

Efficient Mutagenesis of the Rhodopsin Gene in Rod Photoreceptor Neurons in Mice

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ABSTRACT

Dominant mutations in the rhodopsin gene, which is expressed in rod photoreceptor cells, are a major cause of the hereditary blinding disease, autosomal dominant retinitis pigmentosa. Therapeutic strategies designed to edit such mutations will likely depend on the introduction of double-strand breaks and their subsequent repair by homologous recombination or nonhomologous end joining. At present, the break repair capabilities of mature neurons, in general, and rod cells, in particular, are undefined. To detect break repair, we generated mice that carry a modified human rhodopsin-GFP fusion gene at the normal mouse rhodopsin locus. The rhodopsin-GFP gene carries tandem copies of exon 2, with an IScel recognition site situated between them. An IScel-induced break can be repaired either by nonhomologous end joining or by recombination between the duplicated segments, generating a functional rhodopsin-GFP gene. We introduced breaks using recombinant adeno-associated virus to transduce the gene encoding IScel nuclease. We found that virtually 100% of transduced rod cells were mutated at the IScel site, with about 85% of the genomes altered by end joining and 15% by the single-strand annealing pathway of homologous recombination. These studies establish that the genomes of terminally differentiated rod cells can be efficiently edited in living organisms.

INTRODUCTION

Defects in the rhodopsin gene cause the most common form of the most common hereditary blinding disease, retinitis pigmentosa (RP) (1,2). RP is a progressive neurodegenerative disorder that begins with the death of rod photoreceptors, where rhodopsin is expressed, but ultimately destroys rod and cone cells, leading to loss of both dim-light and color vision. All but a handful of the more than 100 rhodopsin alleles that cause retinal degeneration display dominant inheritance (1,2). It is clear that dominance arises from the harmful effects of mutant rhodopsin, rather than from a lack of adequate protein levels, since mice and human patients heterozygous for rare, null alleles of the rhodopsin gene do not suffer from serious degeneration (3-5), even though mice with this genotype express only about half the normal amount of rhodopsin (4). Thus, it is the presence of rhodopsin encoded by the dominant allele that leads to degeneration, although it remains an open question whether its deleterious consequences are due to toxic gain-of-function or interfering dominant-negative effects (6). In either case, correcting the defective allele, or knocking it out, would be expected to preserve retinal function by diminishing the loss of rod cells, which in turn would protect cones and extend the useful lifetime of central vision.

A promising general strategy for editing the genome in living cells is to introduce a double-strand break (DSB) into the target gene and exploit the normal cellular DSB repair processes to bring about the desired change (7-11). Repair of the break by homologous recombination (HR), in the presence of a nonmutant segment of DNA to act as the template for repair, can correct the mutation (11). Alternatively, repair by nonhomologous end joining (NHEJ), which is usually mutagenic, can knock out gene expression (12). Although HR and NHEJ have been well defined in cycling mammalian cells (13), their characterization in mature neurons, which no longer divide, is in its infancy (14). At present, it is uncertain whether terminally differentiated rod photoreceptor neurons possess the requisite capabilities for DSB-dependent genome editing.

It is clear that the developing nervous system is exquisitely sensitive to DSBs, and that DSB repair by HR and NHEJ is critical to prevent cell death by

apoptosis (15). Defects in either repair pathway, or the signaling pathways that control them, can lead to neurological defects and disease in humans and mouse models (15-17). For example, mutations in the ATM gene in humans lead to defects in the signaling response to DSBs, which causes ataxia telangiectasia, a progressive neurodegenerative disease that compromises the function of the cerebellum. Although HR and NHEJ play important roles in the proliferation and early stages of differentiation of the nervous system, their contribution to genome maintenance in the mature nervous system is poorly defined (14). Because terminally differentiated, nondividing nerve cells in the brain are mixed with a comparable number of glial cells that retain mitotic capability, analysis of DSB repair in neurons is inherently challenging. However, extracts from unfractionated and neuron-enriched rat brain samples display NHEJ activity, which declines with age (18,19). These studies suggest that the NHEJ pathway of DSB repair is probably functional in mature neurons.

Studies in cycling cells, the source of virtually all our detailed information on DSB repair in mammalian cells, indicate that HR functions primarily in the S and G2 phases of the cell cycle, when a sister chromatid—the preferred template for HR repair—is available (20-22). Since neurons are trapped in G1/G0, it is unclear *a priori* whether HR is a viable strategy for modifying the rhodopsin gene in rod photoreceptors. The block to HR in G1 in cycling cells appears to be enforced at the step of 5' to 3' resection of the ends, which is required to expose the single-stranded DNA needed for the strand-invasion step in HR (23-25). By contrast, end resection is not needed for NHEJ, which operates throughout the cell cycle (13,20-22).

These considerations led us to expect that DSBs in rod photoreceptor cells would likely be repaired predominantly by mutagenic NHEJ and rarely, if at all, by HR. Thus, we designed a mouse model that would allow us to detect common NHEJ events by PCR-based methods and rare HR events by an exquisitely sensitive fluorescent assay that is capable of providing a reliable readout for one or a few events per retina (26,27). To detect HR repair of DSBs, we replaced one mouse rhodopsin gene with a modified form of the human

rhodopsin gene fused to enhanced GFP (26). This modified rhodopsin-GFP gene carries two copies of exon 2, which flank the recognition sequence for the rare-cutting nuclease, IScel. The duplicate copy of exon 2 shifts the reading frame so that rhodopsin-GFP is not expressed. Break-induced recombination between the duplicated segments will reconstitute a functional rhodopsin-GFP gene, giving rise to a bright fluorescent green outer segment on each corrected rod cell. This recombination substrate is expected to generate green rods by the single-strand annealing (SSA) pathway of HR. SSA, like the Rad51-mediated strand-invasion pathway of HR, begins by resection of ends at a DSB, but completes the repair event by pairing the exposed, single strands at complementary regions, thereby bypassing strand invasion. Thus, by assaying for the SSA pathway of HR, this duplication substrate tests whether end resection occurs during DSB repair in rod cells, which is a prerequisite for genome editing by HR.

Using recombinant adeno-associated virus (rAAV) to introduce the IScel gene, we addressed three questions about DSBs in terminally differentiated rod photoreceptor neurons. Can DSBs be efficiently introduced into the rhodopsin gene? Can NHEJ and SSA repair DSBs in rod cells? And if so, what are their relative frequencies? Here, we show that most, if not all, rAAV-transduced rod cells were cleaved at the target site, with 85% of the DSBs repaired by NHEJ and 15% by SSA. The high frequency of DSB repair by mutagenic NHEJ lays the foundation for plausible therapeutic strategies designed to treat RP by knocking out dominant rhodopsin alleles in rod cells in patients. The surprisingly high frequency of DSB repair by SSA indicates that end resection is unlikely to block HR in rod cell neurons, raising the possibility that correction of mutant rhodopsin alleles by HR may also be achievable. Collectively, these results establish that the genomes of terminally differentiated rod photoreceptor neurons can be efficiently edited in living organisms.

MATERIALS AND METHODS

Generation of ID2-hRho-GFP Knock-In Mice. All animal procedures were carried according to protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee, and in accordance with the Statement for the Use of Animals in Ophthalmic and Visual Research of the Association for Research in Vision and Ophthalmology. Knock-in mice were generated by gene targeting in HPRT⁻ embryonic stem (ES) cells. To make the targeting vector, we modified a previously described segmental replacement vector (26), which carried the human rhodopsin-GFP fusion gene flanked by LoxP and Lox511 sites, by inserting a duplicate copy of exon 2 generated by PCR. The PCR amplified segment comprised 613 bp of rhodopsin sequences, which included exon 2 along with 282 bp of 5' and 162 bp of 3' flanking sequences, and carried an EcoRV site and an IScel site at its 5' end and an EcoRV site at its 3' end. We inserted this PCR segment 706 bp downstream of the endogenous exon 2 into an artificial EcoRV site that was made by point mutation (AATATC to GATATC) to generate the ID2-hRho-GFP gene. To construct the gene-targeting vector, we excised the 8-kb ID2-hRho-GFP gene from its plasmid by NotI digestion and cloned it into the NotI site at 3' end of the HPRT minigene in the first-step targeting vector described previously (26). This cloning step generates a vector that contains the ID2-hRho-GFP gene adjacent to a LoxP-flanked HPRT minigene, with 4.1 kb of upstream and 6.5 kb of downstream sequences from the mouse rhodopsin locus (**Fig. 1A**). We attached the herpes virus TK gene to one end of the construct to allow selection against random integrants. The targeting vector contains a pBluescriptSK plasmid backbone.

We modified the rhodopsin locus in the HPRT-negative ES cell line AB2.2 123, which was derived from mouse strain 129SvEv, by homologous recombination so that the endogenous mouse rhodopsin gene was replaced by an 11.9 kb SacI-HindIII fragment containing the LoxP-flanked HPRT minigene linked to the ID2-hRho-GFP gene (**Fig. 1A**). The Darwin Transgenic Core Facility, Baylor College of Medicine, electroporated ES cells in the presence of 15 μ g linearized targeting vector, and grew the treated cells on mitomycin C inactivated SNLP 76/7-4 Puro feeder cells. Selection for HPRT⁺TK⁻ cells was applied after 24 hours by growth in HAT medium (0.1 mM hypoxanthine/0.4 μ M aminopterin/16 μ M thymidine) plus 0.2 μ M 1-(2-deoxy-2-fluoro-1-

D-arabinofuranosyl)-5-iodouracil (FIAU) for 7 days, followed by growth in HT medium (0.1 mM hypoxanthine/16 μ M thymidine) for 4 days.

We screened colonies that survived selection by Southern blotting after DNA from individual colonies had been digested with a restriction enzyme (BamHI for the 5' end and HindIII for the 3' end), electrophoresed on 0.7% agarose gels, and blotted onto Nylon membranes (Hybond-N+, Amersham Biosciences). To identify correctly targeted clones at the 5' end, we probed blots with the 1.1-kb BamHI-SacI mouse genomic fragment upstream of the 5'-flanking homology used in the targeting vectors. For the 3' end, we used a 1.0-kb BglII-EcoRI DNA probe, which is a subfragment of the 6.5-kb 3'-flanking homology used in the targeting vector. All probes were labeled by random priming in the presence of α -³²P-dCTP (Megaprime DNA labeling system, Amersham Biosciences).

The Darwin Transgenic Core injected modified ES cells into blastocysts from albino C57BL/6-Tyr^{c-Brd} mice (28). We then tested chimeric males for germline transmission by crossing them with C57BL/6-Tyr^{c-Brd} mice and observing coat color and genotyping by PCR (**Fig. 1C**). We generated homozygous HPRT-ID2-hRho-GFP (HPRT-ID2) mice by crossing F1 progeny. We removed the HPRT minigene in two steps. By crossing appropriate mice, we combined the HPRT-ID2 allele with a transgene (GDF-9-iCre) that expresses iCre from the GDF-9 promoter, which drives expression in the female germline (29). Mice expressing the transgene were a gift from Dr. Austin J. Cooney. We then bred female HPRT-ID2/GDF-9-iCre mice to wild type C57Bl/6 mice to generate mice that had eliminated the HPRT minigene. PCR analysis of progeny identified ID2-hRho-GFP mice that were missing the HPRT minigene and the GDF-9-iCre transgene (**Fig. 1E**, pup 4).

See Supplementary Materials and Methods for details of mouse genotyping and histology.

