Correction of the *Crb1^{rd8}* Allele and Retinal Phenotype in C57BL/6N Mice Via TALEN-Mediated Homology-Directed Repair

Benjamin E. Low,¹ Mark P. Krebs,¹ J. Keith Joung,^{2,3} Shengdar Q. Tsai,⁴ Patsy M. Nishina,¹ and Michael V. Wiles¹

¹The Jackson Laboratory, Bar Harbor, Maine

²Molecular Pathology Unit, Center for Computational and Integrative Biology, Massachusetts General Hospital, Charlestown, Massachusetts

³Center for Cancer Research, Massachusetts General Hospital, Charlestown, Massachusetts

⁴Department of Pathology, Harvard Medical School, Boston, Massachusetts

Correspondence: Michael V. Wiles, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609-1500; mvwiles@jax.org.

Submitted: September 16, 2013

Accepted: December 3, 2013

Citation: Low BE, Krebs MP, Joung JK, Tsai SQ, Nishina PM, Wiles MV. Correction of the *Crb1^{rd8}* allele and retinal phenotype in C57BL/6N mice via TALEN-mediated homology-directed repair. *Invest Ophthalmol Vis Sci*. 2014;55:387–395. DOI:10.1167/iov.13-13278

PURPOSE. We directly corrected the mouse *Crb1^{rd8}* gene mutation, which is present in many inbred laboratory strains derived from C57BL/6N and complicates genetic studies of retinal disease in mice.

METHODS. Fertilized C57BL/6NJ oocytes were coinjected with mRNAs encoding a transcription activator-like effector nuclease (TALEN) targeting the *Crb1^{rd8}* allele plus single-stranded oligonucleotides to correct the allele. The oligonucleotides included additional nucleotide changes to distinguish the corrected allele (*Crb1^{em1Mvw}*) from wild-type *Crb1* and to minimize TALEN recutting. Oligonucleotide length, concentration of injected oligonucleotides and TALEN mRNAs were varied to optimize homology-directed repair of the locus. Following microinjection, embryos were carried to term in pseudopregnant females. Correction efficiency was assessed by PCR analysis of the *Crb1^{em1Mvw}* allele. Phenotypic correction was demonstrated by fundus imaging and optical coherence tomography of live mice, and by confocal fluorescence microscopy of retinal flat mounts.

RESULTS. Under optimal conditions, homology-directed repair was observed in 27% (8/30) of live-born animals and showed minimal illegitimate recombination of donor DNA. However, extensive founder mosaicism was evident, emphasizing the need to analyze offspring of founder animals. Unlike C57BL/6NJ mice, which exhibited external limiting membrane fragmentation and regional retinal dysplasia, heterozygous *Crb1^{em1Mvw}/Crb1^{rd8}* mice showed a normal retinal phenotype.

CONCLUSIONS. The C57BL/6NJ-*Crb1^{rd8}* mutation and its associated retinal phenotypes were corrected efficiently by TALEN-mediated homology-directed repair. The C57BL/6NJ-*Crb1^{em1Mvw}* mice generated by this strategy will enhance ocular phenotyping efforts based on the C57BL/6N background, such as those implemented by the International Mouse Phenotyping Consortium (IMPC) project.

Keywords: genetic engineering, TALEN, CRB1 associated disease, KOMP, gene therapy

The use of mouse models to study ocular disease relies heavily on genetically defined inbred strains. One of the most widely used is C57BL/6N, an inbred strain that diverged from C57BL/6J in 1951 when it was provided to the National Institutes of Health by The Jackson Laboratory. The C57BL/6N substrains are available from commercial suppliers and this background has become one of the primary sources of embryonic stem cells used to generate knockout or knockin mice. The C57BL/6N substrain also is the primary strain of the International Knockout Mouse Consortium (IKMC),¹ a large-scale effort to delete every mouse gene systematically, and the International Mouse Phenotyping Consortium (IMPC),² a complementary project aimed to assess physiological attributes and organ systems, including the eye, in each knockout strain. The use of an inbred strain is critical for these studies, as it

allows the effects of different targeted mutations to be compared on a fully defined genetic background. However, as in any inbred strain, C57BL/6N has accumulated mutations due to genetic drift that have become fixed, which may complicate the interpretation of certain phenotypes.³

A prime example of a complicating mutation is *Crb1^{rd8}*, which recently was shown to be homozygous in C57BL/6N and many C57BL/6N-derived strains.^{4–6} The *Crb1* encodes a protein required for the proper formation of adherens junctions at sites of cell-cell contact, regulating cell shape and apical-basal cell polarity. Mutations of human CRB1, as well as *Crb1* orthologs in *Drosophila melanogaster* and mice lead to ocular abnormalities that affect vision adversely.^{7–10} Further, vision is reduced significantly in C57BL/6N versus C57BL/6J as measured by the optokinetic reflex.¹¹ The *Crb1^{rd8}* mutation in mice is associ-

TABLE 1. Number and Percentages of Animals Born With Evidence of TALEN-Mediated Events, HDR Events, and Illegitimate Recombination Events

