14% in sgRNA 3 for BEST1, and 15% in sgRNA 2 for PRPH2. The low rates of indel formation in control fibroblasts transduced with the Cas9/sgRNA system support the idea that most of the indels formed in patient fibroblasts occurred through specific targeting of the mutant allele. Analysis of indel formation rates on known wildtype and mutant reads further showed that with the exception of one guide that selectively cleaved wildtype over mutant, 86-96% of these indels occurred on the mutant allele.

The results for PRPF8 and PRPH2 also suggest that mutations do not need to be in a PAM sequence to be targeted selectively by the Cas9 endonuclease. As long as the mutation site is within the specificity-determining part of the sgRNA, Cas9 can still effectively and specifically target the mutant allele over wildtype.

The majority of indels formed through our allele-specific targeting strategy were out-of-frame deletions: 88% of indels for PRPF8 sgRNA 2, 94% for BEST1 sgRNA 3, and 76% for PRPH2 sgRNA 2 (Figure 4). This phenomenon has been reported in other studies, and the cause of it is unclear, as in theory, in-frame indels should occur in one-third of NHEJ mutagenic events. However, this is a promising sign that an indel formed from NHEJ repair of DSB is likely to produce frameshift mutations in mutant alleles, increasing the likelihood of producing therapeutic genome modifications in GOF alleles.
6.2 Off-Target Cleavage by CRISPR/Cas9

Interestingly, the three guides tested for each target showed similar off-target cleavage efficiencies in patient fibroblasts, suggesting that off-target cleavage rates depend more on the off-target site and less on minor changes to the sgRNA sequence. For instance, all PRPF8 sgRNAs demonstrate some cutting at off-target site 1 (6.5-10.1%), no cutting at sites 2 and 3, and some cutting at site 4 (6.1-12.7%). This implies that while introducing an additional mutation in the sgRNA sequence may improve allele specificity, it does not affect off-target modification rates.

6.3 Allele Specific Targeting in COS-7 Cells

The results seen in Cas9-sgRNA transfected COS7 cells identified sgRNAs with high specificity that also performed well in the fibroblast experiments. For PRPF8, sgRNAs 2 and 3 showed around a two-fold increase in florescence in cells transfected with mutant targets than cells with the wildtype sequence. For BEST1, sgRNA 2 showed the highest level of allele specificity, and for PRPH2, both sgRNAs 2 and 3 showed around a two-fold increase in Cas9 targeting of mutant over wildtype sequence. This suggests that the pCAG-EGxxFP system could be used for an initial screen of sgRNA efficiency and specificity. Of note, sgRNA 1 for PRPF8 and BEST1 mutations did not show a significant difference in Cas9 cleavage between mutant and wildtype targets. However, the introduction of a second mismatch in sgRNA 2 and 3 significantly improved specificity for mutant targets.

Our experiments also demonstrated that manipulation of the pCAG-EGxxFP plasmid produced background fluorescence. Therefore, we support the use of pCAG-EGxxFP reporter plasmid as a relative indicator of the likelihood of an sgRNA’s success. However, it cannot be used to quantitatively compare two sgRNAs.

6.4 Limitations of this Study

The most important limitation of this study is that our experiments were completed in cells from a commercial cell line (COS-7) and primary dermal fibroblasts in vitro, and studies have shown that on and off-target rates of indel formation can differ in primary cell types and
tissues *in vivo*. Therefore, it is not clear that a similar level of allele-specificity could be achieved *in situ*. Additionally, because cells in culture often acquire mutations that affect DNA damage repair pathways, it is unclear whether the spectrum of editing events that would occur *in situ* with this editing technique would mirror the results shown here. The issue of efficiency of allele-specific editing *in vivo* for retinal cells and the spectrum of editing outcomes in the cell types present in the retina will be the subject of future studies.

