Molecular Biology

In vivo genome editing rescues photoreceptor degeneration via a Cas9/RecA-mediated homology-directed repair pathway

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Although Cas9-mediated genome editing has been widely used to engineer alleles in animal models of human inherited diseases, very few homology-directed repair (HDR)–based genetic editing systems have been established in postnatal mouse models for effective and lasting phenotypic rescue. Here, we developed an HDR-based Cas9/RecA system to precisely correct Pde6b mutation with increased HDR efficiency in postnatal rodless (rd1) mice, a retinitis pigmentosa (RP) mutant model characterized by photoreceptor degeneration and loss of vision. The Cas9/RecA system incorporated Cas9 endonuclease enzyme to generate double-strand breaks (DSBs) and bacterial recombinase A (RecA) to increase homologous recombination. Our data revealed that Cas9/RecA treatment significantly promoted the survival of both rod and cone photoreceptors, restored the expression of PDE6B in rod photoreceptors, and enhanced the visual functions of rd1 mice. Thus, this study provides a precise therapeutic strategy for RP and other genetic diseases.

Introduction

Retinitis pigmentosa (RP), an inherited retinal disease characterized by the loss of rod and cone photoreceptors, is the leading cause of progressive vision loss and inherited blindness (1, 2). Photoreceptors are sensory neurons within the retina that convert light signals into electrical signals for visual signal processing (3). Patients with RP display signs of nystagmus (night blindness) due to loss of rods, followed by loss of daytime vision due to secondary cone photoreceptor degeneration. The PDE6B gene, which encodes guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase 6B (PDE6B) for phototransduction, accounts for 4 to 5% of autosomal recessive RP (4). The rodless (rd1) mouse, which shows a point mutation (Y347X, C to A) in the Pde6b gene, is a widely used RP mouse model. The mutant mice exhibit early-onset and severe rod photoreceptor degeneration, leading to progressive visual loss and eventually total blindness (5). Thus, rd1 mice are a suitable model for advancing therapeutic treatments for RP disease (6–8). Several strategies have been developed for rescuing photoreceptor degeneration in rd1 mice, such as small‐fragment target homologous replacement in germ lines (9), adeno‐associated virus (AAV) vectors for gene complementation (6), or small molecules for promoting cell survival (10). However, each of these methods demonstrates substantial shortcomings such as limited efficiency or no gene correction for lasting phenotypic rescue. Moreover, most human genetic diseases are diagnosed at the postnatal stage. Therefore, alternative gene editing strategies with improved gene editing efficiency need to be established for precise gene editing in postnatal animal models.

Clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 has been widely used in genetic engineering (11–19). The CRISPR-Cas9 technique relies on single-guide RNA (sgRNA) to direct Cas9 to generate DNA double-strand breaks (DSBs) at target loci, with the breaks repaired by either nonhomologous end joining (NHEJ) or the homology-directed repair (HDR) pathway, which requires a DNA template (15, 20). NHEJ has been extensively used for gene deletion (21–25). For example, Cas9-mediated NHEJ repair was shown to remove the mutated exon 23 from the dystrophin gene and rescue muscle damage in muscular dystrophy mutant mice (21, 26, 27). However, NHEJ is an error-prone genome editing pathway that can introduce additional insertions and deletions (indel) in DSBs. Conversely, HDR is a high-fidelity pathway for genome editing of germline or proliferating cells, although it is considered inefficient and relies on cell division (9, 23, 28, 29). Cas9-mediated HDR has been used to correct point mutations in the fumarylacetoacetate hydrolase (Fah) gene of an adult mouse model with severe liver damage (22). The reason why Cas9-mediated HDR can correct Fah gene mutations is because the liver has a remarkable capacity to regenerate in adult stages, and thus, the liver cells with the gene correction can proliferate and repopulate the liver. However, most cells in postnatal tissues in mammals, including neurons, lose their proliferation ability along with maturation. Thus, the Cas9-mediated HDR pathway has rarely been applied in postnatal tissues and cells. Therefore, we aimed to design an HDR-based genome editing technique to precisely correct the gene mutation in postnatal rd1 mouse retinai for RP disease therapy.

The Escherichia coli recombinase A (RecA) protein can boost homologous recombination efficiency in mammalian and plant cells (30, 31). RecA is an adenosine 5′-triphosphate (ATP)–dependent
DNA binding protein that catalyzes DNA strand exchange reactions in homologous recombination. These facts prompted us to study whether the addition of bacterial RecA protein in Cas9-mediated genome editing could specifically increase the frequency of HDR. We developed a Cas9/RecA technique, which incorporated Streptococcus pyogenes Cas9 (spCas9) and sgRNA-targeted RecA, to correct the gene mutation in postnatal rd1 mice with enhanced HDR efficiency in vivo. Our research demonstrated that HDR efficiency was enhanced using the Cas9/RecA system both in vitro and in vivo. Furthermore, the system successfully corrected the Pde6b point mutation, significantly rescued photoreceptor degeneration, and improved the visual functions in rd1 mice. Therefore, this study established an improved HDR-based genetic repair system, with the potential use in precise gene therapy for inherited diseases.