Experiment	Live Born/ Zygotes Injected				TALEN Induced Events/Live Born				HDR/Live Born				Illegitimate Recombination/Detectable			
	ng/ μ L TALEN mRNA Microinjected*				ng/ μ L TALEN mRNA Microinjected				ng/ μ L TALEN mRNA Microinjected				ng/ μ L TALEN mRNA Microinjected			
	10	25	50	Total	10	25	50	Total	10	25	50	Total	10	25	50	Total
200-mer	14/84	30/116*	32/99	76/299	6/14	24/30*	30/32	60/76	0/14	8/30*	6/32	14/76	1/14	1/22*	1/26	3/62
sense	17%	26%*	32%	25%	43%	80%*	94%	79%	0%	27%*	19%	18%	7%	5%	4%	5%
200-mer	22/95	36/112*	28/90	86/297	12/22	24/36*	20/28	56/86	1/22	6/36*	0/28	7/86	0/21	3/30*	0/28	3/79
antisense	23%	32%*	31%	29%	55%	67%*	71%	65%	5%	17%*	0%	8%	0%	10%*	0%	4%
Combined*	36/179	66/228*	60/189	162/596	18/36	48/66*	50/60	116/162	1/36	14/66*	6/60	21/162	1/35	4/52*	1/54*	6/141*
	20%	29%*	32%	27%	50%	73%*	83%	72%	3%	21%*	10%	13%	3%	8%*	2%*	4%*
Experiment	ng/ μ L ssODN DNA Microinjected				ng/ μ L ssODN DNA Microinjected				ng/ μ L ssODN DNA Microinjected				ng/ μ L ssODN DNA Microinjected†			
	0.3	2	6	Total	0.3	2	6	Total	0.3	2	6	Total	0.3	2	6	Total
	0.3	2	6	Total	0.3	2	6	Total	0.3	2	6	Total	0.3	2	6	Total
52-mer	14/94	16/93	14/85	44/272	11/14	13/16	12/14	36/44	1/14	1/16	3/14	5/44	ND	ND	ND	ND
sense	15%	17%	16%	16%	79%	81%	86%	82%	7%	6%	21%	11%	ND	ND	ND	ND
52-mer	13/55	13/80	33/93	59/228	10/13	8/13	26/33	44/59	0/13	0/13	0/33	0/59	ND	ND	ND	ND
antisense	24%	16%	35%	26%	77%	62%	79%	75%	0%	0%	0%	0%	ND	ND	ND	ND
Combined*	27/149	29/173	47/178	103/500	21/27	21/29	38/47	80/103	1/27	1/29	3/47	5/103	ND	ND	ND*	ND
	18%	17%	26%	21%	78%	72%	81%	78%	4%	5%	6%	5%	ND	ND	ND	ND*

* Results from the optimal condition of 25 ng/ μ L of TALEN mRNA and 1 ng/ μ L of the 200-mer ssODN.

† Illegitimate recombination values cannot be determined for the 52-mer-derived mice, nor in any mouse that had a correct ssODN-mediated repair.

ated with retinal external limiting membrane (ELM) fragmentation and outer retinal dysplasia.⁸ The unexpected discovery of *Crb1*^{rd8}-associated retinal dysplasia in studies targeting other genes in C57BL/6N led to grave concerns that ocular research in these C57BL/6N-derived strains might be compromised.⁵ These concerns may well extend beyond studies of the eye, as the mouse *Crb1* and its human ortholog are expressed at significant levels in the developing and adult brain.^{7,12} These findings underscore the importance of developing a strategy to correct the *Crb1*^{rd8} allele in the C57BL/6N background.

The direct genetic manipulation of inbred mice via the oocyte provides an experimental system whereby the profound effects of genetic background can be isolated and explored coherently.^{13–15} By avoiding the use of intermediary embryonic stem cells, or cumbersome backcrossing, this direct approach opens the possibility of sequentially modifying existing mouse strains, including those that have been studied extensively previously and/or modified genetically. This reiterative approach provides a means to build directly upon and add to earlier work using the wealth of historical data associated with particular strains. Recently, multiple classes of custom-built nucleases (i.e., zinc-finger nuclease [ZFN], transcription activator-like effector nuclease [TALEN], and clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9) capable of targeting specific DNA sequences directly in the zygote have been developed.^{16–18} These nucleases can be designed to cause precisely targeted double-stranded DNA breaks (DSBs) in the genome, initiating error prone nonhomologous end joining (NHEJ) repair, resulting in insertions or, more commonly, deletions (indels) in the targeted region. In the presence of donor DNA with sequence homology to the target region, integration by a precise homology-directed repair (HDR) pathway can occur.^{19,20} Previous work using site-directed nucleases to mediate HDR in mouse zygotes has been confined to mixed genetic backgrounds.^{18,21,22} While such genetically mixed embryos are robust, it is the genetic

modification of inbred strains and associated transgenics that is required for genetically coherent studies.

Here, we use targeted nuclease-mediated HDR²³ to correct the *Crb1*^{rd8} mutation directly in zygotes of C57BL/6NJ (stock number JR#005304, MGI:3056279), which was established at The Jackson Laboratory in 2005 from cryopreserved embryos of C57BL/6N obtained from the National Institutes of Health. The significant biological roles of *Crb1*, the widespread use of the C57BL/6N strain in mouse model development, and the desire to maintain strain genetic background provided the impetus to correct the *Crb1*^{rd8} mutation by this direct and rapid method. The mice developed and described here will be useful for ocular studies, particularly in conjunction with the IKMC and IMPC initiatives.