7. FUTURE DIRECTIONS

The next step in CRISPR genome editing therapy includes identifying appropriate animal disease models to test this treatment *in vivo*. Dominant mutations can act through haploinsufficiency or GOF mechanism. Thus, to properly test our approach *in vivo*, we must identify a disease model that involves a mutation believed to be GOF or dominant negative. Considerations including presence of viable target cells in the disease model and suitable delivery vectors for the tissue of interest will be critical for the ability to test this form of therapy *in vivo*. Recently work describing the use of a small Cas9 ortholog that is compatible with adeno-associated viral (AAV) vectors has been published, permitting the testing of allele specific targeting for genetic retinal diseases *in vivo*.63

It will also be interesting to explore methodologies to enhance allele specific targeting in the future using recent advances from the CRISPR field. Truncated sgRNAs with 17 or 18 nucleotides of complementarity can reduce off-target modification without sacrificing on-target cleavage.64 Orthogonal CRISPR proteins63,65–68 and engineered variants of Cas969,70 can also recognize different PAM sequences, thus broadening the target range.

Finally, there is still much to work out regarding the mode of action of individual mutations. In some cases, different mutations in the same gene can have different modes of action. For instance, while most mutations in rhodopsin are inherited in an autosomal dominant fashion, and recessive mutations have also been reported.71,72 Thus, enriching our knowledge of the biochemical mechanisms and genotype-phenotype relationships of mutations would improve proper selection of disease models and patients.
8. SUMMARY

Autosomal dominant mutations account for approximately one third of all retinal dystrophies (RetNet database: http://www.sph.uth.tmc.edu/retnet/sum-dis.htm). Diseases caused by GOF mutations cannot be treated with traditional gene supplementation therapy, making the development of a new approach in gene therapy an important endeavor. In these cases, ablation of the mutated gene’s function is essential, and the CRISPR technology offers a novel way to selectively suppress gene expression at the DNA level. The CRISPR/Cas9 system still requires much work to optimize efficacy, safety, and specificity before it can be clinically used to treat patients with IRDs. However, CRISPR holds the potential to address currently unmet needs in clinical medicine and play a key role in the future of precision medicine.
REFERENCES


15. Keaney J, Campbell M HP. From RNA interference technology to effective therapy: how far have we come and how far to go? *Ther Deliv*. 2011:1395-1406.


10. TABLES

Table 1. Six alleles were identified as candidates for targeting by Cas9.

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Gene</th>
<th>RefSeq</th>
<th>Mutation</th>
<th>Mutation Effect</th>
<th>HGMD</th>
<th>PubMed ID</th>
<th>SIFT/PolyPhen/MutationTaster</th>
<th>ExAC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>PRPH2</td>
<td>NM_000322.4</td>
<td>c.631T&gt;C</td>
<td>p.(Phe211Leu)</td>
<td>CM951119</td>
<td>9673478</td>
<td>D/P/P</td>
<td>Absent</td>
</tr>
<tr>
<td>RP</td>
<td>RHO</td>
<td>NM_000539.3</td>
<td>c.1021G&gt;A</td>
<td>p.(Glu341Lys)</td>
<td>CM973327</td>
<td>21094163</td>
<td>T/PosD/P</td>
<td>A=1/G=121,152</td>
</tr>
<tr>
<td>Best</td>
<td>BEST1</td>
<td>NM_004183.3</td>
<td>c.89A&gt;G</td>
<td>p.(Lys30Arg)</td>
<td>CM004422</td>
<td>10798642</td>
<td>D/P/P</td>
<td>G=1/A=121,404</td>
</tr>
<tr>
<td>RP</td>
<td>IMPDH1</td>
<td>NM_000883.3</td>
<td>c.962C&gt;T</td>
<td>p.(Ala321Val)</td>
<td>This Study</td>
<td>This Study</td>
<td>D/P/P</td>
<td>Absent</td>
</tr>
<tr>
<td>RP</td>
<td>PRPF8</td>
<td>NM_006445.3</td>
<td>c.5804G&gt;A</td>
<td>p.(Arg1935His)</td>
<td>This study</td>
<td>This study</td>
<td>NA/P/P</td>
<td>Absent</td>
</tr>
<tr>
<td>RP</td>
<td>SNRNP200</td>
<td>NM_014014.4</td>
<td>c.3260C&gt;T</td>
<td>p.(Ser1087Leu)</td>
<td>CM097780</td>
<td>19878916</td>
<td>D/NA/P</td>
<td>T=1/C=121,392</td>
</tr>
</tbody>
</table>

