RESEARCH ARTICLE

CRISPR-Cas9-Mediated Correction of the 1.02 kb Common Deletion in *CLN3* in Induced Pluripotent Stem Cells from Patients with Batten Disease

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Abstract

Juvenile neuronal ceroid lipofuscinosis (Batten disease) is a rare progressive neurodegenerative disorder caused by mutations in *CLN3*. Patients present with early-onset retinal degeneration, followed by epilepsy, progressive motor deficits, cognitive decline, and premature death. Approximately 85% of individuals with Batten disease harbor at least one allele containing a 1.02 kb genomic deletion spanning exons 7 and 8. This study demonstrates CRISPR-Cas9-based homology-dependent repair of this mutation in induced pluripotent stem cells generated from two independent patients: one homozygous and one compound heterozygous for the 1.02 kb deletion. Our strategy included delivery of a construct that carried >3 kb of DNA: wild-type *CLN3* sequence and a LoxP-flanked, puromycin resistance cassette for positive selection. This strategy resulted in correction at the genomic DNA and mRNA levels in the two independent patient lines. These CRISPR-corrected isogenic cell lines will be a valuable tool for disease modeling and autologous retinal cell replacement.

Introduction

Juvenile neuronal ceroid lipofuscinosis, more commonly known as Batten disease, is an autosomal recessive disorder. Children with Batten disease typically present between 4 and 7 years of age with rapidly decreasing central visual acuity, which results from death of the light-sensing photoreceptor cells of the outer neural retina. Following loss of vision, patients experience severe central nervous system (CNS) deterioration, leading to epilepsy, motor deficits, cognitive regression, and death, which typically occurs within the second to third decade of life.

Batten disease is caused by mutations in the gene *neuronal ceroid lipofuscinosis 3 (CLN3)*. The *CLN3* gene product is a five- to six-transmembrane protein localized to the endosome/lysosome.^{1–4} While the function of the *CLN3* protein is unclear,⁵ mutations in *CLN3* lead to accumulation of autofluorescent lysosomal inclusions.^{6–9} Although >50 different disease-causing mutations in

CLN3 have been reported,¹⁰ 85% of patients have at least one allele harboring a 1.02 kb genomic deletion that causes a frameshift and premature stop codon.^{11–13} However, little is known about the pathophysiologic mechanisms of this fatal disease, and currently no treatment options are available.

Although patient-derived induced pluripotent stem cells (iPSCs) can be used to generate disease-specific cell types that are ideal for evaluating pathophysiology of rare disorders such as Batten disease, selection of appropriate control lines for phenotypic comparison is often challenging. While control lines are typically sourced from sex- and age-matched individuals, the frequency of normal genetic variability in the human population, even among family members, can complicate the assignment of phenotypic behavior to a specific genetic mutation. One way to address this issue is to correct patient-derived iPSCs genetically prior to differentiation, thus creating isogenic controls that only differ from the

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native uncorrected cell lines at the disease-causing loci. Recent advances in genome editing to correct disease-causing variants, specifically components of the CRISPR-Cas9* adaptive immune system, make this approach feasible. This system can be used to target specific genomic loci by using a single-guide RNA (sgRNA) coupled with a human codon-optimized Cas9 nuclease to create double-strand breaks.^{14–16} Co-delivery of sgRNA and Cas9 with a donor repair template carrying wild-type DNA can induce homology-dependent recombination (HDR) and genomic correction.^{17–22} For retinal degenerative blindness, iPSC-derived photoreceptor cells generated from naïve and CRISPR-Cas9-corrected isogenic control lines can be studied side-by-side to evaluate disease phenotypes and mechanisms rigorously.

In this study, we used a CRISPR-Cas9 homology-dependent repair approach to correct the common 1.02 kb genomic deletion mutation in iPSCs derived from two patients with molecularly confirmed Batten disease: a homozygote (1.02 kb del/1.02 kb del) and a compound heterozygote (1.02 kb del/Leu101del3CTC). In both cases, we demonstrate successful genomic correction and restoration of the *CLN3* transcript. Interestingly, correction of this large genomic deletion was significantly more difficult in patient-derived iPSCs obtained from a heterozygous individual than from a homozygous individual. We predict that this is due to greater homology between the HDR cassette and the Leu101del3CTC allele than the HDR cassette and the 1.02 kb deletion allele. This study paves the way for future studies comparing affected and CRISPR-corrected iPSCs to elucidate how mutations in *CLN3* cause retinal cell death and blindness.