RESULTS
Cas9/RecA promoted HDR efficiency in vitro
To study the possibility that RecA can increase the frequency of HDR, we adopted sgRNA-targeted RecA in CRISPR-Cas9 to devise a Cas9/RecA system for precise gene repair. There were four major components in the Cas9/RecA system: (i) RecA-MS2 bacteriophage coat protein complex, (ii) sgRNA with MS2 binding loops, (iii) spCas9, and (iv) single-stranded DNA (ssDNA) donor template (Fig. 1A). We applied spCas9 to generate DSBs via sgRNA guidance, followed by the recruitment of RecA to sgRNA via the MS2 coat protein to promote homologous recombination and increase the probability that ssDNA-guided repair will be enhanced upon enrichment of RecA close to DSBs.

To investigate whether the Cas9/RecA system was able to improve HDR efficiency in vitro, reconstructive human embryonic kidney (HEK) 293FT cells with constitutive expression of blue fluorescent protein (BFP) were used to evaluate HDR efficiency via the fluorescence shift from BFP to enhanced green fluorescent protein (EGFP) (Fig. 1B) (32). In the HEK293FT cell lines, fluorescent BFP can be converted to EGFP by CRISPR-Cas9 editing, as both only have one base pair (196C to T) difference in their coding sequence. Loss of BFP expression signifies that DSBs have been repaired via the NHEJ pathway, whereas an increase in EGFP expression indicates that DSBs have been repaired via the HDR pathway (32). Three components (i.e., Cas9, sgRNA, and ssDNA donor) were transfected into cells to evaluate Cas9-mediated HDR efficiency (Cas9 hereafter). Four components (i.e., Cas9, sgRNA, RecA-MS2, and ssDNA donor) were transfected into cells to evaluate Cas9/RecA-mediated HDR efficiency (Cas9/RecA hereafter). Cells transfected with Cas9 only were used as the control group (Cas9 without ssRNA). For all cell transfections, mCherry plasmid was cotransfected to determine the transfection efficiency. The efficiencies of HDR and NHEJ were quantified as percentages of EGFP+/mCherry+ and BFP ‘EGFP+’/mCherry+”, respectively, via fluorescence-activated cell sorting (FACS) (Fig. 1, C to E). We observed few EGFP” events in the control (Cas9 without ssRNA) group when using Cas9 only. Moreover, the efficiency of NHEJ was not affected by Cas9/RecA treatment in comparison with the Cas9 group (Fig. 1F). Although the efficiency of HDR and NHEJ may be underestimated on the basis of fluorescence shifts from BFP to EGFP, a ~1.7-fold increase of EGFP cells was detected in the Cas9/RecA group compared to the Cas9 group. Therefore, Cas9/RecA significantly improved HDR efficiency in vitro.

In vivo Cas9/RecA precisely repaired Pde6b gene mutation in rd1 mice
To assess the ability of Cas9/RecA to correct the Pde6b nonsense mutation in rd1 mice, we designed sgRNA to target exon 7 of the Pde6b locus using ssDNA donor as the template to change the stop codon (UAA) to tyrosine (UAC) in the Pde6b rd1 mutation (Fig. 2A). Previous findings have reported that rd1 mice may carry additional Thr4 and Gpr179 gene mutations, which may affect visual functions (6, 33). To precisely evaluate Cas9/RecA efficiency and exclude the effects of Thr4 and Gpr179 mutations on rd1 phenotypes, we amplified the Thr4 and Gpr179 genomic sequence. The sequencing and polymerase chain reaction (PCR) results indicated that there were no mutations in Thr4 (fig. S1) and Gpr179 (fig. S2 and table S1). Moreover, rd1 mice carry a murine leukemia virus (Xmv-28) insertion in intron 1 of Pde6b (34) (fig. S3A). We sequenced the complementary DNA (cDNA) fragment spanning the virus insertion site of Pde6b (fig. S3B). The results indicated that virus insertion did not give rise to additional mutations in Pde6b, suggesting that virus insertion did not interrupt Pde6b transcription (fig. S3C).