MATERIALS AND METHODS

Experimental Animals

Mice provided with acidified water, and JL Rat and Mouse/Auto 4F (5K54) diet (LabDiet, St. Louis, MO) were housed in cages exposed to a 12-hour light-dark cycle in The Jackson Laboratory Research Animal Facility. All mice were treated in accordance with the Animal Care and Use Committee at The Jackson Laboratory, and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Gene Correction Single-Stranded Oligonucleotides (ssODN) Design and Rationale

All single-stranded *Crb1*^{rd8} correction DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

TABLE 2. Sequences of ssODNs Designed for *Crb1^{rd8}* Correction by HDR

	Single-Stranded Donor Oligonucleotide (ssODNs) Sequences*
200-mer Sense	5' TTCTACAAATATGGTACTTACTGGCTGTTTGCCATCAAATGCCTGCCACT CCAGCCCCTGTTTGCATGGAGGAACTGTGAAGACAGTTACAGCAGTTATCG GTGTGCCTGTCTGTCGGGATGGTCAGGGACACACTGTGAAATCAACATTGAT GAGTGCTTTTCTAGCCCCTGTATCCATGGCAACTGCTCTGATGGAG
200-mer antisense	5' CTCCATCAGAGCAGTTGCCATGGATACAGGGGCTAGAAAAGCACTCATCA ATGTTGATTTACAGTGTGTCCCTGACCATCCCGACAGACAGGCACACCGAT AACTGCTGTAACTGTCTTCACAGTTTCTCCATGCAAACAGGGGCTGGAGTG GCAGGCATTTGATGGCAAACAGCCAGTAAGTACCATATTTGTAGAA
52-mer sense	5' TGAAGACAGTTACAGCAGTTATCGGTGTGCCTGTCTGTCGGGATGGTCAG GG
52-mer anti-sense	5' CCCTGACCATCCCGACAGACAGGCACACCGATAACTGCTGTAACTGTCTT CA

* The missing base characterizing the *rd8* allele is bold. Five synonymous base substitutions (gray boxes) are designed to prevent further modification by the TALENs following repair and to differentiate the repaired locus from the C57BL/6J wild-type allele.

TALENs and Oligonucleotides

The DNA sequences encoding for the TALEN DNA binding regions were designed using “ZiFit”^{24,25} (sequence available upon request), synthesized by GenScript Corporation (Piscataway Township, NJ), and cloned into the plasmids JDS70 (SNI/A 0.5 Domain) and JDS71 (SHD/C 0.5 Domain), which encode *FokI* monomers (Addgene plasmids 32285, 32287; Addgene, Cambridge, MA).²⁶ The left binding region was designed to bind 5'-TGAAGACAGCTACAGTTC, with an intervening sequence containing the *Crb1^{rd8}* mutation 5'-TTATGGTGTGCCTGTC, and the right binding region to 5'-TCTCGGGATGGTCAGGA. These sequences correspond to the native condition of the *Crb1^{rd8}* locus in the C57BL/6N background. Possible off-target effects were assessed using a scoring function developed previously²⁷ and were deemed to be low.

Pronuclear Microinjections of Zygotes

Four independent experiments were conducted. Fertilized oocytes from approximately 3-week-old superovulated C57BL/6NJ mice were used for microinjection. Plasmids containing TALEN targeted to the *Crb1^{rd8}* region were linearized using *PmeI* and used as a template for capped mRNA synthesized using the T7 promoter (AmpliCap-Max T7 High Yield Message Maker Kit (Cat. No. C-ACM04037; Cellscript, Madison, WI) and A-Plus Poly(A) Polymerase Tailing Kit (Cat. No. C-PAP5104H; Cellscript). The TALEN mRNA and ssODNs at varying concentrations were combined in 10 mM Tris, pH 7.5 plus 1 mM EDTA (TE) and 2 to 5 pL microinjected per oocyte pronucleus. In Experiments 1 and 2, TALEN mRNAs (10, 25, or 50 ng/μL) were coinjected with 1 ng/μL of 200-mer (sense or antisense strand). Following microinjection, embryos (~20/female) were transferred to pseudopregnant females (10–12 weeks, CByB6F1/J) and carried to term. In Experiments 3 and 4, fertilized oocytes were microinjected as before, but with TALEN mRNAs at 50 ng/μL, and coinjected with 0.3, 2, or 6 ng/μL of 52-mer ssODNs (sense or antisense strand).

PCR Analysis for *Crb1^{rd8}* Correction

To ascertain if TALEN-mediated HDR had occurred and incorporated correctly the 200- or 52-mer ssODN (sense or antisense strand), tail DNA was analyzed by PCR amplification. The resulting PCR products were sequenced using the same primers. For the unmodified allele, primer #1303 5'-GGGAAAGCTTCCCAGACTGACAAT and primer #1353 primer 5'-CGACCAGACACCCTTTGTGATAAG gave a product of 659bp. Upon NHEJ deletions (>25–<611 base pairs [bp]), the PCR product band size was visibly reduced. Smaller NHEJ events were detected and verified by sequencing the PCR product using primers #1303 and #1353.

To detect correct HDR events, multiplex PCR was performed to detect specifically the presence of the six synonymous base changes, including the required corrected base, within the context of the *Crb1* region. Multiplex PCR used primers #1303 and #1353, generating a product of 659 bp for an unaltered *Crb1^{rd8}* allele. The PCR primer #1368, 5'-CAGACAGGCACACCGATAACTG and #1353 generate a product only when a correct HDR targeting event occurred in the *Crb1^{rd8}* allele using either the 200-mer or 52-mers, with a band of 330 bp. The PCR amplicons were sequenced to verify the modification.