Materials and Methods

Patients

All patients provided written informed consent for this study, which was approved by the Institutional Review Board of the University of Iowa (project approval #199904167) and adhered to the tenets set forth in the Declaration of Helsinki. The patient-specific cells utilized in this study carry the following molecularly confirmed *CLN3* mutations: 1.02 kb del/1.02 kb del (homozygous patient) and 1.02 kb del/Leu101del3CTC (compound heterozygous patient), as described previously.²³

Generation of patient-specific iPSCs

Three-millimeter skin biopsies were obtained from either the non-sun-exposed upper arm or lower abdomen, and isolated fibroblasts were used for generation of

patient-specific iPSCs, as described previously.^{23–25} Briefly, 250,000 patient-specific dermal fibroblasts were plated in one well of a six-well culture dish and transduced with non-integrating Sendai viral vectors driving expression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* at a multiplicity of infection of 3 (CytoTune-iPS Reprogramming Kit; cat # A16517; Thermo Fisher Scientific, Waltham, MA).^{23–25} iPSC colonies were maintained and expanded in Human Essential 8 media on Laminin-521-coated cell culture surfaces (Thermo Fisher Scientific).²⁴

Cloning of CRISPR-Cas9 and HDR donor constructs

Transgene plasmids were generated that encoded sgRNAs targeting the *CLN3* mutation and the humanized *Staphylococcus aureus* Cas9 nuclease. We also generated bicistronic constructs expressing a *CLN3*-targeted sgRNA and a human codon-optimized *Streptococcus pyogenes* Cas9 nuclease.^{14,22,26,27} The sgRNAs were designed using the Benchling platform (www.benchling.com) or the Optimized CRISPR Design Tool (crispr.mit.edu) for co-expression with *S. aureus* or *S. pyogenes* Cas9, respectively. Oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA), annealed, phosphorylated, and cloned into the *BsaI* sites in the pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA; US::BsaI-sgRNA plasmid; or the *BbsI* sites in pX330-U6-Chimeric_BB-Cbh-hSpCas9. The pX601 and pX330 plasmids (Addgene plasmids #61591 and #42230, respectively) were gifts from Feng Zhang (Massachusetts Institute of Technology and The Broad Institute). A donor repair construct for correcting the 1.02 kb genomic deletion in *CLN3* was synthesized by GenScript (Piscataway, NJ). The donor plasmid carries the corrected *CLN3* sequence and a floxed puromycin-selection cassette flanked by 400–1,000 base pairs (bp) of homologous sequence. In addition, three bases were mutated in an intronic region of the donor construct to introduce a *NcoI* restriction site for downstream restriction fragment length polymorphism (RFLP) analysis, and the PAM sequence was mutated to prevent re-cleavage events following delivery to cells (Fig. 2A).

Determination of on-target cleavage efficiency of single guides

sgRNAs and CRISPR-Cas9 expression constructs were first tested in HEK293T cells via lipofection. HEK293T cells were maintained in standard culture medium (MEM α , Thermo Fisher Scientific; 10% fetal bovine serum; and 0.2% Primocin, Invivogen, San Diego, CA) and passaged using standard culture conditions, as described previously.²² Each plasmid (500 ng) was transfected into 4×10^5 cells with Lipofectamine 2000

*Clustered Regularly Interspaced Short Palindromic Repeats.