To label the rod photoreceptors for cell tracing, rd1 mice were crossed with Nrl-EGFP transgenic mice in which rod photoreceptors were labeled by EGFP fluorescence (35). Consistent with the in vitro experimental design, the retinae electroporated with plasmids expressing Cas9, sgRNA, and ssDNA on postnatal day 0 (P0) were designated as the Cas9 group, and the retinae electroporated with plasmids expressing Cas9, sgRNA, RecA-MS2, and ssDNA were designated as the Cas9/RecA group (fig. S2 and fig. S4). To investigate whether Pde6b genomic DNA and mRNA sequences were corrected in Cas9/RecA retinae, we collected 20 to 40 EGFP” rod photoreceptors from individual retina using glass capillaries on P31 and extracted either genomic DNA or mRNA for further analysis (Fig. 2C). The Pde6b rd1 mutation generated an additional Dde I restriction enzyme site, so we digested the genomic DNA and target fragments from cDNA obtained from Smart-seq2 with DdeI endonuclease to remove the un repaired fragments (36). The sequencing results demonstrated that both the rd1 genomic sequence and mRNA were successfully corrected into the wild-type sequence (Fig. 2, D and E). Correction of Pde6b gene was detected in 10 of 12 sequencing samples from two retinae of two independent mice in the Cas9/RecA group. As a control, 21 retinae from 21 independent rd1 mice in the Cas9 group were sequenced, and the results showed that the wild-type sequence was not detected in that group. To examine the potential off-target loci caused by the Cas9/RecA technique, we performed sequencing on PCR-amplified potentially targeted genomic sites to screen off-target effects of the Cas9/RecA system. We identified no genetic mutations in these potential targeted sites, as predicted by online tools (https://cm.jefferson.edu/Off-Spotter/) (table S2 and fig. S5). Together, these findings suggest that the Cas9/RecA system corrected the Pde6b sequence in vivo without significant off-target effects.

To further explore whether correction of the Pde6b gene can translate into the wild-type PDE6B protein, we performed Western blot analysis in wild-type, Cas9, and Cas9/RecA mice (Fig. 2F). A previous study suggests that the Pde6b rd1 mutation leads to a premature stop codon, and thus, rd1 mice do not exhibit a full-length PDE6B protein (37). In our Western blot results, the Cas9/RecA-treated rd1 mice generated a band whose molecular weight corresponded to the wild-type PDE6B protein, although the protein amount of Cas9/RecA treatment can restore about 2% of PDE6B of wild-type mice (Fig. 2F).
Conversely, there was no detectable wild-type PDE6B band in the Cas9 group samples. These results demonstrate that Cas9/RecA treatment was able to successfully correct the nonsense mutation of the Pde6b gene with enhanced HDR efficiency and restore the expression of the PDE6B protein in vivo.

**Cas9/RecA rescued photoreceptor degeneration in rd1 mice electroporated on P0**

Rd1 mutant mice exhibit early-onset photoreceptor degeneration and vision loss (6). Therefore, we analyzed the number of surviving rod and cone photoreceptors to investigate the feasibility of the Cas9/RecA system for the phenotypic rescue of RP in vivo. The rd1 mouse retinae were electroporated with plasmids as described above on P0, an age at which developmental proliferation of photoreceptors is still ongoing. The rod and cone photoreceptors were quantified on P31 when most rod photoreceptors are lost in rd1 mice (38). The cell number of rod photoreceptors was calculated as the number of EGFP+ cells, and the cone photoreceptors were identified by short-wavelength and mid-wavelength opsin (sw&mwOPN) immunoreactivity, which are classical markers for cone photoreceptors (Fig. 3A) (39). Results revealed that more than fivefold rod photoreceptors survived in Cas9/RecA-treated mice compared to the Cas9 group. The surviving rod photoreceptors in Cas9/RecA-treated mice equaled to 8.2% of wild-type rod photoreceptors in the electroporated area (Fig. 3B). Furthermore,
the number of cone photoreceptors under Cas9/RecA treatment was increased more than fourfold compared with the Cas9 group, suggesting their survival was also improved by the Cas9/RecA system, although this may have been a secondary effect to the surviving rods (Fig. 3C). Together, our results illustrate that Cas9/RecA treatment promoted the survival of both rod and cone photoreceptors.