Screening for Illegitimate Recombination

To monitor for illegitimate recombination, a single nucleotide polymorphism (SNP) assay was designed to recognize differentially the unmodified allele approximately 65 bases central to the 200-mer, enabling the detection of the 200-mer correction ssODN at off-target sites (i.e., illegitimate recombination and legitimate recombination). The 52-mer could not be detected by this assay as an SNP assay could not be designed within its sequence boundaries. The SNP assays were carried out by LGC Genomics LCC (Beverly, MA or Herz, UK) using DNA isolated from mouse tails, a common primer 5'-CAGCCCCTGTTTGCATGGAGGAA paired with labeled primers distinguishing the *Crb1^{rd8}* wild-type sequence 5'-

CCCGASAGACAGGCACACCA, and ssODN modified sequence 5'-CCGASAGACAGGCACACCG.

Confocal Fluorescence Microscopy of Retinal Flat Mounts

Mice were sacrificed by CO₂ asphyxiation and retinal flat mounts were prepared from right eyes as described.²⁸ Following overnight fixation at 4°C in ×0.75 PBS containing 4% wt/vol paraformaldehyde, retinas were stained for four days at 4°C in PBS containing 0.5% Triton X-100, rhodamine phalloidin (1 U/ml; Life Technologies, Grand Island, NY), and 4',6-diamidino-2-phenylindole (DAPI, 10 µg/ml; Life Technologies). After staining, samples were rinsed twice with PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Confocal microscopy was performed at ×63 with an SP5 laser confocal microscope (Leica Microsystems, Buffalo Grove, IL). Laser power in each fluorescence channel was adjusted by attenuation compensation to yield similar object intensities throughout the ELM. Confocal images were analyzed and processed in Imaris 7.6.4 (Bitplane, South Windsor, CT) to highlight the ELM.

Fundus Imaging

Following pupil dilation with 1% atropine (Alcon, Fort Worth, TX), right eyes were examined with a Micron III retinal camera (Phoenix Research Laboratories, Pleasanton, CA). Video images (100 frames) were acquired at approximately 30 frames per second (fps) and processed in Fiji.²⁹ Image stacks were registered using the Image Stabilizer plugin (available in the public domain at http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html) in translation and affine modes with default settings on a cropped subset of the image centered on the optic nerve head. Registration coefficients generated in each mode were applied to the uncropped image stack with the Image Stabilizer Log Applier plugin. Registered image stacks were averaged with Z Project, sharpened in two rounds with the Unsharp Mask function with a 3.0 pixel radius, and mask weights of 0.7 and 0.5, respectively, and adjusted for contrast and brightness. Fundus images were oriented in Fiji with the superior and nasal fundus at the top and right, respectively. The degree of rotation required for orientation was determined from the angle of a line drawn from the pupil center to the nose in an image of the mouse head taken with the Micron III camera immediately before fundus imaging.

Optical Coherence Tomography (OCT)

On the same day as fundus imaging, the same mice were anesthetized with a mixture of 16 mg/mL ketamine (Butler Animal Health Supply, Dublin, OH) and 3.2 mg/mL xylazine (Lloyd, Shenandoah, IA) in 0.9% sodium chloride to yield a final dose of 80 mg ketamine and 16 mg xylazine per kg body weight. Anesthetized mice were examined with an ultrahigh resolution spectral domain OCT instrument (Bioptigen, Morrisville, NC). Ten volume scans (1000 A-scans per B-scan, 100 B-scans, 1.4 mm rectangular volume generated in enhanced depth imaging mode) were acquired for each animal. A custom Fiji macro was used to register, average, and align neighboring B-scans of the 10 image volumes, and generate en face and B-scan stacks of the dataset. En face views were rotated to match to the orientation of the fundus image using the shadows of superficial blood vessels as a guide. The OCT B-scan images were obtained from the same relative position of the image volume in both mice examined.

RESULTS

Strategy to Correct the *Crb1*^{rd8} Mutation in C57BL/6NJ Mice

The *Crb1*^{rd8} mutation is a single bp deletion in exon 9, predicted to cause a frame shift and premature translation stop (Fig. 1A). Our strategy involved introducing a DSB near the *Crb1*^{rd8} mutation using a pair of TALENs microinjected into zygotes. To promote HDR at the DSB site, an ssODNs with homology to the region also was included.^{20,22} Crucial to this process was the use of single-stranded DNA, which has been suggested to be 100-fold less likely to integrate by illegitimate recombination compared to double-stranded DNA.³⁰

For these genomic correction experiments, four single-stranded *Crb1*^{rd8} correction ssODNs (200-mer and 52-mer, in sense and antisense directions) were synthesized with homology centered to the targeted region. In addition to the replacement of the deleted nucleotide ("C/G") in the *Crb1*^{rd8} mutation, the ssODNs were designed to incorporate five synonymous base substitutions inside the TALEN binding regions (Figs. 1A, 1B). These substitutions prevent further modification by the TALENs post HDR repair, and were selected to maintain amino acid usage and codon usage frequency.

Correction of the *Crb1*^{rd8} Mutation

To optimize TALEN-mediated HDR, we varied the TALEN mRNA concentration, as well as the concentration and length of the ssODNs. First, we tested a 200-mer (sense orientation) at 1 ng/µL with TALEN mRNA at 10, 25, or 50 ng/µL. The identical experiment then was performed using the antisense version of the 200-mer ssODN. The third experimental regime maintained the TALEN mRNA at its highest concentration (50 ng/µL), but used three different concentrations of a 52-mer (sense orientation) ssODN (0.3, 2, or 6 ng/µL). The final experiment mirrored the third, except the antisense 52-mer was used. Upon transfer into pseudopregnant dams, approximately 15% to 35% of injected and transferred zygotes survived to wean age (Table 1). The rate of survival was similar across the three concentrations of TALEN-encoding mRNAs, suggesting that the mRNAs were not toxic, even at the highest TALENs concentration of 50 ng/µL.