AAV-IScel Vector Construction and Subretinal Injection. The recombinant adeno-associated virus rAAV-IScel carries AAV2 inverted terminal repeats in pseudotyped AAV5 capsids and expresses IScel from the proximal murine rod opsin promoter (-385 to +86), which drives expression of the recombinant gene product almost exclusively in

photoreceptors (30). We built the plasmid precursor of rAAV-IScel using the pTR-MOPS500(E-)GFP vector (30). We began by inserting a double-stranded oligonucleotide (5'-NotI-EcoRI-BamHI-Sall) in place of the multi-cloning sites between the NotI and Sall sites in phagemid pBlueScript SKII. We used EcoRI and BamHI to excise a 0.87-kb fragment containing the IScel transcription unit with the 3x nuclear localization signal from plasmid pCMV-3x nls-IScel (7), and subcloned it into the modified pBlueScript SKII vector. We then digested the resulting construct with NotI and Sall to liberate the IScel fragment and inserted it into the pTR-MOPS500(E-)GFP vector in place of the existing 1.3-kb NotI-Sall fragment that encoded EGFP. We used the resulting pTR-MOPS500-IScel vector to generate rAAV-IScel (2.25×10^{12} viral particles per ml) by standard procedures (30). The pTR-MOPS500(E-)GFP vector was used to generate rAAV-GFP (1.38×10^{14} viral particles per ml).

For subretinal injections, we followed established procedures (31) with some modification (Supplementary Materials and Methods). We performed subretinal injections under direct observation via a dissecting microscope (SMZ-800 Zoom Stereo Microscope, Nikon Instruments Inc.) at 30x magnification. We injected about 0.7 μ l to 1 μ l of rAAV-GFP or rAAV-IScel subretinally using UltraMicroPump II for mice of 8 to 12 g. After all injections, we treated mice daily with 1% atropine eye drops and neomycin/polymyxin B/bacitracin ophthalmic ointments for 3 days. At 4 to 27 weeks post injection, we prepared retinal whole mounts for examination.

Fluorescence Microscopy. Retinal whole mounts were prepared 4 to 27 weeks after injection from eyes fixed in 4% paraformaldehyde, by removing the cornea, lens, and vitreous and dissecting the retina from the eyecup. We placed the retina on a slide with the photoreceptors up, flattened it by making radial incisions, and mounted it with Gel/Mount (Biomedex, Foster City, CA). To determine the frequency of fluorescent green rods, we captured multiple images from each injected retina using an LSM 510 laser-scanning confocal microscope (Zeiss). The affected area of retinas, as judged by the presence of green rods, ranged from less than 5% to more than 80% of the overall retina in different samples; however, we quantified green rods only in the transduced areas. For rAAV-GFP injected controls, we collected one retina for each time point,

except the 4-week sample, for which we collected two. For rAAV-ISceI injected mice, we examined 2 to 5 retinas for the 6-, 7-, 8-, 10- and 12-week time points and one retina each for the 4-, 5-, 18-, 19-, 24-, 26- and 27-week time points. We examined retinas using either a 40x or a 63x objective and a 10x eyepiece. We manually counted all the green rods in the 50 μm x 50 μm field of the 63x image or in the 230 μm x 230 μm field of the 40x image. We counted several different fields for each retina, adjusted the counts to the 40x image, and averaged them.

PCR analysis of DSB-repair events. We designed nested sets of primers to detect the products of SSA and NHEJ. Primer sites and product sizes are shown schematically in **Figure 3A** (see Supplementary Materials and Methods for primer sequences). PCR analysis of SSA and NHEJ products in bulk samples is given in Supplementary Materials and Methods. For small-pool PCR analysis, we isolated DNA from retinal whole mounts that had examined by confocal microscopy and shown to have a substantial proportion of fluorescent green rods. Slides were washed in 1x PBS for 10 min to loosen the cover slip, and the mounted retinas were scooped up with a razor blade and washed several times to remove mounting media. We extracted DNA from the recovered retinas using the same protocol for isolating tail DNA. We treated individual retinas with 250 μg of proteinase K in 500 μl of lysis buffer containing 50 mM Tris, pH 7.5, 50 mM EDTA, pH 8.0, 100 mM NaCl, 5 mM DTT, 0.5 mM spermidine, and 1% SDS at 55°C overnight with constant agitation. The lysate was then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), and the high molecular-weight DNA was precipitated and spooled out by adding 0.15 volumes of 7.5 M NH_4OAc and 3 volumes of absolute alcohol. The DNA was rinsed successively in 70% alcohol and 100% alcohol, and then air-dried and dissolved in 250 μl TE.

To eliminate unmodified genomes from the analysis, we digested 5 μg of retinal DNA at 37°C with 30 units of ISceI endonuclease for 3 hours, and then added 30 more units of enzyme and digested the sample overnight. We then extracted the digestion mixture once with phenol-chloroform-isoamyl alcohol, precipitated it with alcohol, and dissolved in TE. We then repeated this ISceI-digestion protocol once more. Finally, we

digested this DNA with BamH1, which does not cleave within the amplified region, to reduce viscosity and maintain consistency during serial dilutions.

For small-pool PCR, we initially set up 7 reactions each at DNA concentrations of 100 pg, 50 pg, 25 pg and 12.5 pg per reaction in order to determine the optimum dilution to use for each retina, so that each reaction contained less than 2 amplifiable genomes on average. We used a mixture of three PCR primers, two forward primers (P1 and P6) and a reverse primer (P2), to amplify genomes repaired by SSA and NHEJ (**Fig. 3A**). Primers P1 and P2 yield a 687-bp band for SSA products; primers P6 and P2 yield a variety of bands near 997 bp for NHEJ products, depending on the extent of modification at the repaired junction. Primers P1 and P2 could potentially generate a 1.8-kb band from NHEJ products, but we selected PCR conditions that were not favorable for this long product. We used Roche's Expand Long Template PCR System (Roche, Cat. No. 11 681 842 001) with buffer 2, 1.25 units of enzyme, 0.54 mM dNTPs, and 0.63 μ M for each primer, in a final reaction volume of 15 μ l. The PCR program was 94°C for 2 min for initial denaturation, followed by 15 cycles of 94°C for 10 sec, 60°C for 30 sec, 68°C for 45 sec, then another 15 cycles with 1 sec extra extension time per each additional cycle, and one final cycle at 68°C for 7 min. We divided the PCR products into two portions: one for small-pool PCR analysis and the second for follow-up IScel digestions. For small-pool PCR analysis, samples were separated by electrophoresis on 1.5% TAE agarose gels at 25 V for 18 hours, transferred to a 0.45 μ m nylon membrane (Magnaprobe, GE Water & Process Technologies), and hybridized with α -³²P-dCTP labeled 687-bp PCR DNA, which was generated using primer P1 and P2 to amplify the 687-bp band from plasmid pCR2.1-TOPO-687, which carried the SSA product. The positions of bands were visualized by phosphorimaging.

To determine whether bands near the 997-bp marker arose by amplification of an NHEJ product or a genome with an intact IScel site, we re-amplified the portion of the sample we had set aside, using the same conditions as before, except in a 50 μ l reaction volume and using 25 cycles of amplification in the second step of PCR using the nested primers P8/P3. We then digested the amplified material with IScel, which would cut an intact genome into 312-bp and 644-bp fragments. Cutting was assessed by electrophoresis on an agarose gel.

Analysis of individual green rods. We collected retinas from ID2-hRho-GFP mice injected with AAV-ISceI 7 to 8 weeks post-injection, and dissociated them using a modification of a previously described protocol (32). In brief, we pooled 2 to 3 injected retinas and incubated them for a short time in a pronase solution at 37°C. We dissociated the pronase-treated retinas at room temperature by two cycles of 50 passages through a 1 ml pipette tip, pelleting of the retinal fragments by centrifugation, and removal of the supernatant for the next cycle of treatment. About 0.5 ml of the combined cell suspensions was spread over a 50-mm glass bottom dish (MatTek Corporation). We picked individual green rods under the fluorescent microscope (Nikon Eclipse TE2000-U), using a motorized micromanipulator system (MP-225, Sutter Instrument) and a micropipette with a 5-6 μm inner diameter and 30-degree bend at the tip (Origio Humagen Pipets). We then ejected the picked green rod into a 100- μl PCR tube (Applied Biosystems) containing 2 μl of denaturing reagents. Rhodopsin cDNA was prepared using the QIAGEN OneStep RT-PCR kit. We amplified the cDNA using primers to GFP and exon 1 of human rhodopsin. The initial product was further amplified using nested primers within GFP and exon 1. Products were analyzed by gel electrophoresis, with bands at 960 bp indicating mRNAs with two copies of exon 2 and bands at 791 bp indicating mRNAs with one copy of exon 2, consistent with SSA. We sequenced the bands to verify their structure. A detailed description of this protocol is included in Supplementary Materials and Methods.

RESULTS

The ID2-hRho-GFP mouse model. We generated mice carrying a human rhodopsin-GFP fusion gene with a duplicated copy of exon 2 (ID2-hRho-GFP) by homologous replacement of the endogenous mouse rhodopsin gene in a two-step procedure, as outlined in **Figure 1A**. The targeting construct carries a modified version of the human rhodopsin gene linked to the sequences that flank the mouse rhodopsin gene. We modified the genomic human rhodopsin gene in three ways. First, we fused the gene for enhanced GFP to the C-terminus of the rhodopsin gene, so that the encoded rhodopsin protein would be linked to GFP through the peptide APVAT, as described previously (26). Second, we inserted a 613-nucleotide segment encompassing exon 2 into intron 2 to create an internal duplication, with the two segments separated by 564 nucleotides that include the recognition site for the rare-cutting nuclease IScel. The predicted splicing pattern for the modified rhodopsin would create an mRNA with a termination codon spanning the junction between the two copies of exon 2, which would encode a rhodopsin fragment terminating at amino acid 177 (equivalent to Y178ter). Third, at the 5' junction between mouse and human rhodopsin sequences in the middle of the upstream untranslated region, we inserted a minigene encoding hypoxanthine-guanine phosphoribosyl transferase (HPRT) flanked by LoxP sites to serve as a selectable marker for manipulation of ES cells. Cre-mediated removal of the marker leaves behind a single copy of LoxP, giving rise to an mRNA that is translated about 5-fold less efficiently than an mRNA lacking LoxP (26). We designed this modification to lessen the possibility that the rhodopsin fragment made in the parent mice might cause retinal degeneration on its own, interfering with our assays for DSB repair. From previous results, we knew that expression of the reduced level of human rhodopsin-GFP (as would be generated if a DSB were repaired by HR) would be readily detectable (26,27).

We generated modified mouse ES cells by gene targeting (**Fig. 1A**) and identified clones that were properly targeted at both their 5' and 3' ends by Southern blotting (Fig. 1B). Blastocyst injection of these modified ES cells generated chimeric mice, some of which displayed germline transmission of the

modified human rhodopsin-GFP gene (**Fig. 1C**). Offspring carrying floxed HPRT in the ID2-hRho-GFP allele (HPRT-ID2) were bred to mice with an iCre transgene driven by the GDF-9 promoter, which is expressed in the female germline (29), to generate mice that carried both genes (**Fig. 1D**, pup 1). Female HPRT-ID2/GDF-9-iCre mice were crossed to wild type mice and pups were analyzed by PCR to identify those that were missing HPRT (ID2-hRho-GFP) and that did not carry GDF-9-iCre (**Fig. 1E**, pup 4). These mice were bred to homozygosity to maintain the line. For all experiments, they were crossed to wild type mice to generate heterozygotes that carry one ID2-hRho-GFP allele and one normal mouse allele.