**Cas9/RecA partially rescued photoreceptor degeneration in postmitotic photoreceptors in rd1 mice electroporated on P3**

HDR-mediated repair exists in nondividing neurons to a certain extent (40). To further study the feasibility of Cas9/RecA in correcting the genetic mutation in photoreceptor cells lacking proliferation...
ability, we examined the proliferation ability of these cells at different stages and tested the Cas9/RecA system in rd1 photoreceptors that lost their proliferation ability. Mouse retinas were harvested on P0 and P3 for Ki67 staining, a marker of proliferating cells (Fig. 4A). Most of our plasmids were injected into the central retina area, and electroporation was performed around the region. Our data indicated that the rescued photoreceptors mostly located around the central retina area (fig. S6). Therefore, we analyzed the proliferation ability of photoreceptors within the central retinae. In the P0 rd1 retinae, proliferating EGFP+ photoreceptors were labeled by Ki67. Very few Ki67-positive photoreceptors were found in the P3 central retinae; thus, EGFP+ rod photoreceptors lost most of their proliferation ability in the central retinae by P3. We electro-porated P3 retinae as P0 retinae using the Cas9/RecA system. The number of photoreceptors was analyzed at P31 to evaluate the rescue capability of the Cas9/RecA system (Fig. 4B). Consistent with the findings from the P0 electroporation experiments, our data demonstrated that the Cas9/RecA group had fivefold more rods and threefold more cones that survived compared with the Cas9 group (Fig. 4, C and D). We further compared the Cas9/RecA rescue efficiency between P0 and P3 electroporation. The number of rescued rods and cones in P0 Cas9/RecA electroporated mice was 1.8- and 1.6-fold higher than the P3 Cas9/RecA electroporated mice, respectively. The slight reduction of Cas9/RecA efficiency in P3 electroporation might be due to the lower electroporation efficiency and HDR efficiency in P3 postmitotic retinal neurons. Nevertheless, these results revealed that the Cas9/RecA system efficiently enhanced HDR repair to improve photoreceptor viability, including that of postmitotic photoreceptors.

**Visual restoration of rd1 mice under Cas9/RecA treatment**

Because of a limited number of rescued rods in the Cas9/RecA-treated mice, ex vivo electroretinography (ERG) recordings were conducted around the electroporated area to evaluate the light-induced electrical activity of the rescued photoreceptors and determine the effect of corrected PDE6B on the physiological functions of the retina. After at least 2 hours of dark adaptation, the neural retina was isolated from P14 mice for ex vivo ERG recording. The a- and b-waves of the ERG reflected the activity generated by the photoreceptors and bipolar cell, respectively. To confirm the identity of the waves in ex vivo
ERG, we performed the ex vivo ERG of wild-type mouse retinae in Ames' medium with or without synaptic transmission blockers (STBs; see Materials and Methods). Our data revealed that the a-wave in ex vivo ERG with blockers in room temperature emerged at around 20 ms and decayed with a much slower kinetics, similar to previous reports (41, 42). The b-wave in ex vivo ERG without blockers peaked at around 500 ms after the initial rising of the a-wave. The b-wave could be fully blocked by STBs (fig. S7, A and B). The barium chloride for blocking glial cell response only affected ERG waveforms at high amplitude (>100 μV) (fig. S7, A and C). Because barium chloride could also cause ERG waveform oscillation, we performed the rest of the ERG experiments without

Fig. 4. Partial rescue of photoreceptor degeneration in postmitotic photoreceptors by Cas9/RecA-mediated repair. (A) Ki67 staining in P0 and P3 mouse central retinae. Green, EGFP cells from Nrl-EGFP-labeled cells; red, Ki67 staining. (B) Representative immunofluorescence images of rod and cone photoreceptor markers stained in mouse retinae from wild-type, Cas9/RecA, and Cas9 mice electroporated on P3. Green, EGFP cells from Nrl-EGFP-labeled cells; red, cone markers of swOPN and mwOPN. Scale bars, 10 μm. (C) Quantification results of rod photoreceptors from wild-type (n = 3), Cas9/RecA (n = 17), and Cas9 (n = 8) mice electroporated on P3, and the mouse retinae analyzed on P31. (D) Quantification of double cone photoreceptors from wild-type (n = 3), Cas9/RecA (n = 10), and Cas9 (n = 7) mice electroporated on P3. ***P < 0.001, unpaired Student's t-test.
barium chloride. The rd1 retinae treated with Cas9/RecA electroporation exhibited significantly improved ERG a-wave responses at various light intensities, whereas the rd1 retinae in the Cas9 group showed no detectable ERG signal in response to the light flash (Fig. 5 A to C). The Cas9/RecA-treated rd1 retinae also had detectable b-wave responses (fig. S7C), suggesting that the rescued rd1 rods are functionally connected to bipolar cells.