a RP - Retinitis Pigmentosa; Best - Best Disease

b SIFT: D=Damaging, T=Tolerated, NA= no prediction. PolyPhen: D= Probably Damaging, PosD= Possibly Damaging, NA= No prediction. Mutation Taster: P=Predicted to be disease causing.
Table 2. Rates of indel formation can be further classified as out-of-frame versus in-frame insertions and deletions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>sgRNA ID</th>
<th>Out-of-Frame</th>
<th>In-Frame</th>
<th>% Indels</th>
<th>% Mutant reads</th>
<th>% WT reads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Insertions</td>
<td>Deletions</td>
<td>Insertions</td>
<td>Deletions</td>
<td></td>
</tr>
<tr>
<td>PRPF8</td>
<td>1</td>
<td>1013</td>
<td>5285</td>
<td>57</td>
<td>720</td>
<td>8.4</td>
</tr>
<tr>
<td>PRPF8</td>
<td>2</td>
<td>1512</td>
<td>10452</td>
<td>145</td>
<td>1513</td>
<td>16.3</td>
</tr>
<tr>
<td>PRPF8</td>
<td>3</td>
<td>1548</td>
<td>7106</td>
<td>215</td>
<td>1681</td>
<td>11.6</td>
</tr>
<tr>
<td>BEST1</td>
<td>1</td>
<td>1080</td>
<td>2562</td>
<td>2</td>
<td>279</td>
<td>5.2</td>
</tr>
<tr>
<td>BEST1</td>
<td>2</td>
<td>322</td>
<td>243</td>
<td>3</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>BEST1</td>
<td>3</td>
<td>523</td>
<td>8198</td>
<td>371</td>
<td>1397</td>
<td>14.1</td>
</tr>
<tr>
<td>PRPH2</td>
<td>1</td>
<td>3075</td>
<td>8020</td>
<td>8</td>
<td>6722</td>
<td>10.2</td>
</tr>
<tr>
<td>PRPH2</td>
<td>2</td>
<td>5608</td>
<td>20961</td>
<td>1872</td>
<td>6218</td>
<td>14.8</td>
</tr>
<tr>
<td>PRPH2</td>
<td>3</td>
<td>231</td>
<td>5009</td>
<td>2248</td>
<td>3469</td>
<td>4.7</td>
</tr>
</tbody>
</table>
Table 3. Subanalysis of indel formation rates quantified in known wildtype versus mutant reads.

<table>
<thead>
<tr>
<th>Gene</th>
<th>sgRNA ID</th>
<th>% Indels</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wildtype</td>
<td>Mutant</td>
</tr>
<tr>
<td>PRPF8</td>
<td>1</td>
<td>14.3</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.5</td>
<td>94.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.2</td>
<td>98.8</td>
<td></td>
</tr>
<tr>
<td>BEST1</td>
<td>1</td>
<td>11.5</td>
<td>88.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>83.5</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.3</td>
<td>95.7</td>
<td></td>
</tr>
<tr>
<td>PRPH2</td>
<td>1</td>
<td>1.6</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.9</td>
<td>86.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.5</td>
<td>97.5</td>
<td></td>
</tr>
</tbody>
</table>
11. FIGURES

Figure 1. Proposed mechanism of allele-specific silencing. The sgRNA has perfect complementarity to the mutant allele, resulting in DSB, which when repaired through NHEJ, will usually result in the introduction of an indel into the genome, thereby inactivating the mutant allele. At the wildtype locus, mismatches exist between the sgRNA and the wildtype site, and this DSB/indel formation process does not happen. The net result is that only the wildtype protein is produced.
**Figure 2.** Sequences of guides for candidate alleles. **Red** indicates the patient mutation. **Blue** indicates the additional mutation we introduced into the guide.