To verify that the ID2-hRho-GFP allele was compatible with the development and maintenance of a healthy retina, which is essential for measuring frequencies of DSB repair over time, we examined retinal sections from mice that were heterozygous for ID2-hRho-GFP. The morphology of ID2-hRho-GFP retinas was normal. In addition, the number of nuclei in the outer nuclear layer at one month was normal, and the rate of loss of nuclei was slight and indistinguishable from the rate in mice that expressed nonmutant hRho-GFP (**Fig. S1**). Thus, the ID2-hRho-GFP allele should serve as an acceptable marker for experiments to detect DSB repair: neither the mutant allele nor the corrected version is expected to adversely affect the lifespan of rod cells.

Spontaneous reversion of the ID2 mutation. To establish a baseline against which to measure repair of DSBs by SSA, we examined untreated retinas from ID2-hRho-GFP mice to determine the frequency with which the internal duplications spontaneously resolve into a gene structure that can express hRho-GFP. We prepared whole mounts of 27 retinas from mice at ages ranging from 4 to 52 weeks and scanned them by fluorescence microscopy to identify rods displaying GFP fluorescence, referred to here as “green rods.” We observed a total of 360 events, for an average of 13.3 events per retina. By fluorescence and confocal microscopy, we distinguished three categories of event: tight groups of rods containing from four to more than 30 green rods per cluster (28 events);

doublets of rods, the minimal cluster (200 events); and single rods (132 events) (**Fig. 2A**). Clusters (including doublets), which account for 63% of all events, presumably arose by HR during proliferation of retinal progenitor cells, which divide to generate columns of retinal neurons during differentiation of the retina (33,34). As expected since they were generated in progenitor cells, the numbers of clusters show no age dependence over the range of observation. Surprisingly, however, the same is true for single rods, suggesting that these spontaneous events may occur much more frequently in terminally differentiated cells shortly after the last cell division than at later times in the fully developed cells. In any case, this analysis establishes that spontaneous green rods in the retinas of ID2-hRho-GFP mice are rare. Given that mouse retinas contain about 6.4 million rods (35), spontaneous events that generate green rods (13.3 per retina) occur at frequency of about 2×10^{-6} . This very low frequency defines the baseline against which to compare DSB-induced SSA events.

Repair of DSBs in the ID2-hRho-GFP gene. To induce DSBs in the target rhodopsin gene, we injected rAAV-IScel subretinally in 3 to 4 week old ID2-hRho-GFP mice and followed the appearance of fluorescent green rods for several months by confocal microscopy. Expression of passenger genes from subretinally injected rAAV typically takes up to 3 weeks—a delay thought to be due to the slow conversion of rAAV DNA to a transcriptionally active, double-stranded form in rod cells (30). As shown in **Figure 2B**, green rods first appeared at about 4 weeks post injection and continued to increase in frequency over time. To measure the efficiency of rAAV transduction, we injected rAAV-GFP and followed appearance of rods expressing GFP (**Fig. 2C**). We calculated frequencies as the number of green rods per area observed in the 40x objective field ($230 \mu\text{m} \times 230 \mu\text{m}$), which contains about 23,000 rod photoreceptors (35), and plotted them as shown in **Figures 2D** and **2E**. GFP expression after subretinal injection of rAAV-GFP (**Figure 2E**) indicates that 22% of rods were transduced by 6 months after treatment. At that time, 3.3% of rods expressed hRho-GFP (**Figure 2D**), indicating that about $3.3/22 = 15\%$ of transduced rods

had been modified to express hRho-GFP. Thus, the frequency of green rods among transduced cells is more than 70,000-fold above the frequency of spontaneous green rods. Because rAAV vectors have been shown to persist and express their passenger genes robustly for at least 6 months in the mouse retina (36), the simplest explanation for the plateau in the number of events seen in **Figure 2D** is that by six months ISceI has cleaved the genome in all the rod cells where it is expressed. Those events that have not undergone repair by SSA have been repaired by mutagenic NHEJ, leading to loss of the ISceI site, an explanation confirmed by the analysis of the products presented below.

Products of ISceI-induced DSBs. We designed the ID2 target so that delivery of a DSB at the ISceI site between the duplicated segments would stimulate recombination via SSA, giving rise to green rods. In addition, we expected mutagenic NHEJ to generate alterations at the site of ISceI cleavage. To determine which of these potential products had been generated by treatment of ID2-hRho-GFP mice with rAAV-ISceI, we analyzed ISceI-resistant genomes, since both SSA and NHEJ products would eliminate the ISceI site. Digestion with ISceI was carried out to remove the background of ISceI-sensitive genomes that come from non-rod cells (about 22% of retinal cells (35)) and from rod cells that had not been transduced.

We subjected DNA from treated retinas to 2 or 3 cycles of ISceI digestion and PCR amplification of the region around exon 2 (**Fig. S2**). To enrich for the products of SSA (**Fig. 3A**), we used primers P1/P2 in the initial round of amplification and primers P1/P3 subsequently. The 657-bp band corresponding to the putative SSA product was purified from the gel after the final cycle and cloned (**Fig. S2**). DNA sequence analysis revealed that the cloned DNA matched the expected product of recombination between the two copies of exon 2, as illustrated in **Figure 3A**. These results confirm that SSA between duplicated segments occurs in rod cells. To verify that the genomes in green rods had structures consistent with their generation by SSA, we isolated 13 individual green rods from dissociated retinas and analyzed their hRho-GFP mRNA by RT-

PCR, using primers in exon 1 and GFP, as shown for 8 rods in **Figure 4**. As authenticated by DNA sequencing, rods that expressed hRho-GFP gave a 791-bp band with one copy of exon 2, whereas rods that did not express hRho-GFP gave a 960-bp band with two copies of exon 2 (**Fig. 4**).

To enrich for the products of NHEJ (**Fig. 3A**), we amplified IScel-resistant DNA using primers P4/P5 in the initial round of amplification and primers P6/P7 subsequently. To ensure that we could detect the maximum size NHEJ-induced deletions compatible with our primers (300 bp in either direction from the IScel site), we cloned the unfractionated amplification products after the last cycle of PCR. We analyzed 128 colonies by PCR (**Fig. S3**). Except for the few with apparent insertions, the PCR products from these colonies seemed to have very minor changes in the size, with no obvious deletions. We obtained sequences for 37 clones. 14 retained the intact IScel site, indicating that some uncleaved genomes evaded our isolation procedure. Among the remainder, 19 had small deletions ranging from 1 to 13 bp and 4 had insertions (**Fig. 5**, column A). The two longer insertions, which were 42 and 48 bp, were derived from the inverted terminal repeats of rAAV. The size of the deletions, the 0-3 nucleotides of microhomology at deletion junctions, and the proportion of insertions are all typical characteristics of NHEJ (37). Moreover, the modifications all overlap the site at which IScel is predicted to introduce a DSB. These results confirm that IScel cleaves rod cell genomes and that NHEJ occurs in rod cells.

In principle, IScel-treated ID2-hRho-GFP mice could also give rise to green rods by NHEJ, if sufficient DNA sequence were deleted at the break to inactivate the upstream or downstream copy of exon 2 (889 bp and 457 bp, respectively). Primers P6/P7 (**Fig. 3A**) could have detected deletions up to 300 bp on either side of the IScel site, but uncovered deletions that extended no more than 50 bp into the flanking sequences. In the small-pool PCR experiments described below, primers P6/P2 could have detected a 600-bp deletion into the downstream copy of exon 2, but, once again, no significant deletions were observed in more than 200 NHEJ junctions (see **Fig. 3B**). Thus, NHEJ does not account for a significant proportion of the green rods found after IScel cleavage.

Relative frequencies of DSB repair by NHEJ and SSA. Although the above studies confirm that DSBs can be repaired by NHEJ and by the SSA pathway of HR in terminally differentiated rod cells, they do not address the issue of the relative frequencies with which these pathways repair a DSB. To measure their relative frequencies, we chose to use small-pool PCR to assess individual genomes. We isolated DNA from three retinal whole mounts that displayed large areas of green rods at 10, 12, or 24 weeks after subretinal injection of rAAV-ISceI. We extensively digested the DNA with ISceI to cleave genomes that retained the ISceI recognition sequence (that is, genomes from cells that had not been transduced by rAAV or had not been repaired by SSA or NHEJ), and then diluted the resulting DNA so that each PCR reaction contained on average fewer than 2 intact genomes. We separated the PCR products (generated by a mixture of primers P1, P2, and P6) by gel electrophoresis, as shown in **Figure 3B**, and counted the number of bands at the position of the SSA marker (687 bp, primers P1/P2) as SSA events, and the number at the position of the NHEJ marker (997 bp, primers P6/P2) and at higher positions as presumptive NHEJ events.

Each sample with a band at the position of the NHEJ marker was re-amplified and digested with ISceI to determine whether the band was a true NHEJ event or was derived from an intact background genome (**Fig. 3C**). This analysis identified a small percentage of background genomes, which were eliminated from further consideration (Table 1). For several NHEJ events, we amplified the DNA and analyzed it by sequencing. As expected, bands at the position of the NHEJ marker gave small deletions, whereas slower migrating bands yielded insertions. We also reamplified several bands at the position of the SSA marker and confirmed by sequencing that they had been generated by SSA. Table 1 summarizes the data obtained from three retinas. This analysis indicates that NHEJ accounted for about 85% of DSB-repair events, whereas SSA accounted for about 15%.

Absolute frequency of NHEJ. Ultimately, the ability to use mutagenic NHEJ (and HR) for therapeutic genome editing depends on the overall efficiency with which changes can be introduced into the retina. To measure the absolute frequency of rods with NHEJ events, we isolated DNA from a retina 24 weeks after injection of rAAV-ISceI (Retina A in Table 1). We selected this retina because about 60-80% had been exposed to rAAV-ISceI, as judged by the distribution of green rods. We amplified the DNA by PCR using primers P6/P7, which does not distinguish between unaltered genomes and NHEJ-induced mutant genomes (**Fig. 3A**). The PCR products were cloned and analyzed. Among 146 clones, we found 20 that contained NHEJ junctions (**Fig. 5**, column B), which corresponds to 13.7% with a standard deviation of 3% expected from Poisson statistics. Since ISceI is expressed only in rod cells, which constitute 78% of retinal cells (35), the overall frequency of rod genomes with engineered NHEJ mutations is $17.6 \pm 3.8\%$ for the whole retina. Given the relative frequencies of NHEJ (85% of all events that lead to loss of the ISceI site) and SSA (15% of those events) (Table 1), we can estimate that the overall frequency of rod genomes with SSA events is about $17.6\% \times 0.15/0.85 = 3.1\%$ in this retina. Making a small adjustment (dividing by 103.1%) to account for the SSA events, which were excluded by the PCR strategy, gives absolute frequencies for this retina of about 17% of rods with NHEJ events and about 3% with SSA events.