To identify whether the ERG signals in the Cas9/RecA-treated rd1 mice are driven by rescued rods or cones, we analyzed the ERG response of transducin α subunit knockout mice (Gnat1<sup>−/−</sup>), in which the rod photoreceptors are not functional. The result revealed that the Cas9/RecA-treated rd1 mice group had an ERG response with the 20-ms light flash of 3.04 × 10⁵ photons/μm², while Gnat1<sup>−/−</sup> mice showed no response at the same light intensity,
indicating that Cas9/RecA could partially rescue the function of rods (fig. S8). To evaluate cones’ contribution to Cas9/RecA-treated rd1 mice ERG, we bred rd1 mice with cDTA mice, in which a cone–diphtheria toxin A (cDTA) transgene was used to eliminate cones from the retinae. The rd1/cDTA retinae under Cas9/RecA treatment did not show any ERG response (fig. S8). We noticed a much more severe rod degeneration in rd1/cDTA mice compared with rd1 only mice, suggesting that loss of cones promotes a more severe rod degeneration in rd1/cDTA retina (fig. S9). Therefore, rd1/cDTA mice are not a good model to evaluate cones’ ERG contribution. Because more than 50% cones survived in the Cas9/RecA-treated rd1 mice compared to wild-type mice (Figs. 3C and 4D), and because rd1 mice cones have normal phototransduction components, it is reasonable to speculate that cones also contributed partially to the ERG signal at high light intensity. The responses of the Cas9/RecA retinae did not reach the same level as those of the wild-type mice, indicating that Cas9/RecA treatment partially restored, but did not fully rescue, the response to light stimulation (Fig. 5D). The mice that received P3 electroporation also displayed a similar improvement in ERG, although the effect was not as strong as that of P0 electroporation (Fig. 5E).

To assess whether the Cas9/RecA-treated mice regained the light response ability, we used the pupillary light reflex (PLR) to analyze the rescue efficiency in rd1 mice at P35. PLR is mediated by intrinsically photosensitive melanopsin-containing retinal ganglion cells (ipRGCs) that also receive inputs from rod-cone photoreceptors. Along with the loss of photoreceptors, PLR was greatly attenuated in rd1 mice. Wild-type mice exhibited light-dependent constrictions at different light intensities, whereas the retina in the Cas9 group exhibited limited eye constrictions due to loss of photoreceptors (fig. S6). Quantification indicated that the Cas9/RecA-treated rd1 mice showed a significantly enhanced PLR ability from 2.55 × 10^4 to 1.70 × 10^6 photons/μm^2·s (Fig. 6B). As the PLR analyses of wild-type, Cas9, and Cas9/RecA mice were conducted under the same conditions, the enhanced PLR response of Cas9/RecA mice was likely the result of the greater number of surviving photoreceptors under this treatment. However, the PLR responses from Cas9/RecA-treated mice were much weaker than those from wild-type mice. Thus, this study provides evidence that Cas9/RecA treatment partially rescued the PLR of rd1 mice.

**DISCUSSION**

In this study, we established a novel HDR-based Cas9/RecA system, which allowed us to precisely correct the Pde6b mutation and rescue photoreceptor degeneration in rd1 mice in vivo. Through this approach, we achieved improved efficient gene correction in Pde6b and restored the PDE6B protein in rd1 mice in a short-term study. Furthermore, the degeneration of photoreceptors was rescued, and visual function was sustained in rd1 mice. In this study, RecA was incorporated into the CRISPR-Cas9 system to increase HDR efficiency. The major functions of RecA are to polymerize ssDNA to form a nucleoprotein filament, determine the homology between the filament and neighboring double-stranded DNA molecules, and enhance the exchange of strands (43). On the basis of these findings, the Cas9/RecA system exhibited higher HDR efficiency than Cas9-mediated HDR in our in vitro and in vivo studies.

Compared with the NHEJ pathway, which exhibits error-prone repair and poor high-fidelity edits, the HDR pathway has a wider application in disease treatment. HDR-based genome editing is advantageous over NHEJ, as it enables precise introduction of any desired gene engineering, such as point mutations and deletions. Here, as an example of editing point mutations, the Pde6b mutation was successfully corrected by the HDR-based Cas9/RecA system. Although CRISPR-Cas9 can potentially generate DSBs at unwanted off-target sites, we did not detect any off-target sequences under Cas9/RecA treatment. This may be because, in our study, HDR was highly specific to the Pde6b sequence in the homology arms of the template DNA. Moreover, the high HDR efficiency of the Cas9/RecA system in the in vitro and in vivo gene editing of Egfp and Pde6b strongly suggests that the broad applicability of Cas9/RecA for many other genes or loci. However, it should be noted that HDR efficiency may vary from target to target. Many factors, such as sequences in and around the target, sgRNA sequences, and distances between insertion sites and DSBs, may affect HDR efficiency of the Cas9/RecA system.