(Thermo Fisher Scientific) according to the manufacturer's instructions. Forty-eight hours post transfection, genomic DNA (gDNA) was isolated (Machery-Nagel Nucleospin Tissue Kit; Clontech, Mountain View, CA), and loci of interest were polymerase chain reaction (PCR) amplified using the AccuPrime Taq DNA Polymerase High Fidelity Kit (Thermo Fisher Scientific) and locus-specific primers (Table 1). PCR reactions were column purified using the MinElute PCR Purification Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Two hundred nanograms of each purified PCR reaction were heteroduplexed in 20 μ L reactions with 1 $\times$ Buffer 2 (New England Biolabs, Ipswich, MA) as follows: 95°C for 10 min, 95–85°C at -2°C/s , 85°C for 1 min, 85–75°C at -0.3°C/s , 75°C for 1 min, 75–65°C at -0.3°C/s , 65°C for 1 min, 65–55°C at -0.3°C/s , 55°C for 1 min, 55–45°C at -0.3°C/s , 45°C for 1 min, 45–35°C at -0.3°C/s , 35°C for 1 min, 35–25°C at -0.3°C/s , 25°C for 1 min, 25–4°C at -0.3°C/s , 4°C hold. Heteroduplexes were treated with 1 μ L of T7E1 Nuclease (New England Biolabs) for 30 min at 37°C and subsequently resolved via gel electrophoresis using 2% pre-cast agarose E-gels (Thermo Fisher Scientific). To assess the target efficiency of individual sgRNAs, the amplified target locus was TA cloned using the PCR2.1 TOPO TA cloning kit according to manufacturer's instructions (Thermo Fisher Scientific). Colonies were picked and Sanger sequenced with M13(–20)F or M13R primer (Functional Biosciences, Inc., Madison WI). Sequences were aligned and read using LaserGene SeqMan Pro alignment software

(DNASTAR, Madison, WI). For each sgRNA, at least 80 clones were evaluated.

Delivery of CRISPR-Cas9 machinery to *CLN3*-patient-specific iPSCs

Large-scale endonuclease-free CRISPR-Cas9 plasmids were prepared using the Machery–Nagel NucleoBond Xtra Maxi kit (Clontech) according to manufacturer's instructions. One microgram of pX601-AAV-sg1*CLN3* was transfected into 1×10^6 *CLN3*-patient-specific iPSCs via NEON transfection using Buffer R according to the manufacturer's instructions (Thermo Fisher Scientific). Guide-expressing and HDR plasmids were delivered in 1:2 molar ratios to iPSCs. Three days post transfection, cultures were placed under puromycin (0.05 $\mu\text{g/mL}$) selection. Puromycin was increased to 0.2–0.5 $\mu\text{g/mL}$, and surviving clones were selected for further analysis. GDNA from individual colonies was isolated using the DNeasy Blood & Tissue Kit (Qiagen). For screening of clones using restriction enzyme digest, 200 ng of gDNA was PCR amplified using BIOLASE DNA polymerase (Bioline, Taunton, MA) and primers that span the introduced NcoI restriction site (Table 1). PCR products were digested with the NcoI enzyme (New England Biolabs) and resolved on a 2% E-gel. To remove the puromycin-resistance cassette, 2 μg of a plasmid expressing Cre recombinase (Addgene plasmid #31308 was a gift from Gerhart Ryffel) was transfected into clones 1 and 6 (1×10^6 cells) via NEON transfection. Two hundred nanograms of gDNA was

Table 1. List of CRISPR guides and primers used to confirm correction of *CLN3*

Purpose	Oligo name	Sequence	Expected gel size (bp)
CRISPR guides	sg1	TGGAGGAGATGGGAGAAAATGG	N/A
	sg12	CTGGACTCTGATAGCCCCAGGT	N/A
T7E1 assay	<i>CLN3</i> F1	GATGGGAGACTGGGACAATTTG	Unmodified = 625; +sg1 = 572, 53; +sg12 = 439, 187
	<i>CLN3</i> R1	GTCCACCCTCATCTACTTCTA	
NcoI digestion	<i>CLN3</i> F2	GGTTCTCGTCAGTGGGATTT	PCR = 538; Digestion = 273, 265
	<i>CLN3</i> R2	CCTCCCAGGTTCAAGAGATTC	
Genomic sequencing	<i>CLN3</i> F2	GGTTCTCGTCAGTGGGATTT	HDR cassette inserted = 1,625
PCR hom Δ	<i>CLN3</i> R3	CCCGGTAGATAACTTCGTATAGC	No HDR cassette = no band
Genomic sequencing	<i>CLN3</i> F3	TCTGCGGTCTCACTCTATTCT	Corrected = 1,149; Uncorrected = no band
PCR comp. het.	<i>CLN3</i> R4	CTGATGAGATGCTAGCGAAGAC	
Genomic PCR	<i>CLN3</i> F4	TCAGAGCATTTGTGGGAAGAG	Corrected = 1,746; Uncorrected = 708
	<i>CLN3</i> R1	GTCCACCCTCATCTACTTCTA	
RT-PCR hom Δ	<i>CLN3</i> F2	GGTTCTCGTCAGTGGGATTT	Corrected = 321; Uncorrected = 104
	<i>CLN3</i> R5	CAGGAGATGTGAGCAACAAGAA	
RT-PCR comp. het.	<i>CLN3</i> F5	CGACATCCTTAGCCACAAGAG	Corrected = 524; Uncorrected = 307
	<i>CLN3</i> R5	CAGGAGATGTGAGCAACAAGAA	
RT-PCR control	<i>POLR2A</i> F	TAACCTGCTGCGGATGATCTGGAA	150
	<i>POLR2A</i> R	GATGTTGAAGAGCAGCGTGGCATT	

PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; hom, homozygous; comp. het., compound heterozygous.