These absolute values depend critically on the fraction of the retina that was exposed to rAAV-ISceI, which varies from injection to injection. We can adjust these values to allow comparisons between retinas by taking into account the area exposed to rAAV-ISceI. Since 60-80% of the area of Retina A was exposed, the adjusted frequencies per exposed portion are 21-28% for NHEJ events and 3.8-5.0% for SSA events, with the total frequency of ISceI-induced events in the range of 25-33% of rod cells in the exposed area. The value calculated for SSA events is in reasonable agreement with the frequency (3.3%) derived from the experiments shown in **Figure 2B** and **2D**, which measured frequencies in exposed areas of the retina. In those experiments we also estimated that about 22% of rod cells were transduced. The similar values for

transduction efficiency (22%) and proportion of modified rods in transduced areas (25-33%) suggest that the ID2-hRho-GFP gene is cleaved in virtually all rAAV-ISceI transduced rods.

DISCUSSION

Diseases that are caused by dominant alleles—like RP due to dominant rhodopsin mutations—present a special challenge for gene therapy (6). One cannot simply replace a missing function by adding in a good copy of the gene, as can be done for recessive diseases. One general therapeutic strategy for dealing with dominant mutations is to use a gene-specific damaging agent to knock out the defective gene by NHEJ or to stimulate its correction by HR (38). However, we know very little about NHEJ and HR in terminally differentiated neurons, or about the efficiency with which gene-specific damage can be induced. Using a mouse model with an internal duplication of rhodopsin exon 2 (ID2-hRho-GFP), we show here that mutations can be induced in virtually 100% of transduced rod cell neurons, with about 85% due to DSB repair by NHEJ and 15% by the SSA subpathway of HR. These results indicate that therapeutic gene modification in rod cells is feasible.

In the experiments reported here we designed a substrate with an internal duplication to set up a competition between DSB repair by NHEJ and SSA, in order to probe the repair capabilities of rod photoreceptors. In the absence of the duplication, as occurs in native rhodopsin alleles, and in the absence of an HR-promoting sister chromatid, which is lacking in terminally differentiated cells, mutagenic NHEJ would be expected to repair virtually all DSBs. Thus, the limit to rhodopsin mutagenesis depends on the proportion of rod cells in which DSBs can be introduced, which depends in turn on the efficiency of rod-cell transduction and on the efficiency of cleavage in transduced cells. We show here that essentially all the rod cells transduced with rAAV-IScel acquire DSBs in the ID2-hRho-GFP target gene. This very high cleavage efficiency is likely due to the persistence of rAAV vectors, which have been shown to express their passenger genes robustly in the mouse retina for at least 6 months (36). Although cleavage efficiency is excellent, the transduction efficiency is only about 25%. However, recent reports suggest that transduction efficiencies can be improved using other serotypes of AAV (39,40), an observation we have confirmed in our system (unpublished observations).

The high efficiency of DSB repair by mutagenic NHEJ suggests that it could serve as the basis for strategies designed to knock out dominant rhodopsin alleles in rod cells. The key will be to design a nuclease that can cleave within the coding region of the rhodopsin gene. Over the past decade substantial progress has been made in the development of zinc-finger nucleases (ZFNs), which can be engineered to cleave specific target genes (41). ZFNs have proven exceptionally versatile, specific, and selective agents for introducing gene-specific DSBs into the genomes of cultured cells and a variety of organisms (11,38,41-45). Head-to-head comparisons with IScel, the gold standard for cleaving genomic DNA, have shown that ZFNs can cut as efficiently as IScel (10). By designing a ZFN to cleave near the beginning of the rhodopsin gene, repair of the break by NHEJ could be used to shift the reading frame (two-thirds of the time on average) to generate a fragmentary product. Gene knockout by NHEJ repair of ZFN-induced DSBs has been used to generate several mutant cell lines and organisms (41,42). The efficient DSB-induced mutagenesis we observe in rod cells suggests that ZFN-directed gene knockout may be a promising avenue to explore as a potential therapy for RP patients with defective rhodopsin alleles.

It is no surprise that NHEJ is the primary mechanism for DSB repair in terminally differentiated rod cells, as it is in cycling mammalian cells (13). However, it is surprising that 15% of IScel-induced DSBs in terminally differentiated rod neurons are repaired by the SSA subpathway of HR. Previous studies in cycling cells have shown that strand-invasion, the main pathway of HR, occurs predominantly in S and G2, when the sister chromatid can serve as the template for repair (20,21,25,46,47). In G1, strand invasion is blocked at the step of 5' to 3' resection of the ends, which is required to expose the single-stranded DNA tails that are necessary for invading an homologous duplex (13). This cell-cycle control of end resection is exerted by cyclin-dependent kinase (CDK), which mediates phosphorylation of CtIP in S and G2 to activate it for end resection (23-25). Although the strand-invasion and SSA pathways of HR differ in their downstream events, they both begin with end resection (13). Our studies in ID2-hRho-GFP mice show that end resection is robust in rod cells, occurring at

15% of DSBs. It is also extensive. To expose complementary exon-2 sequences on either side of the DSB, so that the resulting single-stranded tails can pair by SSA to bridge the break, requires that several nucleotides of DNA be removed from each end. Efficient SSA in rod cells indicates that end resection is not an insurmountable barrier to the strand-invasion pathway of HR in rod cells. These results raise the possibility that repair of DSBs by HR, using an added nonmutant segment of DNA as a template for repair, might also be feasible as an approach to therapy for RP. Studies in cycling cells have shown that DSB-induced gene correction from an added DNA segment can occur at frequencies approaching 20% (11). In addition, it may be possible to improve the efficiency of HR-mediated gene editing in rod cells by knocking down the activities of key NHEJ repair proteins, as has been done in cultured cells (48-51).

In this study we have focused on the basic parameters of DSB repair in a specific class of neurons, the rod photoreceptors responsible for dim-light vision. We concentrated on these neurons because dominant rhodopsin mutations expressed in rod cells trigger the neurodegenerative events that cause the most common form of the hereditary blinding disease, RP. The highly efficient introduction of DSBs into rod cell genomes, coupled to their repair by mutagenic NHEJ, offers a promising approach to therapy of RP in human patients. While our measurements focus on DSB repair in rod photoreceptors, it is likely that the results can be extrapolated to DSB repair capabilities in other classes of mature neurons, which are poorly defined at present (14). Thus, this study lays the groundwork for any long-term efforts to use DSBs to edit genomes in mature neurons.

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REFERENCES

1. Sohocki, M.M., Daiger, S.P., Bowne, S.J., Rodriguez, J.A., Northrup, H., Heckenlively, J.R., Birch, D.G., Mintz-Hittner, H., Ruiz, R.S., Lewis, R.A. *et al.* (2001) Prevalence of mutations causing retinitis pigmentosa and other inherited retinopathies. *Hum Mutat*, **17**, 42-51.
2. Rivolta, C., Sharon, D., DeAngelis, M.M. and Dryja, T.P. (2002) Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns. *Hum Mol Genet*, **11**, 1219-1227.
3. Rosenfeld, P.J., Cowley, G.S., McGee, T.L., Sandberg, M.A., Berson, E.L. and Dryja, T.P. (1992) A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa. *Nat Genet*, **1**, 209-213.
4. Lem, J., Krasnoperova, N.V., Calvert, P.D., Kosaras, B., Cameron, D.A., Nicolo, M., Makino, C.L. and Sidman, R.L. (1999) Morphological, physiological, and biochemical changes in rhodopsin knockout mice. *Proc Natl Acad Sci U S A*, **96**, 736-741.
5. Humphries, M.M., Rancourt, D., Farrar, G.J., Kenna, P., Hazel, M., Bush, R.A., Sieving, P.A., Sheils, D.M., McNally, N., Creighton, P. *et al.* (1997) Retinopathy induced in mice by targeted disruption of the rhodopsin gene. *Nat Genet*, **15**, 216-219.
6. Wilson, J.H. and Wensel, T.G. (2003) The nature of dominant mutations of rhodopsin and implications for gene therapy. *Mol Neurobiol*, **28**, 149-158.
7. Rouet, P., Smih, F. and Jasin, M. (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol*, **14**, 8096-8106.
8. Brenneman, M., Gimble, F.S. and Wilson, J.H. (1996) Stimulation of intrachromosomal homologous recombination in human cells by electroporation with site-specific endonucleases. *Proc Natl Acad Sci U S A*, **93**, 3608-3612.

9. Bibikova, M., Beumer, K., Trautman, J.K. and Carroll, D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science*, **300**, 764.
10. Porteus, M.H. and Baltimore, D. (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science*, **300**, 763.
11. Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D. and Holmes, M.C. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, **435**, 646-651.
12. Perez, E.E., Wang, J., Miller, J.C., Jouvenot, Y., Kim, K.A., Liu, O., Wang, N., Lee, G., Bartsevich, V.V., Lee, Y.L. *et al.* (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol*, **26**, 808-816.
13. Kass, E.M. and Jasin, M. (2010) Collaboration and competition between DNA double-strand break repair pathways. *FEBS Lett*, **584**, 3703-3708.
14. McKinnon, P.J. (2009) DNA repair deficiency and neurological disease. *Nat Rev Neurosci*, **10**, 100-112.
15. Lee, Y. and McKinnon, P.J. (2007) Responding to DNA double strand breaks in the nervous system. *Neuroscience*, **145**, 1365-1374.
16. Abner, C.W. and McKinnon, P.J. (2004) The DNA double-strand break response in the nervous system. *DNA Repair (Amst)*, **3**, 1141-1147.
17. Rass, U., Ahel, I. and West, S.C. (2007) Defective DNA repair and neurodegenerative disease. *Cell*, **130**, 991-1004.
18. Ren, K. and Pena de Ortiz, S. (2002) Non-homologous DNA end joining in the mature rat brain. *J Neurochem*, **80**, 949-959.
19. Sharma, S. (2007) Age-related nonhomologous end joining activity in rat neurons. *Brain Res Bull*, **73**, 48-54.
20. Wyman, C. and Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well. *Annu Rev Genet*, **40**, 363-383.

21. Rothkamm, K., Kruger, I., Thompson, L.H. and Lobrich, M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol*, **23**, 5706-5715.
22. Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. and Takeda, S. (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J*, **17**, 5497-5508.
23. Sartori, A.A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J. and Jackson, S.P. (2007) Human CtIP promotes DNA end resection. *Nature*, **450**, 509-514.
24. Huertas, P. and Jackson, S.P. (2009) Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. *J Biol Chem*, **284**, 9558-9565.
25. Ira, G., Pellicoli, A., Balijja, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N.M. *et al.* (2004) DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature*, **431**, 1011-1017.
26. Chan, F., Bradley, A., Wensel, T.G. and Wilson, J.H. (2004) Knock-in human rhodopsin-GFP fusions as mouse models for human disease and targets for gene therapy. *Proc Natl Acad Sci U S A*, **101**, 9109-9114.
27. Wensel, T.G., Gross, A.K., Chan, F., Sykoudis, K. and Wilson, J.H. (2005) Rhodopsin-EGFP knock-ins for imaging quantal gene alterations. *Vision Res*, **45**, 3445-3453.
28. Zheng, B., Sage, M., Sheppard, E.A., Jurecic, V. and Bradley, A. (2000) Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications. *Mol Cell Biol*, **20**, 648-655.
29. Lan, Z.J., Xu, X. and Cooney, A.J. (2004) Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol Reprod*, **71**, 1469-1474.