The Cas9/RecA system used Cas9 to induce DSBs and RecA to elevate the rate of homologous recombination. Therefore, Cas9/RecA likely promoted the HDR efficiency in postmitotic neurons. We observed enhanced HDR efficiency on P3 photoreceptors that exited the cell cycle. However, our results demonstrated more
surviving rod photoreceptors in P0 mice than in P3 mice. This could be attributed to the higher HDR efficiency, proliferation ability, and electroporation efficiency of P0 photoreceptors. More cone photoreceptors also survived after Cas9/RecA treatment. The expression of PDE6B was restricted in rod photoreceptors but not in cone photoreceptors. Therefore, the survival of cones in rd1 mice was possibly a secondary effect of the survival of rod photoreceptors. Our data also found that the remaining rod photoreceptors were responsible for the ERG response in rescued rd1 mice (fig. S8). Because more cones survived in Cas9/RecA-treated rd1 mice than in Cas9-treated rd1 mice (figs. 3C and 4D), and cones are functionally normal in rd1 mice (at P14), it is reasonable to speculate that cones also contributed partially to the ERG signal at high light intensity. To precisely evaluate whether cones also contributed partially, further ERG studies need to be done in rd1/Gnat1<sup>−/−</sup> double knock-out mice by Cas9/RecA editing.

Previous studies have shown that the efficient delivery of donor template by virus-mediated single-cell labeling of endogenous proteins via HDR (vSLENDR) can promote HDR in postmitotic cortical neurons (40) and many DNA template copies were delivered in neurons via AAV. In the Cas9/RecA system, we locally delivered more DNA template copies at the target loci than achieved by endogenous HDR because of the recruitment of RecA. Thus, both vSLENDR and Cas9/RecA can efficiently improve HDR efficiency by increasing the copy number of the DNA template. We believe, therefore, that Cas9/RecA HDR efficiency could show further improvement if vSLENDR and Cas9/RecA were combined, delivering RecA and DNA template by AAV. Because the efficiency of HDR is substantially lower than NHEJ, alternative strategies have been developed for targeted sequence insertion via NHEJ using engineered nucleases, where the exogenous DNA fragment is directly ligated at DSBs (25, 44). The homology-independent targeted integration (HITI) NHEJ-based strategy has been used to correct Mertk mutations in an RP disease rat mutant model (25). HITI has more flexibility than Cas9/RecA in gene editing; however, HITI function may not be effective for correction of point mutations, as it relies on the NHEJ repair pathway. Therefore, Cas9/RecA is an effective method for precise gene correction.

Together, our study established Cas9/RecA-mediated genome editing as a tool for precise gene modification. The Cas9/RecA system could potentially act as a therapeutic approach to correct gene mutations for RP and many other inherited diseases. The continued development of this technology to characterize and enhance the safety and efficiency of gene editing will help to realize its promise in treating genetic diseases.

**DNA construction**

The Cas9 expression cassette was cloned from plasmid pX330 (Addgene, 42230) and inserted into FUGW via Bam HI and Eco RI. Genes for RecA-2a-mCherry and sgRNA containing MS2-binding aptamers (sgRNA-ms2) were synthesized in GenScript. RecA-2a-mCherry was cloned into plasmid MS2-P65-HSF1_GFP (Addgene, 61423) via Bam HI and Eco RI. EF1a-MS2-RecA-2A-mCherry was then amplified and inserted into a vector containing sgRNA-ms2 to obtain the plasmid pU6-sgRNA-ms2-EF1a-MS2-RecA-2a-mCherry. RecA was deleted to obtain pU6-sgRNA-ms2-EF1a-MS2-2a-mCherry, which was used as the control plasmid.

**Fluorescence-activated cell sorting**

After the cell transfection experiment, cells were digested into a single-cell suspension and collected before acquisition on a flow cytometer. Cells were sorted and counted on a BD FACScan flow cytometer (FACSaria II) (BD Biosciences). We used the 561- to 614-nm channel to detect mCherry signal, the 355- to 448-nm channel for BFP, and the 488- to 513-nm channel for EGFP. Data were acquired with BD CellQuest Pro software (BD Biosciences) and analyzed with FlowJo flow cytometry analysis software (Tree Star).

**In vitro gene editing**

The HEK293FT cells stably expressing BFP were used as reporter cells for the editing experiments. Reporter cells were placed in 24-well plates 24 hours before transfection. Cell transfection was performed using Lipoctamine 3000 in accordance with the manufacturer’s instructions. Briefly, DNA was initially mixed with reagent p3000 in Opti-MEM and then mixed thoroughly with Lipofectamine 3000 at room temperature. The mixture was then added into each well after 10 min of incubation. Fluorescence status was measured on day 3 after transfection for editing ratio analysis.