<table>
<thead>
<tr>
<th></th>
<th>BEST1</th>
<th>PRPF8</th>
<th>PRPH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT seq</td>
<td>GCTGGCGGGGCAGCATCTAC</td>
<td>AATCAGGATGAGACGGGAGA</td>
<td>TAGGATTGCAGCAGCTGAAA</td>
</tr>
<tr>
<td>sgRNA 1</td>
<td>GCTGGCGGGGCAGCATCTAC</td>
<td>AGG</td>
<td>TAGGATTGCAGCAGCTGAGA</td>
</tr>
<tr>
<td>sgRNA 2</td>
<td>GCTGGCGGGGCAGCATCTC</td>
<td>AGG</td>
<td>TAGGATTGCAGCAGCTGAGG</td>
</tr>
<tr>
<td>sgRNA 3</td>
<td>GCTGGCGGGGCAGCATCTC</td>
<td>AGG</td>
<td>TAGGATTGCAGCAGCTGAGG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>RHO</th>
<th>IMPDH1</th>
<th>SNRNP200</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT seq</td>
<td>CACCTGGCTCTCCGCTCCGCTCT</td>
<td>TGG</td>
<td>TGCTTCTTTCTGTTAGTTGGC</td>
</tr>
<tr>
<td>sgRNA 1</td>
<td>CACCTGGCTCTCCGCTCCGCTCT</td>
<td>TGG</td>
<td>TGCTTCTTTCTGTTAGTTGGC</td>
</tr>
<tr>
<td>sgRNA 2</td>
<td>CACCTGGCTCTCCGCTCCGCTCT</td>
<td>TGG</td>
<td>TGCTTCTTTCTGTTAGTTGGC</td>
</tr>
<tr>
<td>sgRNA 3</td>
<td>CACCTGGCTCTCCGCTCCGCTCT</td>
<td>TGG</td>
<td>TGCTTCTTTCTGTTAGTTGGC</td>
</tr>
</tbody>
</table>
Figure 3. (A) Schematic of pCAG-EGxxFP plasmid design. The target sequence is inserted into the plasmid, in between regions of homology. If the target is cleaved by Cas9 nuclease, homologous recombination takes place to reconstitute the EGFP expression cassette. (Adapted from Mashiko et al., Develop Growth Differ. 2014;56:122-129.) (B) Testing of Cas9 cutting efficiency was done in COS-7 monkey cells with constructed EGxxFP plasmids with allele target sequence compared to wildtype sequence. When the EGxxFP plasmid is cut by the Cas9 nuclease system, EGFP is produced and fluorescence is quantified by FACS. The experiments were carried out in triplicates and repeated three times. These graphs show the aggregate of our results for each allele. (Black columns represented WT allele, and white columns represent mutant.)
Figure 4. Pie charts of rates of insertions and deletions (indels) in patient and control genomes after transduction of Cas9-sgRNA system. (A) Experimental design of patient fibroblast study. Theoretically, patient fibroblasts have half wildtype and half mutant alleles. After Cas9 transduction of fibroblasts, indels are formed in the mutant alleles, reducing their percentage to below 50%. (B) In patient and control fibroblasts transduced with sgRNAs for PRPF8. (C) In patient and control fibroblasts transduced with sgRNAs for BEST1. (D) In patient and control fibroblasts transduced with sgRNAs for PRPH2.
A