The DNA isolated from tail tips was interrogated to identify NHEJ indels and the presence of HDR-corrected alleles (data summarized in Table 1). Across all treatment groups for the 200-mers, the rate of all TALEN-mediated events detected was 72%, including two apparent biallelic correction events. The sense 200-mer resulted in higher HDR rates (18%, 14 of 76 animals) compared to 8% (7 of 86 animals) from the antisense 200-mer.

As Figure 2 illustrates, the TALEN pair used here (plus donor ssODN) provided detectable events in 43% to 94% of founder animals under all various conditions used. The HDR was variable and detected only under some of the conditions examined. The optimal condition for HDR using this TALEN/ssODN combination was 25 ng/µL of each TALEN, combined with 1 ng/µL of the sense 200-mer, resulting in 27% HDR (8 of 30 animals born). The identical condition using the antisense 200-mer gave a similar result (17% HDR, 6/36 animals) as the sense 52-mer at 6 ng/µL coinjected with 50 ng/µL TALEN (3 of 14 animals, 21% HDR). No HDR was detected from the antisense 52-mer experiments.

Using the shorter 52-mer *Crb1*^{rd8} correction ssODNs, the sense strand gave 11% HDR (5 of 44 animals) and the antisense gave 0% (0 of 59 animals); that is, a combined HDR rate of 5% for the 52-mer ssODN, in contrast to that of the 13% (21 of 162 animals) with the 200-mers; that is, the longer ssODN mediate

FIGURE 1. Outline of approach showing the critical portion of the *Crb1^{rd8}* allele sequence before and after gene correction using any of four ssODNs. (A) The *Crb1^{rd8}* allele resulted from a single base deletion (arrow) causing a frame shift and functional null mutation (putative translation product in gray). A TALEN pair was designed to bind at sites flanking the mutation (indicated by gray boxes). Aligned below the *Crb1^{rd8}* nucleotide sequence is the central core sequence of the ssODNs (shown 5'–3') used to facilitate gene correction (full 200-mer and 52-mer sequences in Table 2). Homology is indicated by periods, while base changes are shown in red text; the base repairing the point mutation also is boxed. (B) The corrected gene product is predicted to be identical to the wild-type CRB1 protein. The final repaired gene sequence closely matches the wild-type version of the *Crb1* gene at the nucleotide level, except for the five synonymous base substitutions (all in red).

precise modification more efficiently. However, over the range examined, ssODN concentrations used did not impact the HDR rate (Fig. 2, Table 1).

Both 200-mer and 52-mer sense ssODNs provided a higher level of HDR rate compared to the antisense sequence, accounting for 73% (19 of 26 animals) of all HDR events (Fig. 2, Table 1). The significance of this finding is unclear, given the small sample size, but it may relate to microhomologies at the site of the DSB, and/or the nature of the TALEN overhang and its interaction with the DNA repair machinery.

Germline Transmission

A total of 26 potential founder mice was identified as candidates based on evidence for HDR correction of the *Crb1* allele by multiplex PCR and PCR amplicon sequencing of tail-derived DNA. However, due to the obfuscating effect of multiple alleles in these mosaic animals, the actual correction could not be verified until N1 progeny were produced and analyzed. One mouse was euthanized before sexual maturity due to hydrocephaly, but the remaining 25 founder candidates were bred to C57BL/6NJ to determine heritability for the modified allele, and to segregate and detect founder gamete mosaicism.^{27,31}

“Perfect” oligonucleotide-mediated repair as judged by the presence of all the synonymous substitutions was evident in litters from 13 of the 25 tested candidate mouse lines. Furthermore, two lines resulted in offspring that contained the corrected allele, but lacked one of the five synonymous substitutions (away from the correcting base), showing evidence of oligonucleotide truncation at the 3' end, yet functional corrections nonetheless. Another four lines carried the corrected allele, but they also carried a secondary nearby

mutation in *Crb1* nullifying the functional *rd8* correction. Finally, six lines failed to produce carriers, although three of these lines were nonproductive or subfertile (yielding zero, or only one or two offspring).

As mentioned above, partial insertions of the ssODN were identified in the offspring of some founders. Two lines contained the correction and 5' substitutions, but lacked the 3' G to C substitution (Fig. 1). This truncation has no impact on the predicted functional correction of *Crb1*. However, five lines produced offspring that contained the corrected base and at least some or all of the ssODN substitutions, but also had secondary indels that ultimately nullified the correction. One of these nullified lines actually had littermates with the complete insertion (“perfect” correction), which clearly demonstrated the confounding effects of germline mosaicism.

Two founder animals that showed clear biallelic (i.e., homozygous) *Crb1^{rd8}* allele correction based on their tail-derived DNA proved to be germline mosaics, resulting in a mixture of unaltered and *Crb1^{rd8}* allele-corrected offspring. This phenomenon appears to be a common occurrence with the nuclease-mediated HDR modification method used here^{27,31} and serves as a cautionary tale; that is, apparent biallelic founders should not be used experimentally as they may be mosaic and must be bred to resolve this condition.