**In vivo electroporation**

Newborn mouse pups (P0 or P3) were anesthetized by chilling on ice, and a small incision was made in the eyelid and sclera near the lens with a needle. DNA solutions (5 μg/μl of each plasmid) in phosphate-buffered saline (PBS) were injected into the subretinal space through the incision using a Hamilton syringe with a 33- or 34-gauge blunt-ended needle under a dissecting microscope. For P0 mouse pups, 0.5 μl of DNA was injected; for P3 mouse pups, 0.8 μl of DNA was injected. Customized tweezer-type electrodes briefly soaked in PBS were placed to softly hold the heads of the pups, and five square pulses of 50-ms duration with 950-ms intervals were applied using a constant voltage power supply triggered by a pulse generator.

**Off-target detection**

An online database (https://cm.jefferson.edu/Off-Spotter/) was used to predict the potential off-target sites. Several of the most likely sites (table S2) were selected from many potential off-target sites. The selected sites had to satisfy the following conditions: The sites must be located in the coding region of the exon in the GRCm38 genome database, the five bases at the 3’ terminal (seed region) of sgRNA must be free from mismatch, and the number of mismatched bases must be greater than or equal to four. The proximal regions of these selected potential off-target sites were amplified by PCR, followed by sequencing. The results were compared with wild-type controls to confirm whether real off-target sites existed.

**MATERIALS AND METHODS**

**Animals**

The rd1 (C57BL/6), Nrl-EGFP (C57BL/6), and cDTA (C57BL/6) mice were gifts from K.-W. Yau (Johns Hopkins University). All animal experiments (no. USTCACUC1801027) were approved by the Institutional Animal Care and Use Committee at the University of Science and Technology of China. All adult mice were housed three to five per cage and maintained under a 12-hour light/dark cycle with ad libitum access to food and water. The mice used in this study were of both sexes. Mouse pups from birth were designated as P0. All the primers for mouse genotyping and DNA or cDNA sequencing were shown in table S1.

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Smart-seq2
Collected EGFP+ cells were mixed with 4 μl of cell lysis buffer containing 0.1 μl of ribonuclease (RNase) inhibitor, 1.9 μl of a 0.2% (v/v) Triton X-100 solution, 1 μl of oligo-dT30VN and 1 μl of deoxynucleotide triphosphate mix. The mixture was incubated at 72°C for 3 min, and then 5.7 μl of reverse transcription (RT) buffer (0.5 μl of SuperScript II reverse transcriptase, 0.25 μl of RNase inhibitor, 2 μl of 5× SuperScript II first-strand buffer, 0.5 μl of 100 mM dithiothreitol, 2 μl of 5 M betaine, 0.06 μl of 1 M MgCl2, 0.1 μl of 100 μM template-switching oligos, and 0.29 μl of nuclelease-free water; Thermo Fisher) was added into the tube for RT. RT-PCR was done at 40°C for 90 min, and 10 cycles were done at 50°C and 42°C switch with each temperature for 2 min. After an enzyme inactivation step at 72°C for 15 min, the cDNAs were preamplified with IS PCR primer for ~20 cycles using KAPA HiFi HotStart ReadyMix (Roche). Amplified cDNAs were purified with DNA beads.

Preparation of retinal sections and immunostaining
Electroporated retinas were harvested at P31 after electroporation and dissected under a fluorescent microscope (Leica, spm8) to select EGFP+ retinae. Dissected retinae were fixed with 4% paraformaldehyde in PBS for 2 hours at 4°C, cryoprotected in PBS containing 30% sucrose for several hours at 4°C, and embedded in optimum cutting temperature compound (Sakura, Torrance, CA, USA) on dry ice. Cryosections (18 μm) were cut on a cryostat (Leica, CM3050S). Sections were stained with anti-swOPN (1:500; Millipore, catalog no. AB5405), anti-mwOPN (1:500; Millipore, catalog no. AB5407), and anti-GNAT1 antibodies (1:200; Santa Cruz Biotechnology, sc-389). Sections were incubated with secondary antibody Alexa Fluor 568 goat anti-rabbit IgG (H+L) (1:500; Thermo Fisher, catalog no. A11036) for fluorescence detection.