PCR amplified using the Expand Long Template PCR system (Roche, Indianapolis, IN) and primers that span the 1.02 kb deletion and puromycin cassette (Table 1). PCR products were sequence confirmed, as described above.

Reverse transcription PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using 100 ng of RNA template. Human *CLN3* transcript was amplified using BIOLASE DNA polymerase and *CLN3* transcript-specific primers (Table 1). PCR bands were excised from the gel, purified (QIAquick Gel Extraction Kit; Qiagen), subcloned using the PCR2.1 TOPO TA cloning kit, and sequence confirmed as above.

Off-target analysis

Guides exhibiting the most efficient on-target cleavage were subjected to off-target analysis. The 50 most likely off-target sites for sg1*CLN3* were determined using the Benchling platform (www.benchling.com). The top 10 predicted off-target sites and all predicted off-target sites that fell within protein coding regions were evaluated for off-target cleavage. GDNA from sgRNA-treated *CLN3* patient cells and untreated control *CLN3* patient cells was PCR amplified (Table 2) and subjected to T7E1 nuclease assays as described above.

Results

CRISPR-Cas9 cleaves *CLN3* locus in patient-derived iPSCs

To test the efficiency of our engineered CRISPR-Cas9 constructs for targeting the 1.02 kb genomic deletion in *CLN3*, we initially evaluated nonhomologous end joining (NHEJ) in HEK293T cells treated with CRISPR-Cas9. We tested 24 guides, 20 of which utilize *S. pyogenes* (SpCas9) PAM sites and four of which utilize those sites from *S. aureus* (SaCas9), targeting both upstream and downstream of the deletion (Fig. 1A). Initial evaluation included cleavage assessment via T7E1 assays of nine guides employing SpCas9 PAM sites downstream of the deletion mutation. No discernable cleavage was detected in these assays (Supplementary Fig. S1A and B; Supplementary Data are available online at www.liebertpub.com/crispr). When we tested an additional 15 guides (11 of which employed SpCas9 PAM sites and four of which employed those from SaCas9) targeting both upstream and downstream of the deletion, we observed cleavage from 11 of the 15 guides (Supplementary Fig. S1A and C). Indel frequencies were quantified via subcloning and Sanger sequencing for three SpCas9 guides (sg2, sg4, and sg8) and three SaCas9 guides (sg1, sg10, and sg12). Interestingly, we observed a trend toward increased efficiency when using SaCas9 compared to SpCas9 (Fig. 1B; blue vs. red bars). We selected the two guides with the highest percent NHEJ (sg1, 36%;