Illegitimate Recombination Rate

A major concern using nuclease-mediated HDR gene correction is the potential for illegitimate recombination of donor DNA. We attempted to estimate the frequency of illegitimate recombination by examining DNA from all founder animals and from the offspring of founders in which the 200-mers was used. The SNP genotyping strategy focused on the unique base

FIGURE 2. Distribution of events in founder mice. The percent of TALEN-mediated events in live-born founders is shown as a function of experimental condition. Values in *square brackets* indicate concentration in ng/μL. Complete data are shown in Table 1. Illegitimate recombination values were not determined for the 52-mer-derived mice, nor in any mouse that had a correct ssODN-mediated repair.

changes incorporated in the ssODN. The developed SNP assay was not able to distinguish between an off-target event and an ssODN HDR correction, and so evidence of illegitimate recombination was obtained only from offspring without HDR of the *Crb1^{rd8}* allele (Fig. 2, Table 1). Under this series of conditions, illegitimate recombination events were detected at 0% to 10%, with no readily apparent trends. Under our optimal conditions (sense 200-mer with 25 ng/μL TALEN mRNAs), illegitimate recombination was detected at approximately one-sixth the level of HDR. We also noticed that off-site ssODN sequences occurred only in mice that also had *Crb1^{rd8}* TALEN-targeted mediated events (i.e., none was detected in mice with no evidence of an NHEJ event). Overall, the frequency of detectable off-target ssODN sequences for all experiments combined was only 4% (6 of 141 animals, Table 1). By way of comparison, the TALEN NHEJ event efficiency was 74% (196 of 265 animals) and the cumulative rate of HDR was 9.8%, resulting in 26 total potential founder animals (Table 1).

This SNP strategy only identifies illegitimate 200-mer ssODN sequences in the absence of the corrected allele. Based on this, we predicted that at most 1 of the 21 *Crb1^{rd8}* allele candidate HDR mice made here would contain an illegitimate ssODN insertion. To test this, we bred the *Crb1^{rd8}* corrected founder animals and examined their offspring for the presence of the 200-mer sequences in an illegitimate genomic context. To date, only 1 line out of 14 tested has shown evidence of illegitimate recombination (1/76), confirming a low frequency of off-target modification even among this corrected population. If needed, the off-target events could almost certainly be eliminated by further breeding. However, it is clear that more research is needed to reduce illegitimate events to near zero to minimize undesired mutations.

Correction of *Crb1^{rd8}* Mutation Ocular Phenotypes

We examined founders and their offspring from a C57BL/6NJ backcross to determine if the associated ocular phenotypes also were corrected. The primary *Crb1^{rd8}* phenotype, a recessive trait, is characterized by fragmentation of the ELM, a retinal structure formed by adherens junctions between photoreceptor and Müller cells.⁸ Hence, its phenotypic repair would be predicted to be evident in heterozygous animals. Phalloidin staining of retinal flat mounts revealed a patchy disruption of F-actin structures at the ELM in C57BL/6NJ mice (30 days old, 3 males; 99 days old, 2 males), consistent with ELM fragmentation (Fig. 3A). In striking contrast, a corrected founder (99-day-old male, Fig. 3B) showed no ELM fragmentation and the F-actin structures were similar to that of wild-type C57BL/6J mice (30 days old, 1 female, 2 males; 99 days old, 2 males; Fig. 3E). Offspring derived from this founder that were heterozygous for the corrected *Crb1* allele (31 days old, 3 males; Fig. 3C) had a normal ELM phenotype, while littermates homozygous for the (uncorrected) *Crb1^{rd8}* allele had a fragmented phenotype (1 female, 2 males; Fig. 3D).

The *Crb1^{rd8}* allele also is associated with outer retinal dysplasia, which is correlated with a progressive degeneration of photoreceptor cells.^{8,10} This phenotype varies with strain background and is diminished substantially in C57BL/6 substrains.^{8,32} Therefore, to test whether the dysplastic phenotype was corrected, we examined offspring of a mating between a corrected founder and STOCK-*Crb1^{rd8}*/J (JR#018392) mice, which exhibit robust dysplasia. Fundus imaging (Figs. 3F, 3I) and OCT (Figs. 3G, 3H, 3J, 3K) revealed retinal dysplasia in homozygous *Crb1^{rd8}* (30 days old, 3 males) offspring (Figs. 3F-H), but no detectable dysplasia in a heterozygous corrected (1 female) littermate (Figs. 3I-K). Together, these data showed that TALEN mRNA-mediated HDR

FIGURE 3. Correction of *Crb1*^{rd8}-associated phenotypes. (A–E) ELM fragmentation in C57BL/6NJ (A), a corrected *Crb1*^{em1Mvw}/*Crb1*^{rd8} founder (B), *Crb1*^{em1Mvw}/*Crb1*^{rd8} (C), and *Crb1*^{rd8}/*Crb1*^{rd8} (D) offspring from a cross of the founder with C57BL/6NJ, and C57BL/6J (E) control mice. The heterozygous *Crb1*^{rd8} founder and offspring are normal, while homozygous animals show ELM fragmentation. Retinal flat mounts at a postnatal age of 99 days (31 days for offspring) were stained with rhodamine phalloidin. The thresholded surface highlights F-actin structures at the ELM. Images of offspring, C57BL/6NJ, and C57BL/6J are representative of at least three littermates. Samples at P31 were similar to those at P99. Scale bar: 50 μ m. (F–K) Brightfield fundus (F, I), OCT en face projection of the outer nuclear layer (G, J) and OCT B-scan (H, K) of the right eyes of *Crb1*^{rd8}/*Crb1*^{rd8} (F–H) and *Crb1*^{em1Mvw}/*Crb1*^{rd8} (I–K) littermates at P31 from a cross between a *Crb1*^{em1Mvw}/*Crb1*^{rd8} founder and STOCK-*Crb1*^{rd8}/J. Spots corresponding to dysplastic lesions in the outer retina were observed in homozygous *Crb1*^{rd8} offspring, but not in corrected *Crb1*^{em1Mvw}/*Crb1*^{rd8} offspring. Arrowheads in (F–H) indicate the same dysplastic lesion.

using ssODN genotypically and phenotypically correct the *Crb1*^{rd8} mutation in C57BL/6NJ mice.