Electroretinography
Dark-adapted P14 mice were sacrificed by CO2 asphyxiation or cervical dislocation in dim red light, after which the eyes were enucleated. The neuroretinae were removed from the eyecup for ERG recordings and then transferred (photoreceptor side up) to a recording chamber with a 100-μm hole connected to the bottom chamber. For ERG recordings, we used Ames’ solution (120 mM NaCl, 22.6 mM Na2CO3, 3.1 mM KCl, 0.5 mM KH2PO4, 1.5 mM CaCl2, 1.2 mM MgSO4, and 6 mM glucose) for both chambers. To isolate the photoreceptor component (a-wave) of the retina, we added STBs [20 μM L-(-)-2-amino-4-phosphonobutyric acid (L-AP4) to the solution to block on-bipolar cell signals, 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block AMPA/kainate receptors, and 20 μM ε-2-amino-5-phosphonovalerate (p-AP5) to block N-methyl-D-aspartate receptors]. Barium chloride (200 μM) was added to block Müller cell signals. We stimulated the photoreceptors with calibrated 20-ms flashes of 470 nm, and light intensities included 12, 39.5, 236, 1.06 × 104, 3.04 × 105, 2.90 × 106, 1.61 × 107, 8.74 × 107, and 4.48 × 108 photons/μm2.s. The signals were amplified, low-pass–filtered at 20 Hz (eight-pole Bessel), and digitized at 100 Hz for further analysis.

Pupillary light reflex
Mice were kept in 12:12-hour light/dark cycles, and after more than 1-hour dark adaptation, the PLR was performed from 2 hours after lights on to 2 hours before lights off. To measure the PLR of the Cas9 group and Cas9/RecA mice, we built a pupillometer with a miniature, infrared charge-coupled device camera and 850-nm light-emitting diode light for video recording via a Ganzfeld sphere; used a 550-nm light flash for 3 s with light intensities of 271, 3932, 25,505, 25,5047, 1,700,310, and 19,447,300 photons/μm2.s; and compared the proportion of pupillary before and after light stimulation.

Western blotting
Whole retinal extracts were fractionated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using transfer apparatus, according to the manufacturer’s protocols (Bio–Rad). After incubation in 5% nonfat milk and TBST [10 mM tris (pH 8.0), 150 mM NaCl, and 0.5% Tween 20] for 60 min at room temperature, the membrane was washed once with TBST and incubated with antibodies against PDE6B (1:1000; Thermo Fisher) or actin (1:2000; Thermo Fisher) at 4°C for 12 hours. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (1:1000; Jackson ImmunoResearch) for 2 hours at room temperature. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer’s protocols.

Statistical analyses
Cell numbers and data from ERG and PLR were analyzed using unpaired Student’s t tests. FACs was analyzed using paired Student’s t tests. P < 0.05 indicated statistical significance. The error bars represent ±SEM. No statistical methods were used to predetermine sample size. The mice were randomly assigned to the different experimental groups.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/4/eaav3335/DC1
Fig. 51. TH4 sequencing in rd1 mice.
Fig. 52. Gpr179 sequencing in rd1 mice.
Fig. 53. Examination of potential mutations induced by virus insertion in Pde6b cDNA from the rd1 mice.
Fig. 54. Representative images of retinal electroporation.
Fig. 55. Sequencing results at predicted off-target sites.
Fig. 56. Representative section images of mouse retinae.
Fig. 57. Visual function of wild-type and rd1-Cas9/RecA mice evaluated by ERG with different blockers at P14.
Fig. 58. Visual function of wild-type, cDTA, Gnat1−/−, rd1-Cas9/RecA, rd1-Cas9, and rd1/cDTA-Cas9/RecA mice evaluated by ERG with STBs in different intensities at P14.
Fig. 59. Immunostaining with rod photoreceptor marker in rd1 and rd1/cDTA mice at P20.
Table S1. DNA sequences of primers and oligos.
Table S2. Potential off-target sites from mouse coding sequence predicted by the Off-Spotter database.

REFERENCES AND NOTES
1. S. van Soest, A. Westerveld, P. T. V. M. de Jong, E. M. Bleeke-Wagemakers, A. A. B. Bergen, Retinitis pigmentosa: Defined from a molecular point of view. Surv. Ophthalmol. 43, 321–334 (1999).
2. M. Kalloniatis, E. L. Fletcher, Retinitis pigmentosa: Understanding the clinical presentation, mechanisms and treatment options. Clin. Exp. Optom. 87, 65–80 (2004).
3. L. Lagnado, The Wellcome Prize Lecture. Visual signals in the retina: From photons to synapses. Exp. Physiol. 85, 1–16 (2000).
4. M. Danciger, J. Blaney, J. R. Heckenlively, S. G. Jacobson, D. B. Farber, Mutations in the PDE6B gene in autosomal recessive retinitis pigmentosa. Genomics 30, 1–7 (1995).
conducting the project in the early stage. L.L. collected the Nr1-EGFP rods for DNA sequencing. H.Z. and B.J. contributed to the ERG experiment. All authors edited and proofread the manuscript. Competing interests: T.X., Z.Q., T.C., and Y.C. are inventors on a pending patent related to this work filed by the University of Science and Technology of China (no. 201811144025.3, filed on 29 September 2018). The authors declare no other competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the lead contact T.X.

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