**Control Fibroblasts**

PRPF8 g1

% Total Indels: 1.4%

98.6%

PRPF8 g2

% Total Indels: 0.08%

99.9%

PRPF8 g3

% Total Indels: 1.5%

98.5%

**PRPF8 Patient Fibroblasts**

PRPF8 g1

% Total Indels: 8.4%

0.8%

12.5%

6.2%

PRPF8 g2

% Total Indels: 16.3%

1.2%

1.8%

1.8%

PRPF8 g3

% Total Indels: 11.6%

1.8%

1.8%

1.7%

B

C

**BEST1 g1**

% Total Indels: 0.3%

0.3%

0.3%

0.3%

99.7%

99.7%

99.7%

**BEST1 Patient Fibroblasts**

**BEST1 g1**

% Total Indels: 5.2%

4.9%

1.7%

0.4%

44.1%

50.7%

50.7%

**BEST1 g2**

% Total Indels: 0.7%

0.7%

0.7%

0.7%

48.2%

51.1%

51.1%

**BEST1 g3**

% Total Indels: 14.1%

11.4%

1.9%

0.5%

40.9%

45.0%

45.0%

D

**PRPH2 g1**

% Total Indels: 0.05%

0.05%

0.05%

0.05%

99.9%

99.9%

99.9%

**PRPH2 Patient Fibroblasts**

**PRPH2 g1**

% Total Indels: 10.2%

4.6%

2.9%

1.8%

43.8%

45.9%

45.9%

**PRPH2 g2**

% Total Indels: 14.8%

8.9%

2.4%

1.5%

48.0%

57.2%

57.2%

**PRPH2 g3**

% Total Indels: 4.7%

1.0%

2.1%

1.5%

46.4%

48.9%

48.9%
**Figure 5.** Rates of off-target editing as approximated by the Surveyor assay (g1 = sgRNA OT1 = off-target site 1, etc.).

<table>
<thead>
<tr>
<th>Background: BEST1 patient fibroblasts</th>
<th>Background: PRPH2 patient fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BEST1 g1</strong></td>
<td><strong>PRPH2 g1</strong></td>
</tr>
<tr>
<td>OT1</td>
<td>OT2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| **BEST1 g2**                          | **PRPH2 g2**                           |
| OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 |
| 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |

| **BEST1 g3**                          | **PRPH2 g3**                           |
| OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 | OT1 | OT2 | OT3 |
| 0   | 0   | 11.2| 0   | 0   | 0   | 0   | 0   | 0   | 11.2| 0   | 0   | 0   | 4.9 | 0   | 10.6|

<table>
<thead>
<tr>
<th><strong>PRPF8 patient fibroblasts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRPF8 g1</strong></td>
</tr>
<tr>
<td>OT1</td>
</tr>
<tr>
<td>7.6</td>
</tr>
</tbody>
</table>
## 12. SUPPLEMENTAL MATERIALS

### Table S1. Primers for sgRNAs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>sgRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHO</td>
<td>1</td>
<td>CACCGACCTGGCTCGTCTCGTCTGCTT</td>
<td>AAACAGACGAAAGACGAGCCAGGTC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGACCTGGCTCGTCTCGTCTT</td>
<td>AAACAAACGAAAGACGAGCCAGGTC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGACCTGGCTCGTCTCGTCTT</td>
<td>AAACAGACGAAACGAGCCAGGTC</td>
</tr>
<tr>
<td>BEST1</td>
<td>1</td>
<td>CACCGCTGCCGGCCAGCATGCTCTAC</td>
<td>AAACCTAGATGTGCTGCCCGAGCCAGC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGCTGCCGGCCAGCATGCTCTAC</td>
<td>AAACAGAAGATGCTGCCCGAGCCAGC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGCTGCCGGCCAGCATGCTCTAC</td>
<td>AAACAGAAGATGCTGCCCGAGCCAGC</td>
</tr>
<tr>
<td>IMPDH1</td>
<td>1</td>
<td>CACCGGACTACCCTCTGCTCGTCTTCA</td>
<td>AAACACTGAGACCGAGGATTGTAGTCC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGGACTACCCTCTGCTCGTCTTCA</td>
<td>AAACACTGAGACCGAGGATTGTAGTCC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGGACTACCCTCTGCTCGTCTTCA</td>
<td>AAACACTGAGACCGAGGATTGTAGTCC</td>
</tr>
<tr>
<td>PRPF8</td>
<td>1</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td>SNRNP200</td>
<td>1</td>
<td>CACCGGCCCTTCTTCTTTGTAGTCTCCTG</td>
<td>AAACGCCAACAATGCAAAAGAAGCC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGGCCCTTCTTCTTTGTAGTCTCCTG</td>
<td>AAACGCCAACAATGCAAAAGAAGCC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGGCCCTTCTTCTTTGTAGTCTCCTG</td>
<td>AAACGCCAACAATGCAAAAGAAGCC</td>
</tr>
<tr>
<td>PRPH2</td>
<td>1</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CACCGAGATTGAGATGGAGATGGAGA</td>
<td>AAACCTTCCCATCTCATCTGTGACT</td>
</tr>
<tr>
<td>EMX1</td>
<td></td>
<td>CACCGGCACCCTCAATTGAGATGGG</td>
<td>AAACCCCTGTAGTGAGGTTGAC</td>
</tr>
</tbody>
</table>