Table 2. List of off-target primers

Name	Sequence	Expected gel size (bp)
sg_1OT1F	CACCTTCTTTCTCTGGGCTAAG	Unmodified = 746; Modified = 432, 313
sg_1OT1R	GGCCTTGACTTTCTGTCATCTA	
sg1_OT2F	CAGTGCCGTGACTACTATCTTC	Unmodified = 564; Modified = 284, 279
sg1_OT2R	CTCAACTCTGGTTTGGGAGTAA	
sg1_OT3F	GCTGTTGTAGCCTCCTGATT	Unmodified = 602; Modified = 345, 258
sg1_OT3R	CCCGTGATCCTCTTCTTTAT	
sg1_OT4F	GACGACCATAGGCATGTAGAAC	Unmodified = 437; Modified = 357, 81
sg1_OT4R	CTGAGCTTGAAGCGTAGAGAAA	
sg1_OT5F	GGGTTGTCAAGCGGAGTAATA	Unmodified = 610; Modified = 357, 252
sg1_OT5R	TTGCGCCAAGGAATTCAAATAG	
sg1_OT6F	CCTACGCATGGTCTCACATT	Unmodified = 466; Modified = 394, 72
sg1_OT6R	CTTCCTCTGCCTACGTTCTATC	
sg1_OT7F	TAGGTTGGAAGGGTTAAGTGG	Unmodified = 426; Modified = 214, 212
sg1_OT7R	GGCAAGCAAGCAAGGAATAAA	
sg1_OT8F	CTTTGTGTCAAGGCCAGATA	Unmodified = 653; Modified = 422, 232
sg1_OT8R	GCCAGAATATTGAGTGGAGGAG	
sg1_OT9F	CAGTGGACTCCTTCCAAACA	Unmodified = 710; Modified = 509, 200
sg1_OT9R	GGAAGCCCTGCTCTGATATAC	
sg1_OT10F	ACTTGGTGCCTGAGAATATG	Unmodified = 751; Modified = 481, 272
sg1_OT10R	GGGTACTTGCTCTTCTGTTT	
sg1_OT11F	GCTCTCTCCATTGTCCATCAA	Unmodified = 758; Modified = 454, 304
sg1_OT11R	ATGCCACCACCGACTTATAC	
sg1_OT15F	GTCCCTTGCTGAACAAGATA	Unmodified = 677; Modified = 444, 233
sg1_OT15R	CCTGAGCTGCCTCATTGTAATA	
sg1_OT37F	CACATGCACCTGCGAACTA	Unmodified = 492; Modified = 89, 203
sg1_OT37R	AGAGAGGGAGAGAGAGAGAAGC

FIG. 1. CRISPR-Cas9 targets the *CLN3* locus in patient-derived induced pluripotent stem cells (iPSCs). **(A)** Schematic representing the *CLN3* locus and the location of guides employing SpCas9 (red arrows) or SaCas9 (blue arrows) PAM sites that were screened for nonhomologous end joining (NHEJ) activity. **(B)** Histogram representing the percent NHEJ in HEK293T cells transfected with CRISPR-Cas9 plasmids expressing selected guides and SpCas9 (red bars) or SaCas9 (blue bars). Bars represent the average from duplicate experiments. Error bars represent the standard error of the mean. **(C)** Sequence alignment of selected clones from HEK293T cells transfected with sg1*CLN3* or sg12*CLN3*. Arrows indicate predicted cleavage sites; the target sequences are underlined. Red letters indicate insertions, and red dashes indicate deletions. **(D)** Representative gel images showing T7E1 assays in iPSCs derived from a patient homozygous for the 1.02 kb deletion mutation. Asterisk indicates major cleavage product. **(E)** Sequence alignment from selected TA clones from patient-derived iPSCs transfected with sg1*CLN3*. Arrow indicates predicted cleavage site; the target sequence is underlined. Red letters indicate insertions, and red dashes indicate deletions.

sg12, 34%) to carry forward in experiments in patient-derived iPSCs (Fig. 1B and C). When we delivered the sg1*CLN3*-SaCas9 or sg12*CLN3*-SaCas9 plasmids into iPSCs derived from a patient homozygous for the 1.02 kb deletion, we observed 8.8% or 0% NHEJ, respectively, as determined by Sanger sequencing (Fig. 1D and E). Therefore, sg1*CLN3*-SaCas9 was employed in subsequent experiments.

CRISPR correction of the 1.02 kb deletion in *CLN3* in homozygous patient-specific iPSCs

CRISPR-mediated HDR requires co-delivery of a wild-type DNA donor template with a sgRNA and Cas9. We designed a *CLN3* HDR donor cassette to include 400–1,000 bp of homologous sequence upstream (left arm) and downstream (right arm) of exons 7 and 8 (Fig. 2A). This donor cassette also included synonymous codon changes within intron 6 that mutate the seed and PAM

sites to prevent re-cleavage events, as well as create an NcoI recognition site that allowed for downstream RFLP analysis. A loxP-flanked puromycin resistance cassette under the control of the mPGK promoter was added to aid in positive selection of corrected cells (Fig. 2A).