The corrected mouse strain is formally called C57BL/6NJ-*Crb1*^{em1Mvw}/Mvw, and after five backcrosses to C57BL/6NJ and interbreeding to homozygosity, is available through The Jackson Laboratory (JR#022521).

DISCUSSION

We showed that precise gene correction, with accompanying phenotypic correction, can be achieved at a high frequency directly in inbred mice. Further, our data strongly suggested that this occurs with limited, low levels of illegitimate recombination or off target events. That this was successful in an inbred mouse strain is of particular importance, as it demonstrates that this powerful approach can be used to dissect gene interactions in defined genetic contexts.^{13,14} Also crucially, these data open avenues for the sequential, reiterative genetic modification of complex transgenic inbred strains, building upon previous historical data; speeding model development, characterization, and their more rapid availability to the scientific community.

Evidence of mosaicism in founder animals, post nuclease modification, has been reported previously; however, to our knowledge founder mosaicism in relation to HDR has not. Knowledge that these events identified in tail DNA may not match the whole organism and its gametes/germline requires the implementation of breeding strategies with characterization of the offspring. This phenomenon currently precludes the direct phenotyping of founder animals with the assurance that all somatic tissues are modified similarly. Therefore, we strongly recommend backcrossing mice derived using these

technologies. This allows for the absolute confirmation of the mutation of interest and also increases the probability that possible illegitimate recombination or other off-target events will segregate out, diminishing their potentially confounding impact. Founder mosaicism probably is due to delayed action of the introduced TALEN mRNAs, with NHEJ and HDR events occurring cell independently at two-cell, four-cell or even later stages of development. A possible solution to this technical challenge is to sidestep translation of the introduced nucleases mRNAs by directly microinjecting them as proteins together with donor DNAs.

The correction of the *Crb1*^{rd8} mutation in C57BL/6N inbred mice, a strain used to generate many animal models, is a proof-of-concept of this powerful approach. The corrected strain can be used to correct and maintain models being developed by the IKMC, alleviating or controlling the effects of the C57BL/6N *Crb1*^{rd8} gene defect, which they all carry. For example, if an enhanced dysplastic retinal phenotype is observed in a knockout strain obtained from the current IKMC and IMPC pipeline, the strain may be intercrossed with the corrected *Crb1* strain to assess whether the deleted gene is epistatic to *Crb1*. This approach is preferred to intercrossing with C57BL/6J, which has a wild-type *Crb1* allele, but which possesses many other gene variations compared to C57BL/6N⁴ that may modify the knockout phenotype in an unpredictable fashion. Although it could be argued that *Crb1*^{rd8} is relevant only to those interested in modifiers of the retinal phenotype, the mutation also may have significant effects in the developing and adult brain.¹² Further, *Crb1* normally is expressed in the telencephalic ventricle, rostral migratory stream, and olfactory bulbs,¹² and its absence could have profound effects on how smell is perceived and used in mice. The corrected strain provides a simple and elegant system to recapitulate the wild-

type *Crb1* allele in the C57BL/6N background to elucidate the role of *Crb1* throughout the mouse.

The TALEN-mediated HDR strategy developed in this study may be applied to other inbred strains, allowing correction of the *Crb1*^{rd8} mutation while maintaining the strain background. This approach might be useful for modifying complex genetic backgrounds initially derived by crossing C57BL/6N with additional strains, where intercrossing as described above would introduce unnecessary complication and time. The strategy used here illustrates how gene correction can be performed, in principle, for other genes of interest, providing more complete and conclusive results than standard transgenics or embryonic stem cell-based approaches. However, given that every inbred strain carries its own set of mutations, which cumulatively result in the characteristics of that strain, it is reasonable to ask whether correction of mutations in any given strain is a sensible pursuit. Arguably, in this case, in which the *Crb1*^{rd8} allele has profound phenotypic effects in the eye that can influence the interpretation of a large body of research, correction of *Crb1*^{rd8} in C57BL/6N is desirable.

Our work contributes to a growing number of reports that nuclease-mediated HDR with TALEN or particularly CRISPR/Cas9 technologies can yield targeted gene changes in mice with high efficiency.^{17,18,21,22} These methods are poised to revolutionize our ability to generate mouse models carrying subtle genetic changes as informed by human genome-wide association studies, multiple mutations to study gene interaction in disease, or markers to interrogate biological pathways. While we have demonstrated that the nuclease-mediated HDR process works well in inbred mice, there still is considerable room for improvement. Increasing the frequency of HDR and, very importantly, eliminating mosaicism and partial off-target modifications should be key areas of future research focus to enhance the use of nuclease-mediated approaches in developing mouse models.

Acknowledgments

The authors thank the Jackson Laboratory Microinjection group, especially Pete Kutny, for continually providing excellent microinjection services, Cindy Avery for excellent technical assistance and animal husbandry, Wanda Hicks for help with fundus imaging, and Keith Sheppard for streamlining OCT image processing, and Jürgen Naggert and Gabriele Proetzel for critical reading and comments on the manuscript.

Supported by National Institutes of Health Grant OD011190 (MVW), National Institutes of Health Grant EY011996 (PMN), National Institutes of Health Research Fellowship under the Ruth L. Kirschstein National Research Service Award 1F32GM105189 (SQT), and by National Institutes of Health Cancer Center Support (CORE) Grant 5P30CA034196 (The Jackson Laboratory).