### Table S2. Primers for PCR amplification of targets for insertion into pCAG-EGxxFP plasmid.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHO</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
<tr>
<td>BEST1</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
<tr>
<td>IMPDH1</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
<tr>
<td>PRPF8</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
<tr>
<td>SNRNP200</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
<tr>
<td>PRPH2</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
<td>CTGAACCGATCCCTCCCTTCTGCTCATGCCAT</td>
</tr>
</tbody>
</table>
Table S3. Primers for PCR amplification of targets for NGS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPH2</td>
<td>GTTCCAGAGGCAAGGGTTG</td>
<td>CTCCGTCTGGTGTCGTAAC</td>
</tr>
<tr>
<td>BEST1</td>
<td>TGGCCATGACCACTCACTTACA</td>
<td>GACTTTTCTCTCCCCAGCC</td>
</tr>
<tr>
<td>PRPF8</td>
<td>AGCTGACCTACCCCACCTTT</td>
<td>TTCTTGCCG TAGTCAGCCA</td>
</tr>
<tr>
<td>EMX1</td>
<td>AGGTGAAGGTGTGGTCCAG</td>
<td>AGTGGCGAGGTCCAGCTT</td>
</tr>
</tbody>
</table>

Table S4. Primers for PCR amplification of off-target sites.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Off-target site</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPH2</td>
<td>1</td>
<td>GAAGCCCTGTCTGGCACAT</td>
<td>TCAGTGTTCACCAGGCAGAG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CAGCACCCTCACACAGTTCT</td>
<td>TGAGGACACAGGTCTTAG</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>GCAATGTCCCCATCTCCATCCTC</td>
<td>AGTGGCCCCCTGGTACTGTC</td>
</tr>
<tr>
<td>BEST1</td>
<td>1</td>
<td>GGGCAACTCTTGACACCTC</td>
<td>CTCGGAGCTGTCAACCCAA</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>GGATTTCTTCAGCAGACAGGC</td>
<td>AGCACAGCTTTGAGGGGAC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TGACTCAGACACAGAAGGGG</td>
<td>CCCGCCCTAAACCTGTA</td>
</tr>
<tr>
<td>PRPF8</td>
<td>1</td>
<td>GGCCTTAGCAGTTTGCTCCC</td>
<td>GTAGAGAAAGCGGTGGGGAC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>AAAGCAGCAGCTGTATGCCC</td>
<td>ACCATTGGGTGTTTCCCCTG</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>AGTGCGGTGGTGGGTGCTGT</td>
<td>CCGCAAATGCTGTAATGTC</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CCCTGCAAAGGTGTGTTGTA</td>
<td>TCTAAAGGGCATAACCCCT</td>
</tr>
</tbody>
</table>

39