To demonstrate successful correction of the 1.02 kb deletion mutation, we co-delivered sg1*CLN3*-Cas9 and *CLN3* HDR donor plasmids via electroporation to homozygous patient-specific iPSCs. Following positive selection with puromycin, we isolated, expanded, and analyzed 15 resistant clones via PCR using a primer specific to exon 6 of endogenous *CLN3* and a primer downstream of the NcoI restriction site located within the HDR donor cassette (Fig. 2B). Subsequent treatment of PCR products with NcoI demonstrated that 10 clones displayed positive digestion, indicating successful HDR template incorporation (Fig. 2B, red labels). Clone 1 appeared to have biallelic correction and was chosen

FIG. 2. CRISPR-Cas9 homology-dependent recombination (HDR) correction of homozygous *CLN3* patient iPSCs. **(A)** Schematic depicting wild-type *CLN3* alleles, those carrying the common 1.02 kb deletion mutation, and the HDR donor construct containing wild-type *CLN3* sequence upstream (left arm) and downstream (right arm) of exons 7 and 8, an engineered *NcoI* restriction site, and a loxP-flanked puromycin-resistance cassette. **(B)** *NcoI* digestion of genomic polymerase chain reaction (PCR) products from unaffected iPSCs, *CLN3* Hom Δ iPSCs, and 15 *CLN3* Hom Δ patient iPSC clones treated with sg1*CLN3*, SaCas9, and the HDR donor cassette. Positively digested clones are labeled in red. **(C)** Genomic PCR of *CLN3* in unaffected (lanes 1 and 5), *CLN3* Hom Δ iPSCs (lanes 2 and 6), and CRISPR-treated *CLN3* Hom Δ iPSCs from clones 1 (lane 3) and 6 (lane 7) before treatment with Cre recombinase (No Cre) and from clones 1 (lane 4) and 6 (lane 8) following exposure to Cre (+Cre). **(D)** Reverse transcription (RT)-PCR analysis of *CLN3* in unaffected (lane 1), *CLN3* Hom Δ iPSCs (lane 2), and CRISPR-treated *CLN3* Hom Δ iPSCs from clones 1 and 6 before (lanes 3 and 5) and after (lanes 4 and 6) treatment with Cre recombinase. Diagrams below each gel image depict the location of primers. Amplification of *POLR2A* was used as a reference gene.

for further investigation. Additionally, we assessed correction in clone 6, which appeared to have monoallelic incorporation of the HDR cassette (Fig. 2B). To confirm that the 1.02 kb deletion was corrected, PCR was performed with one primer in exon 6 and one in the puromycin cassette. Bidirectional Sanger sequencing of the clone 1 amplicon confirmed genomic restoration of *CLN3* exon 7, intron 7, exon 8, and intron 8 (Supplementary Fig. S2A and B).

PCR of genomic DNA using primers targeting regions within intron 6 (outside left arm) and intron 8 (right arm) of *CLN3* demonstrated amplification of only the wild-type product in control unaffected iPSCs (Fig. 2C, lanes 1 and 5). Amplification of the 1.02 kb deletion product was evident in untreated *CLN3* Hom Δ iPSCs, as well as CRISPR-treated clone 6 iPSCs, which underwent monoallelic correction (Fig. 2C, lanes 2 and 6–8). PCR of CRISPR-treated clones 1 and 6 prior to exposure to Cre recombinase produced an amplicon that included the puromycin cassette (Fig. 2C, lanes 3 and 7), which did not amplify following treatment with Cre (Fig. 2C, lanes 4 and 8). In contrast to clone 6, only the wild-type band amplified in genomic DNA isolated from CRISPR-treated clone 1, which indicates biallelic correction (Fig. 2C, lane 4).

Initial reverse transcription (RT)-PCR analysis of cDNA from clones 1 and 6 did not reveal restoration of wild-type *CLN3* transcript (Fig. 2D, lanes 3 and 5). We hypothesized that the presence of the puromycin-resistance cassette could be interfering with transcription of *CLN3*. To this end, we treated clones 1 and 6 with Cre recombinase to remove the floxed puromycin cassette, which resulted in restoration of wild-type *CLN3* transcript in both clones, as demonstrated by RT-PCR using primers spanning exons 6–9 (Fig. 2D, lanes 4 and 6). This restored band was consistent with that produced in unaffected control iPSCs (Fig. 2D). Sanger sequencing of clone 1 confirmed correction of *CLN3* transcript that included exons 7–8, which were absent prior to CRISPR correction in patient cells homozygous for the 1.02 kb genomic deletion mutation (Supplementary Fig. S2C).