30. Flannery, J.G., Zolotukhin, S., Vaquero, M.I., LaVail, M.M., Muzyczka, N. and Hauswirth, W.W. (1997) Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus. *Proc Natl Acad Sci U S A*, **94**, 6916-6921.
31. Pang, J.J., Chang, B., Kumar, A., Nusinowitz, S., Noorwez, S.M., Li, J., Rani, A., Foster, T.C., Chiodo, V.A., Doyle, T. *et al.* (2006) Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. *Mol Ther*, **13**, 565-572.
32. Lolley, R.N., Lee, R.H., Chase, D.G. and Racz, E. (1986) Rod photoreceptor cells dissociated from mature mice retinas. *Invest Ophthalmol Vis Sci*, **27**, 285-295.
33. Turner, D.L., Snyder, E.Y. and Cepko, C.L. (1990) Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron*, **4**, 833-845.
34. Morrow, E.M., Furukawa, T. and Cepko, C.L. (1998) Vertebrate photoreceptor cell development and disease. *Trends Cell Biol*, **8**, 353-358.
35. Jeon, C.J., Strettoi, E. and Masland, R.H. (1998) The major cell populations of the mouse retina. *J Neurosci*, **18**, 8936-8946.
36. Mao, H., Thomas Jr, J., Schwein, A., Shabashvili, A., Hauswirth, W.W., Gorbatyuk, M.S. and Lewin, A. (2010) AAV Delivery of Wild-Type Rhodopsin Preserves Retinal Function in a Mouse Model of Autosomal Dominant Retinitis Pigmentosa. *Hum Gene Ther*.
37. Roth, D.B. and Wilson, J.H. (1986) Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol*, **6**, 4295-4304.
38. Wilson, J.H. (2008) Knockout punches with a fistful of zinc fingers. *Proc Natl Acad Sci U S A*, **105**, 5653-5654.
39. Allocca, M., Mussolino, C., Garcia-Hoyos, M., Sanges, D., Iodice, C., Petrillo, M., Vandenberghe, L.H., Wilson, J.M., Marigo, V., Surace, E.M. *et*

- al.* (2007) Novel adeno-associated virus serotypes efficiently transduce murine photoreceptors. *J Virol*, **81**, 11372-11380.
40. Petrs-Silva, H., Dinculescu, A., Li, Q., Min, S.H., Chiodo, V., Pang, J.J., Zhong, L., Zolotukhin, S., Srivastava, A., Lewin, A.S. *et al.* (2009) High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. *Mol Ther*, **17**, 463-471.
 41. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. and Gregory, P.D. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*, **11**, 636-646.
 42. Santiago, Y., Chan, E., Liu, P.Q., Orlando, S., Zhang, L., Urnov, F.D., Holmes, M.C., Guschin, D., Waite, A., Miller, J.C. *et al.* (2008) Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc Natl Acad Sci U S A*, **105**, 5809-5814.
 43. Carroll, D. (2008) Progress and prospects: zinc-finger nucleases as gene therapy agents. *Gene Ther*, **15**, 1463-1468.
 44. Meyer, M., de Angelis, M.H., Wurst, W. and Kuhn, R. Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc Natl Acad Sci U S A*, **107**, 15022-15026.
 45. Geurts, A.M. and Moreno, C. Zinc-finger nucleases: new strategies to target the rat genome. *Clin Sci (Lond)*, **119**, 303-311.
 46. Lieber, M.R. (2008) The mechanism of human nonhomologous DNA end joining. *J Biol Chem*, **283**, 1-5.
 47. Aylon, Y., Liefshitz, B. and Kupiec, M. (2004) The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. *EMBO J*, **23**, 4868-4875.
 48. Clikeman, J.A., Khalsa, G.J., Barton, S.L. and Nickoloff, J.A. (2001) Homologous recombinational repair of double-strand breaks in yeast is enhanced by MAT heterozygosity through yKU-dependent and -independent mechanisms. *Genetics*, **157**, 579-589.
 49. Allen, C., Kurimasa, A., Brenneman, M.A., Chen, D.J. and Nickoloff, J.A. (2002) DNA-dependent protein kinase suppresses double-strand break-

- induced and spontaneous homologous recombination. *Proc Natl Acad Sci U S A*, **99**, 3758-3763.
50. Pierce, A.J., Hu, P., Han, M., Ellis, N. and Jasin, M. (2001) Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev*, **15**, 3237-3242.
 51. Delacote, F., Han, M., Stamatou, T.D., Jasin, M. and Lopez, B.S. (2002) An *xrcc4* defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. *Nucleic Acids Res*, **30**, 3454-3463.

FIGURE LEGENDS

Figure 1. Generation of human rhodopsin-GFP mouse. (A) Strategy for replacing the mouse rhodopsin gene with the ID2-hRho-GFP gene. The mouse rhodopsin gene and flanking sequences are shown in dark gray, with exons indicated by rectangles and introns in lines. The human rhodopsin-GFP fusion gene is shown in black except for the duplicated segments containing exon 2 and the fused GFP (G) gene, which are shown in white. LoxP sites (inverted triangles marked with P) flank the HPRT minigene (light gray), which was inserted at the boundary between the human and mouse rhodopsin sequences. The 3' boundary between human and mouse DNA is marked by a Lox511 site, which is not shown as it played no role in these experiments. (B) Southern-blot analysis of ES cells. Restriction enzymes used to digest genomic DNA for analysis of the 5'- and 3'-ends of the modified locus are shown in parentheses, and fragment sizes are indicated in kb. (C) PCR analysis of tail DNA from HPRT-ID2 targeted mice. PCR product sizes are indicated in kb. (D) Breeding scheme to generate mice carrying the HPRT-ID2 and GDF-9-iCre genes. Heterozygous HPRT-ID2 mice were crossed with GDF-9-iCre mice. Examples of mice with each of the four expected gene combinations are shown. (E) Breeding scheme to remove the HPRT minigene. A female mouse heterozygous for both the HPRT-ID2 gene and the GDF-9-iCre transgene was crossed to a wild-type (C57BL/6) mouse. Examples of mice with four different gene combinations are shown. Pup 4 contains the ID2-hRho-GFP gene, but not the GDF-9-iCre transgene or the HPRT minigene.

Figure 2. Fluorescent green rods in retinal whole mounts from heterozygous ID2-hRho-GFP mice. (A) Confocal images of retinal whole mounts from heterozygous ID2-hRho-GFP mice. (a) Images of clusters of green rods. (b) Image of rod doublets. As was typical of doublets, the two rods were closely apposed and were difficult to distinguish except by rotation of the projection angle through the image stacks. In this image the two rods are slightly separated at one end. (c) Images of individual rod cells. All images were taken at the same magnification;

scale bar is 10 μm . (B) Confocal images of retinal whole mounts after subretinal injection of rAAV-ISceI. For retinas expressing hRho-GFP, confocal images were captured at the indicated times post injection using a 40x objective and a 10x eyepiece. Scale bar is 20 μm . (C) Confocal images of retinal whole mounts after subretinal injection of rAAV-GFP. For retinas expressing GFP, images were captured at the indicated times using a 63x objective and a 10x eyepiece. Scale bar is 20 μm . (D) Counts of fluorescent green rods after subretinal injection of rAAV-ISceI. (E) Counts of fluorescent green rods after subretinal injection of rAAV-GFP. For D and E, counts are expressed as number of green rods per unit area (which corresponds to the size of the field observed with a 40x objective). Error bars indicate standard deviations.

Figure 3. Analysis of SSA and NHEJ events. (A) Scheme for PCR analysis of SSA and NHEJ events. The duplicated copies of exon 2 along with their flanking introns are shown in white and marked by horizontal arrows. The ISceI cleavage site is indicated by a vertical arrow. Small numbered arrowheads indicate PCR primers. Primer pairs and their expected PCR fragment sizes are listed beneath the initial ID2-hRho-GFP gene and the structures of the genes resulting from SSA- and NHEJ-mediated repair of ISceI-induced DSBs. (B) Small-pool PCR of DNA from a single AAV-ISceI injected retina. A mixture of primers (P1/P6/P2) was used to amplify DNA samples, and the PCR fragments were separated by gel electrophoresis and visualized by Southern blotting. Size markers for products of NHEJ (997 bp, primers P6/P2) and SSA (687 bp, primers P1/P2) are present in the first lanes of each blot. We counted bands at the position of the SSA marker as arising from SSA events, and bands at the position of the NHEJ marker and elsewhere as arising from NHEJ events (or background genomes with intact ISceI sites). Each distinct band was counted once, regardless of its intensity. (C) Distinguishing NHEJ events from background genomes with intact ISceI sites. Samples with bands at 997 bp were re-amplified with nested primers P8/P3, subjected to ISceI digestion, and displayed on agarose gels. The first lane shows the 644-bp and 312-bp bands expected for digestion of a PCR product

(956 bp) with an intact ISceI site. The rest of the samples are shown in pairs, with the undigested control on the left and the ISceI-digested sample on the right. In two instances on this gel, the amplified sample was cleaved with ISceI; these samples were designated ISceI+ (see Table 1).

Figure 4. Analysis of individual green rods. (A) Fluorescence micrographs of an individual green rod cell being selected and removed using a micropipette. (B) PCR amplification of hRho-GFP cDNA from initial RT-PCR reactions. Lane 1, 960-bp size marker for NHEJ and background events, which contain two copies of exon 2. Lane 2, 791-bp size marker for SSA events, which contain one copy of exon 2. Lanes 3 to 10, PCR products from individual green rods. Lane 11, PCR product from control fluorescent rod isolated from retina of heterozygous hrhoG(H) mouse, which carries one copy of a wild type human rhodopsin-GFP fusion gene (26). Lane 12, nonfluorescent retinal cell isolated from an rAAV-ISceI injected retina. Sequencing results confirmed that samples 3 through 10 all contain a single copy of exon 2, as expected for SSA events.

Figure 5. Sequence analysis of 43 NHEJ clones. Both DNA strands for the ISceI site are shown at the top in black with the sites of cleavage indicated by triangles on the top and bottom strand. An adjacent EcoRV site used in construction of the duplication is shown in gray. For deletions and insertions, only the top strand is shown. The column labeled A shows the results of analysis of 23 ISceI-resistant clones isolated after the treatment described in Figure S2. The column labeled B shows the results of analysis of 20 ISceI-resistant clones isolated by screening a population of cloned PCR fragments from genomic DNA that had not been pre-digested with ISceI. The number of clones with the same sequence is shown adjacent to the sequence. Deleted nucleotides are indicated as dots. Junctional homologies for deletions are indicated by bold nucleotides and have arbitrarily been shown to the right of the deleted nucleotides. The number of inserted nucleotides is indicated above the triangles, which indicate the place in the sequence where they were inserted. The inserted sequences are: 1 bp, A; 1 bp,

C; 42 bp, CCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC;
and 48 bp,
GTCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCA.

Table 1. Small-Pool PCR Analysis of Injected Retinas.

Retina ^a	PCR Samples	Lanes with No Bands ^b	Genomes per Reaction ^b	ISceI+ ^c	NHEJ ^d	HR ^d
A1	90	15	1.8	2	76 (85%)	13 (15%)
A2	90	13	1.9	7	74 (84%)	14 (16%)
B	90	51	0.6	1	34 (83%)	7 (17%)
C	90	59	0.4	6	23 (82%)	5 (18%)

^aThree different retinas were used. A1 and A2 are replicates of a single retina.

^bThe number of lanes that did not show a product was used to estimate the average number of intact, amplifiable genomes per reaction, using the Poisson Distribution.

^cISceI+ indicates the number of samples that had upper bands that were cleavable with ISceI, indicating that they were not the products of NHEJ events.