Disclosure: **B.E. Low**, P; **M.P. Krebs**, None; **J.K. Joung**, See below; **S.Q. Tsai**, None; **P.M. Nishina**, None; **M.V. Wiles**, P

JKJ has a financial interest in Transposagen Biopharmaceuticals. JKJ's interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

References

- Bradley A, Anastassiadis K, Ayadi A, et al. The mammalian gene function resource: the International Knockout Mouse Consortium. *Mamm Genome*. 2012;23:580–586.
- Brown SDM, Moore MW. The International Mouse Phenotyping Consortium: past and future perspectives on mouse phenotyping. *Mamm Genome*. 2012;23:632–640.
- Taft RA, Davisson M, Wiles MV. Know thy mouse. *Trends Genet*. 2006;22:649–653.
- Keane TM, Goodstadt L, Danecek P, et al. Mouse genomic variation and its effect on phenotypes and gene regulation. *Nature*. 2011;477:289–294.
- Mattapallil MJ, Wawrousek EF, Chan CC, et al. The Rd8 mutation of the *Crb1* gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Invest Ophthalmol Vis Sci*. 2012;53:2921–2927.
- Chang B, Hurd RE, Wang J, Nishina PM. Survey of common eye diseases in laboratory mouse strains. *Invest Ophthalmol Vis Sci*. 2013;54:4974–4981.
- den Hollander AI, Ten Brink JB, de Kok YJM, et al. Mutations in a human homologue of *Drosophila crumbs* cause retinitis pigmentosa (RP12). *Nat Genet*. 1999;23:217–221.
- Mehalow AK, Kameya S, Smith RS, et al. CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina. *Hum Mol Genet*. 2003;12:2179–2189.
- Bulgakova NA, Knust E. The Crumbs complex: from epithelial-cell polarity to retinal degeneration. *J Cell Sci*. 2009;122:2587–2596.
- Aleman TS, Cideciyan AV, Aguirre GK, et al. Human *CRB1*-associated retinal degeneration: comparison with the *rd8* *Crb1*-mutant mouse model. *Invest Ophthalmol Vis Sci*. 2011;52:6898–6910.
- Simon MM, Greenaway S, White JK, et al. A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome Biol*. 2013;14:R82.
- den Hollander AI, Ghiani M, de Kok YJM, et al. Isolation of *Crb1*, a mouse homologue of *Drosophila crumbs*, and analysis of its expression pattern in eye and brain. *Mech Dev*. 2002;110:203–207.
- Gerlai R. Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? *Trends Neurosci*. 1996;19:177–181.
- Montagutelli X. Effect of the genetic background on the phenotype of mouse mutations. *J Am Soc Nephrol*. 2000;11: S101–S105.
- Festing M. The choice of animal model and reduction. *Altern Lab Anim*. 2004;32(suppl 2):59–64.
- Jacob HJ, Lazar J, Dwinell MR, Moreno C, Geurts AM. Gene targeting in the rat: advances and opportunities. *Trends Genet*. 2010;26:510–518.
- Carlson DE, Fahrenkrug SC, Hackett PB. Targeting DNA with fingers and TALENs. *Mol Ther Nucleic Acids*. 2012;1:e3.
- Wang H, Yang H, Shivalila CS, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153:910–918.
- Gaj T, Gersbach CA, Barbas CF III. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31:397–405.
- Orlando SJ, Santiago Y, DeKolver RC, et al. Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. *Nucleic Acids Res*. 2010;38:e152.
- Meyer M, Ortiz O, De Angelis MH, Wurst W, Kühn R. Modeling disease mutations by gene targeting in one-cell mouse embryos. *Proc Natl Acad Sci U S A*. 2012;109:9354–9359.
- Wefers B, Meyer M, Ortiz O, et al. Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. *Proc Natl Acad Sci U S A*. 2013;110: 3782–3787.
- Bedell VM, Wang Y, Campbell JM, et al. In vivo genome editing using a high-efficiency TALEN system. *Nature*. 2012;491:114–118.

24. Sander JD, Maeder ML, Reyon D, Voytas DF, Joung JK, Dobbs D. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res.* 2010;38:W462–W468.
25. Sander JD, Zaback P, Joung JK, Voytas DF, Dobbs D. Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. *Nucleic Acids Res.* 2007;35:W599–W605.
26. Sander JD, Cade L, Khayter C, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol.* 2011;29:697–698.
27. Doyon Y, Vo TD, Mendel MC, et al. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat Methods.* 2011;8:74–79.
28. Krebs MP, White DA, Kaushal S. Biphasic photoreceptor degeneration induced by light in a T17M rhodopsin mouse model of cone bystander damage. *Invest Ophthalmol Vis Sci.* 2009;50:2956–2965.
29. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9:676–682.
30. Zorin B, Hegemann P, Sizova I. Nuclear-gene targeting by using single-stranded DNA avoids illegitimate DNA integration in *Chlamydomonas reinhardtii*. *Eukaryot Cell.* 2005;4:1264–1272.
31. Tesson L, Usal C, Meqinoret S, et al. Knockout rats generated by embryo microinjection of TALENs. *Nat Biotechnol.* 2011;29:695–696.
32. Chu XK, Wang Y, Ardeljan D, Tuo J, Chan CC. Controversial view of a genetically altered mouse model of focal retinal degeneration. *Bioengineered.* 2013;4:130–135.