^dNHEJ events equal the number of distinct bands around the 997-bp marker, minus the number of ISceI+ samples. HR events equal the number of bands at the 687-bp marker. In neither case, have we made an attempt to correct for samples that may contain two distinct genomes that give the same size band.

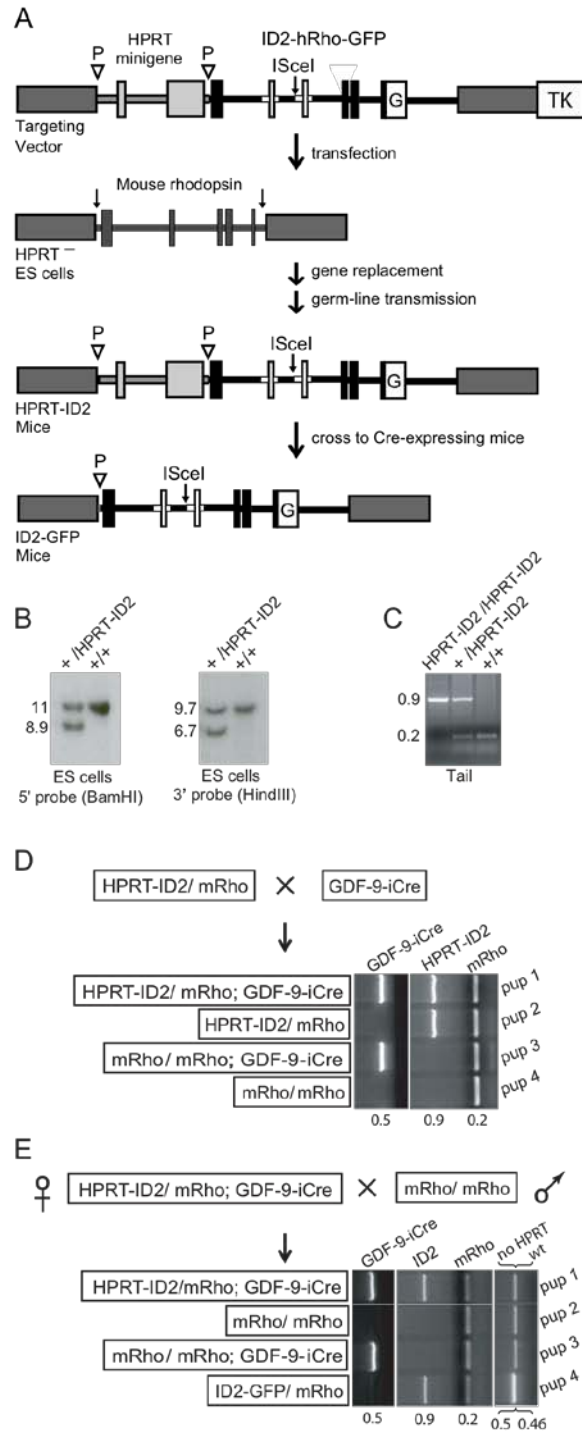
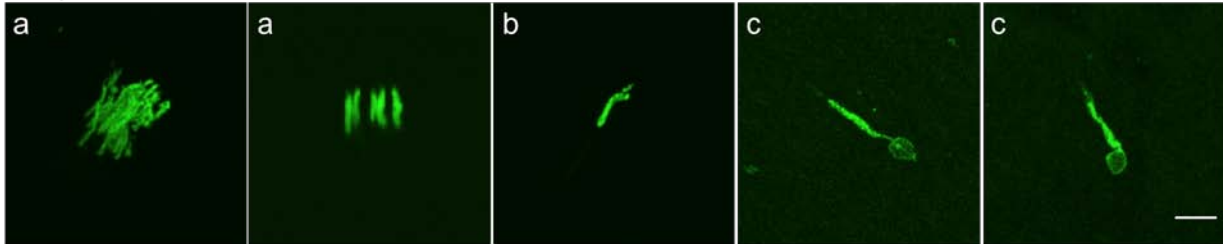
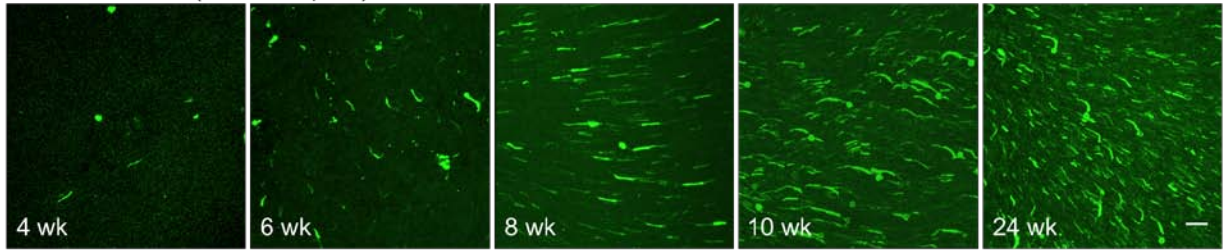


FIGURE 1

A Spontaneous



B rAAV-ISceI (DSB Repair)



C rAAV-GFP (Transduction Efficiency)

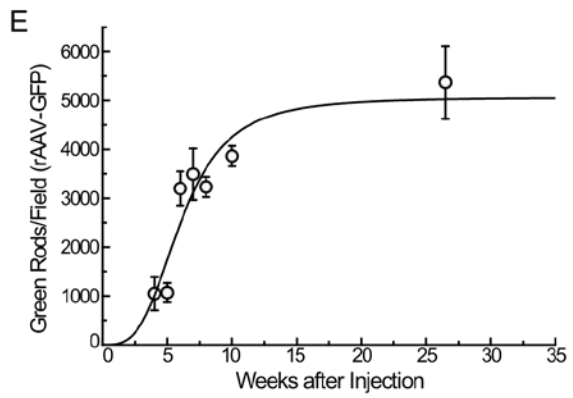
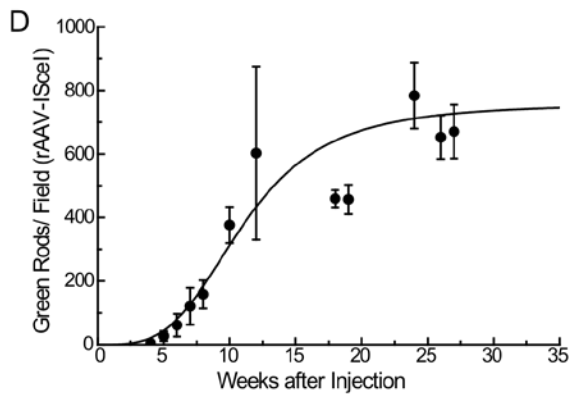
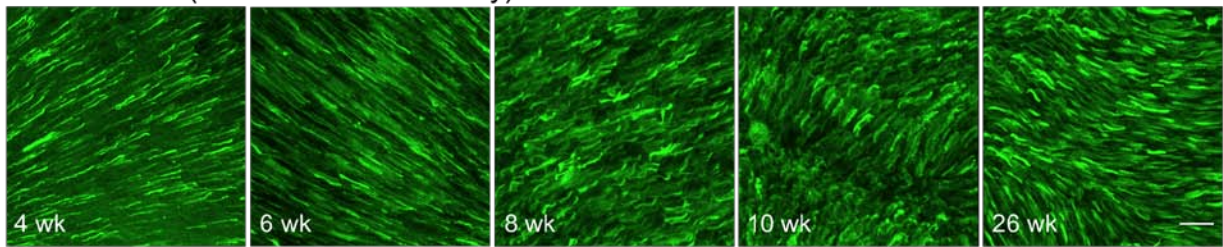


FIGURE 2

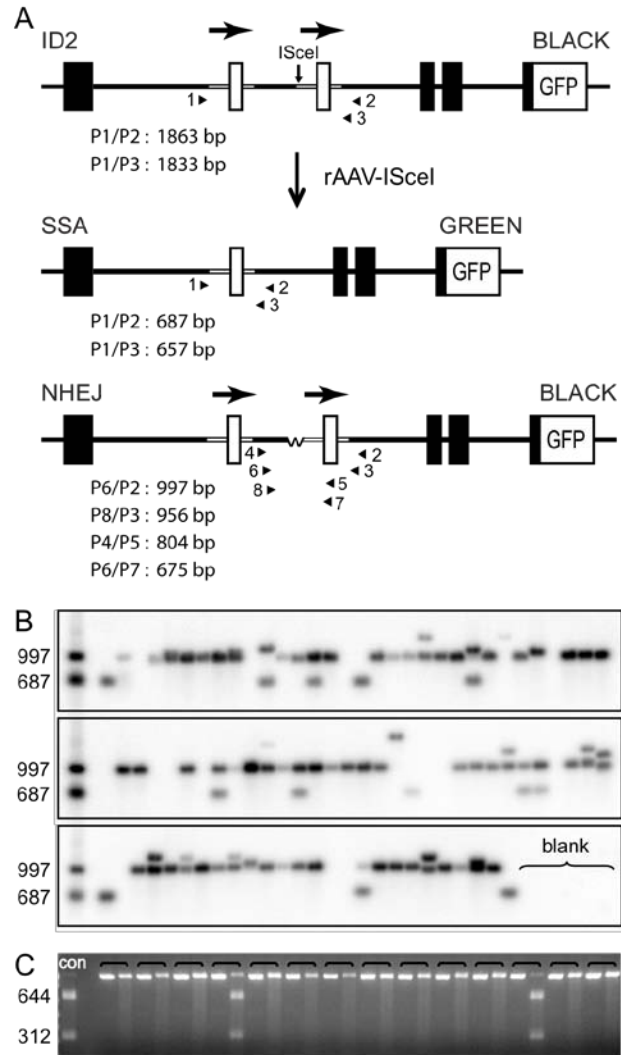


FIGURE 3

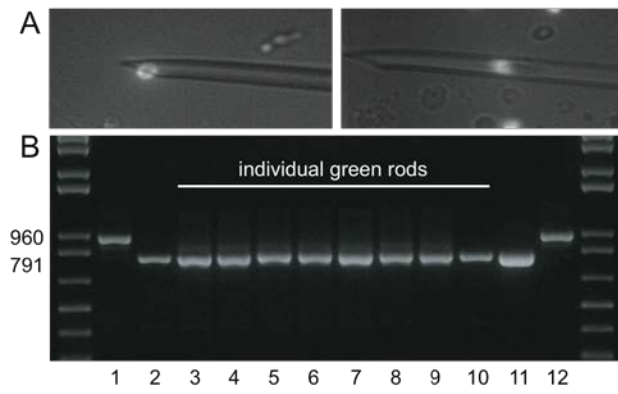


FIGURE 4

I_SceI Site

..GATATCTAGGGATAACAGGGTAAT..
 ..CTATAGATCCCTATTGTCCCATTA..
 ▲

DELETIONS	A	B
..GATATCTAGGGAT.ACAGGGTAAT..	1	3
..GATATCTAGGGATAAC.GGGTAAT..		1
..GATATCTAGGG..AACAGGGTAAT..	4	2
..GATATCTAGGGATA..AGGGTAAT..		1
..GATATCTAGGG...ACAGGGTAAT..	6	1
..GATATCT....ATAACAGGGTAAT..	1	
..GATATCTAG.....ACAGGGTAAT..		1
..GATATCTAG.....CAGGGTAAT..		1
..GATATCT.....ACAGGGTAAT..	1	1
..GATATC.....AACAGGGTAAT..		1
..GATATCTAGGGA.....TAAT..	4	2
..GATATCTAG.....GGGTAAT..		1
..G.....AACAGGGTAAT..		1
..GATAT.....GGTAAT..	2	
..GATATCTAGGGATA....41bp....		1
.....46bp.....		1
..GATATCTAGGG.....49bp....		1
INSERTIONS	1 bp	
..GATATCTAGGGATAACAGGGTAAT..	▲	1
	1 bp	
..GATATCTA..GATAACAGGGTAAT..	▲	1
	42 bp	
..GATATCTA..GATAACAGGGTAAT..	▲	1
	48 bp	
..GATATCTAGGGATAACAGGGTAAT..	▲	2

FIGURE 5

SUPPLEMENTARY INFORMATION

Efficient Mutagenesis of the Rhodopsin Gene in Rod Photoreceptor Neurons in Mice

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SUPPLEMENTARY MATERIALS AND METHODS

Mouse Genotyping. Mice were cared for and handled in accordance with an approved protocol from the Animal Research Committee of Baylor College of Medicine and in compliance with National Institutes of Health guidelines for the care and use of experimental animals. We determined mouse genotypes by PCR analysis of tail DNA. To assay for the knock-in allele (HPRT-ID2 or ID2-hRho-GFP), we used primers that annealed at sites on either side of GFP (5'-GTTCCGGAAGTGCATGCTCACCAC in human exon 5 and 5'-GGCGCTGCTCCTGGTGGG in the human 3' untranslated region). PCR using these primers generates a 933-bp product from HPRT-ID2 and from ID2-hRho-GFP. Each primer has a single-base mismatch to the corresponding mouse sequence, but still gives rise to a 194-bp PCR product from the native mouse rhodopsin locus (Fig. 1C). To assay for the GDF-9-iCre transgene, we used forward (5'-TCTGATGAAGTCAGGAAGAACC) and reverse (5'-GAGATGTCCTTCACTCTGATTC) primers to generate a 0.5-kb PCR product (1) (Fig. 1D). To assay for the HPRT minigene, we used a forward primer (5'-GGGAAGAGATGGGATAGGTGAG) that anneals to the 5' untranslated region of the mouse rhodopsin gene, and a reverse primer (5'-GCCTTCTGTGCCATTCATG) that anneals to exon 1 of human rhodopsin. Under the conditions used for amplification, these primers generate no product if the HPRT gene is present; in its absence, they generate a 495-bp product (Fig. 1E). The reverse primer contains two mismatches with the mouse rhodopsin sequence, but still give a faint 455-bp PCR product (Fig. 1E). These two products differ in size because the ID2-hRho-GFP allele retains one LoxP site that remains after Cre-mediated removal of the HPRT minigene.

Histology. We removed eyes from euthanized mice and fixed them in 4% paraformaldehyde (EM Science) in PBS for 1 h at room temperature with gentle shaking. For sections, we soaked eyes in 30% sucrose in PBS at room temperature with gentle shaking until the eyes sank, and froze them in 75% Tissue-Tek OCT (Sakura Finetek, Torrance, CA) and 25% Immu-Mount (Shandon, Pittsburgh) in liquid nitrogen. We cut 10-20 μ m thick sections with a Microm HM500 microtome (Microm Instruments, Heidelberg, Germany), air-dried and kept them at -20°C until use. For retinal whole

mounts, we removed the cornea, lens, and vitreous and dissected the retina from the eyecup. We placed the retina on a slide with the photoreceptors up, flattened it by making radial incisions, mounted it with Gel/Mount (Biomedica, Foster City, CA), and examined it by widefield or confocal fluorescence microscopy (LSM 510, Zeiss).

To count nuclei in outer nuclear layers, we stained eye sections using TO-PRO-3 iodide (Molecular Probes) in 0.1 M phosphate buffer plus 0.5% Triton x-100, 0.1% NaN₃, and 1% sheep serum for 1 h at room temperature. We then washed sections with PBS three times for 10 min each, rinsed with water, and mounted them with Gel/Mount. We captured images from several different locations in the retina, excluding areas around the optic nerve and the periphery. We counted 60-100 columns of nuclei for each retina and averaged them for each time point.

Subretinal Injection. For subretinal injections, we followed established procedures (2) with some modification. We used an UltraMicroPump II and Micro4 Controller (World Precision Instruments, UMP2-1) fitted with footswitch (WPI, 15867) to inject specified volumes. We filled the 100 microliter Nanofil syringe (WPI, NANOFIL-100) mounted on the pump first with 60 µl of sterile filtered light paraffin oil (EMD, PX0047-1) and then with a solution of rAAV containing 0.1 mg/ml fluorescein. We connected the syringe to the NanoFil Injection Holder (WPI, NFINHLD) through SilFlex tubing (WPI, SILFLEX) and then mounted a blunt-ended 35-gauge needle (WPI, NF35BL) on the holder for injection. The injection rate was 170 nl/sec at setting G. We used heterozygous ID2 mice between 3- 3¹/₂ weeks of age in all the injections. In preparation for injections, we treated mice with 1% atropine eye drops 1 h before anesthetization by intraperitoneal injection of ketamine (72 mg/kg) plus xylazine (4 mg/kg), followed by topical administration of 2.5% phenylephrine hydrochloride eye drops. Prior to injection, we administered 2.5% hydroxypropyl methylcellulose to keep the eyes moist. Before the actual injection, we used a 30-gauge disposal needle to open an aperture within the pupil area through the superior cornea.

Analysis of products generated by ISceI injection. We designed nested sets of primers to detect the products of HR and NHEJ, which are the major pathways of DSB

repair. Primer sites and product sizes are shown schematically in Figure 4A. Primer sequence are: P1, 5'-CCTACCGGGTTCCCAGTTCAATC; P2, 5'-GCGCTGGAAGCCTCTAGTTTC; P3, 5'-CAGGAACCATTGCCACGTCC; P4, 5'-GGCAGCCCCCTCTAACCTTG; P5, 5'-GCGTTGCCTTCACCTGGGTC; P6, 5'-GGTCCCCTCAGCCTCTAGG; P7, 5'-GTACGTGGTGGTGTGTAAGCCC; and P8, 5'-CCTCTAGGCGATTGTCTCC.

To amplify repaired regions of chromosomes for sequence analysis, we digested retinal DNA samples repeatedly with IScel before and after each PCR reaction in order to reduce background and enhance the repaired species (Fig. S2). At each step, we removed protein by treatment with phenol-chloroform-isoamyl alcohol and desalted the samples with QIAGEN spin columns.

We cloned presumptive NHEJ products after three rounds of PCR amplification and IScel digestion; we cloned HR products after two rounds. For NHEJ events, we used the Expand Long Template PCR System (Roche, Cat. No. 11 681 842 001) for the first PCR reaction (primers P4 and P5), using buffer 1, 3.75 units of enzyme, 0.35 mM dNTPs, and 0.3 μ M of each primer in a 50 μ l reaction with 1 μ l of DNA. The PCR program was 94°C for 2 min for initial denaturation, followed by 15 cycles of 94°C for 10 sec, 65°C for 30 sec, 68°C for 45 sec, then another 25 cycles with extension time of 1 minute, and was finished off with 1 cycle of 68°C for 10 min. For the second and third PCR reactions, we used nested primers (primers P6 and P7) and reduced the extension time to 35 sec for the first 15 cycles, to 50 sec for the next 25 cycles, and to 5 minutes for the final cycle. We digested the PCR products with IScel and then phenol extracted and desalted them prior to cloning into the TOPO-TA cloning vector pCR 2.1-TOPO (Invitrogen). To analyze the NHEJ events, we picked a total of 128 colonies and screened them by electrophoresis of PCR products (primers P6 and P7) on 1.5% agarose gels for 25 to 40 hours at low voltage with recirculating 1x TAE buffer. We selected 20 colonies that appeared to have longer or shorter products upon visual inspection and sequenced them. We randomly selected an additional 20 colonies and sequenced them. Roughly equal proportions of modified genomes were found by each method: 12/20 versus 11/20.

For HR events, we used primers P1 and P2 for the first round of PCR and primers P1 and P3 for the second round. We used the same PCR protocol for both amplifications under conditions similar to those for NHEJ amplifications, except that we used buffer 2 with final concentration of 0.5 mM dNTPs. The PCR program was 94°C for 2 min for initial denaturation, followed by 15 cycles of 94°C for 10 sec, 60°C for 30 sec, 68°C for 30 sec, then another 25 cycles with 45 sec extension time, and was finished off with 1 cycle of 68°C for 7 min. The gel-purified 657-bp band was cloned into plasmid vector pCR 2.1-TOPO and shown to be the expected HR event by DNA sequencing.

Analysis of individual green rods. We collected retinas from ID2-hRho-GFP mice injected with AAV-ISceI 7 to 8 weeks post-injection and dissociated them as previously described (3). We pooled 2 to 3 injected retinas and kept briefly in ice in 0.5 ml solution A [neurobasal medium Hibernate-A (Invitrogen) supplemented with 1x B27-supplement and 0.5 mM Glutamax (Gibco)] until all retinas were collected. We incubated the retinas at 37°C for 11 min in 0.5 ml of solution A containing 1 mg/ml pronase (Roche) and 2 mM EGTA. We then removed the pronase medium and replaced it with 0.5 ml solution A containing 2 mM EGTA, 10 U/ml DNase I (RNase free, Roche) and 0.5% BSA. We removed and replaced the medium three times to wash the pronase from the intact retinas. We dissociated the pronase-treated retinas at room temperature by repeated passage through a 1 ml pipette tip (cut and smoothed to 4 mm inner diameter). After 50 passages, the retinal fragments were centrifuged at 100 × g for 10 seconds using a tabletop centrifuge. We removed about ¾ of the supernatant, transferred it to a new eppendorf tube, added fresh medium, and repeated the dissociation process once more.

We diluted the combined supernatants with solution A, and spread about 0.5 ml of the cell suspension over a 50-mm glass bottom dish (MatTek Corporation). We picked Individual green rods under the fluorescent microscope (Nikon Eclipse TE2000-U), using a motorized micromanipulator system (MP-225, Sutter Instrument) and a micropipette with a 5-6 µm inner diameter and 30-degree bend at the tip (Origio Humagen Pipets). We initially scanned for Green rods using a 20× Apo objective and then sucked them into the micropipette under a 63× objective and a FITC/TRITC dual cube filter (Nikon). We ejected the picked green rod into the lid of a regular 100 µl PCR

tube (Applied Biosystems) containing 2 μ l of denaturing reagents [RNaST lysis buffer, which contains 2 U/ μ l RNase inhibitor (Ambion), 0.135 M NaCl; 9 mM Tris-HCl (pH 8.0), 4.5 mM dithiothreitol] (4). After closing the lid, we centrifuged the tube briefly in a “Quick-Spin” Minifuge (ISC BioExpress) to pellet the cell into the buffer. We immediately froze the tube on dry ice and stored it at -80°C for further analysis.

We prepared rhodopsin cDNA from the mRNA in individual green rods, using the QIAGEN OneStep RT-PCR kit. We used a reverse primer to GFP (CGAGCTGGACGGCGACGTAAACG) to make first strand cDNA, and added a forward primer to exon 1 of human rhodopsin (CTCTCTGCATGGATACTTCGTCTTCG) to amplify the cDNA. We set up the RT-PCR reactions according to the manufacturer’s suggested protocol in a 50- μ l final volume, using 400 μ M of each dNTP and 0.6 μ M of each primer. The PCR program was 50°C for 30 min for cDNA synthesis; 95°C for 15 min; followed by 15 cycles of 94°C for 30 sec, 60°C for 30 sec, and 1 min at 72°C ; then another 15 cycles with 1 sec extra extension time per each cycle; and 1 final cycle at 72°C for 10 min. We further amplified the initial product using nested primers in exon1 and GFP (forward: CACAGGATGCAATTTGGAGGGCTTC, reverse: GGTGGTGCCCATCCTGGTTCG). Products were analyzed by gel electrophoresis, with bands at 960 bp indicating mRNAs with two copies of exon 2 and bands at 791 bp indicating mRNAs with one copy of exon 2, consistent with SSA. We sequenced the bands to verify their structure.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Counts of nuclei in the outer nuclear layer. Nuclei in individual columns in the outer nuclear layers of heterozygous ID2-hRho-GFP mice were counted in retinal sections prepared at the indicated ages. These data are plotted relative to previously published data for heterozygous hrhoG(H) mice, which carry a LoxH site in the upstream untranslated region of the hRho-GFP gene and express human rhodopsin-GFP at the same level as an allele with a LoxP site (5). Heterozygous ID2-hRho-GFP mice, open circles; heterozygous hrhoG(H) mice, filled circles. Error bars indicate standard deviations.

Figure S2. PCR strategy for isolation of genomes modified at the ISceI site. Ten weeks after subretinal injection of rAAV-ISceI, retinal DNA was isolated and subjected to cycles of ISceI digestion (to remove background) and PCR amplification (to amplify NHEJ or HR products). After each ISceI digestion and PCR amplification, proteins were removed by treatment with phenol-chloroform-isoamyl alcohol (Phenol-IAC) and the DNA was isolated using QIAGEN spin columns (QIAquick). Gels showing the status of the DNA after each cycle of digestion and PCR amplification are shown beneath for both NHEJ and HR. Gels 1, 5, 7, 8, and 12 show DNA before (U) and after (C) ISceI digestion. The left lanes in gels 2, 3, 4, 6, 9, 10, and 11 show size controls or DNA ladders. The sizes of relevant fragments in kb are shown to the right of each gel. For NHEJ, the first PCR amplification used primers P4 and P5, and later amplifications used primers P6 and P7 (Fig. 4A). For HR, the first PCR amplification used primers P1 and P2; the second amplification used primers P1 and P3 (Fig. 4A). PCR-amplified DNA was cloned after the final ISceI digestion.

Figure S3. Analysis of products enriched for NHEJ events. After the final ISceI digestion (Fig. S2), the mixture of products in solution (not gel-extracted DNA) was cloned into pCR2.1-TOPO vectors. The resulting colonies were screened using primers P6 and P7 (Fig. 4A). Gel images show positions of the PCR products from 64 of the 128 total colonies screened relative to the wild-type

control (675 bp), which is shown in the first and last lanes of each gel. Except for the larger bands in lanes numbered 6, 15, and 16, there were no substantial size differences among the screened colonies. 20 colonies that appeared to have slight differences in migration were isolated from the numbered lanes and sequenced. 20 additional colonies from the remaining 64 colonies were picked randomly and sequenced. The type of event and the number of deleted or inserted nucleotides is shown below each lane. N, wild type IScel sequence; M, mixed band with no clear sequence; d, deletion; i, insertion.

SUPPLEMENTARY REFERENCES

1. Lan, Z.J., Xu, X. and Cooney, A.J. (2004) Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol Reprod*, **71**, 1469-1474.
2. Pang, J.J., Chang, B., Kumar, A., Nusinowitz, S., Noorwez, S.M., Li, J., Rani, A., Foster, T.C., Chiodo, V.A., Doyle, T. *et al.* (2006) Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. *Mol Ther*, **13**, 565-572.
3. Lolley, R.N., Lee, R.H., Chase, D.G. and Racz, E. (1986) Rod photoreceptor cells dissociated from mature mice retinas. *Invest Ophthalmol Vis Sci*, **27**, 285-295.
4. Li, Y., Thompson, H., Hemphill, C., Hong, F., Forrester, J., Johnson, R.H., Zhang, W. and Meldrum, D.R. (2010) An improved one-tube RT-PCR protocol for analyzing single-cell gene expression in individual mammalian cells. *Anal Bioanal Chem*, **397**, 1853-1859.
5. Chan, F., Bradley, A., Wensel, T.G. and Wilson, J.H. (2004) Knock-in human rhodopsin-GFP fusions as mouse models for human disease and targets for gene therapy. *Proc Natl Acad Sci U S A*, **101**, 9109-9114.

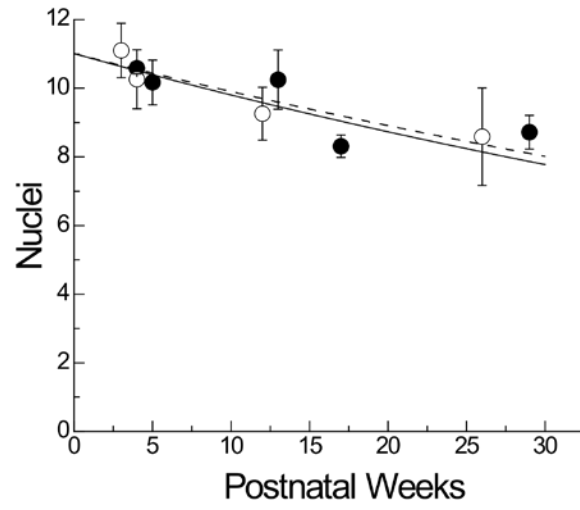


FIGURE S1

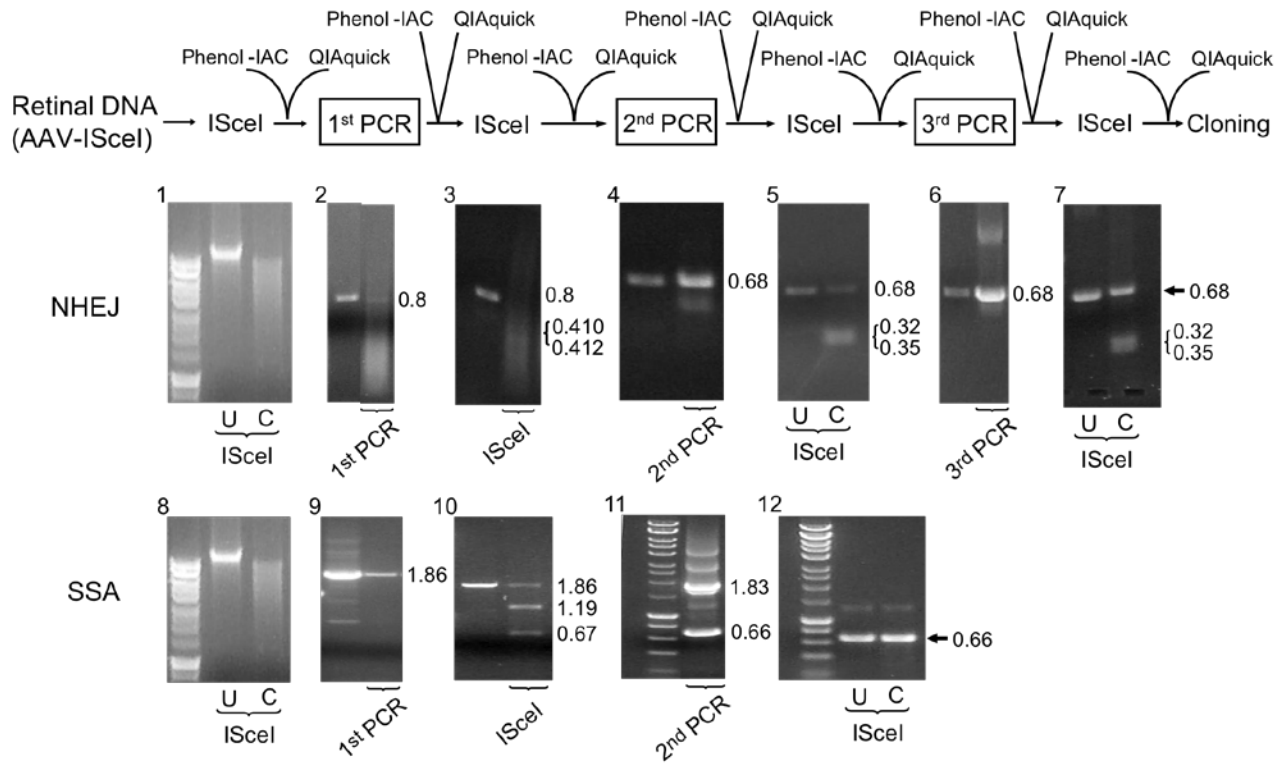


FIGURE S2

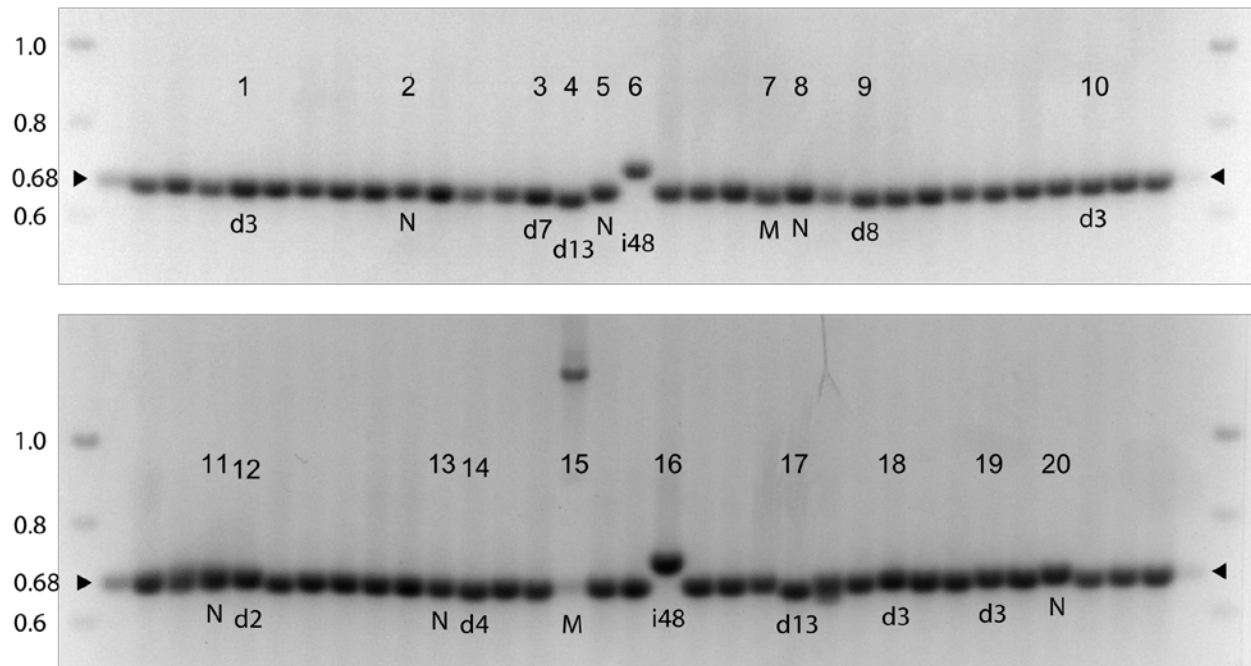


FIGURE S3