CRISPR correction of the 1.02 kb deletion in *CLN3* in compound heterozygous patient-specific iPSCs

To determine if the same HDR strategy and reagents developed for patients who are homozygous for the common 1.02 kb deletion mutation could also be used to correct iPSCs derived from heterozygous individuals, a patient harboring the common 1.02 kb deletion on one allele and the Leu101del3CTC mutation on the other allele was targeted (Fig. 3A). The Leu101 amino acid residue is

located within a transmembrane domain near the amino terminus of the CLN3 protein.^{2–4} Deletion of this residue may lead to misfolding and/or mislocalization of the mature protein. As the repair template developed has greater homology to the Leu101del3CTC allele than the deletion allele (i.e., due to inclusion of wild-type exons 7 and 8, our HDR construct shares 37% homology to the 1.02 kb deletion allele and 67% homology to the Leu101del3CTC allele), we hypothesized that while the strategy was still sound, the efficiency of HDR cassette incorporation and genomic correction would be lower in cells derived from compound heterozygotes.

To test this hypothesis, we co-delivered sg1*CLN3*-Cas9 and *CLN3* HDR donor plasmids via electroporation, as described above. Following puromycin selection, correction was assessed in genomic DNA from 16 clones via RFLP analysis using NcoI. Seven positive clones displayed incorporation of the HDR construct, which was a similar result to that observed with the homozygous patient (Fig. 3B, red labels). However, the NcoI digest does not differentiate among the three possible HDR outcomes: (1) recombination on the Leu101del3CTC allele, (2) recombination on the 1.02 kb deletion allele, or (3) biallelic recombination. Therefore, we analyzed genomic DNA with primers that span the deleted sequence and puromycin cassette, which revealed that each iPSC clone amplified the 1.02 kb deletion mutation band, indicating either that the donor template was incorporated into the Leu101del3CTC allele in a homogenous population or positively digested iPSC clones represent a mixed population of cells (Fig. 3C). To investigate the latter, we amplified the locus using one primer upstream of the Leu101del3CTC mutation (in exon 5) and one in exon 7 (data not shown), followed by TA cloning and Sanger sequencing of iPSC clones 1, 3, 4, and 16, which displayed positive NcoI digestion. All sequences recovered from iPSC clones 1, 3, and 4 harbored the Leu101del3CTC mutation and the engineered NcoI restriction site, indicating the 1.02 kb deletion mutation was not corrected in these cell clones. In contrast, sequencing analysis of iPSC clone 16 revealed that 43% (6/14) of the sequences recovered did not carry the Leu101del3CTC mutation upstream of the engineered NcoI site, indicating correction of the 1.02 kb deletion mutation allele, and that clone 16 contained a heterogeneous population of cells. To obtain a pure population of corrected iPSCs, clone 16 was plated at a limiting dilution, and an additional 24 clones were expanded and analyzed via PCR using the primers indicated in Figure 3C. In one iPSC clone (i.e., 16.20), the 1.02 kb deletion product did not amplify indicating successful correction (Fig. 3D and Supplementary Fig. S3). Interestingly, clone 16.20 also did not

display amplification of the wild-type *CLN3* product prior to treatment with Cre recombinase, suggesting biallelic incorporation of the HDR construct. We hypothesize that the PCR amplicon following incorporation of the HDR template carrying the puromycin selection cassette was too large to amplify using the previous reagents. To confirm this, we performed long template PCR to amplify the product containing the puromycin cassette (Fig. 3D, lane 3). As shown in Figure 3E (lane 4), wild-type *CLN3* transcript, confirmed via Sanger Sequencing, was detected post-Cre recombinase treatment.

Sg1*CLN3*-Cas9 does not induce off-target cleavage events

To evaluate off-target modifications associated with the use of sg1*CLN3*, we evaluated cleavage in each CRISPR-treated patient line at the top 10 off-target sites, as predicted by the Benchling program. We did not observe any modification, as indicated by T7E1 assays (Fig. 4). Differential intensity in cleavage products was observed at OT1; this is predicted to be due to unequal loading of DNA used in sgRNA-treated samples in the assay. We cannot, however, rule out the possibility that there is cleavage at this locus. The OT1 target is located within an intron of a predicted RefSeq gene of unknown function (AC114813.1), and thus we do not predict that modification of this locus would be detrimental. Monitoring this site in subsequent experiments will evaluate any adverse affects in treating cells. We extended our assessment to three additional exonic off-targets within the top 50 predicted sites (OT11, OT15, and OT37). T7E1 assays did not reveal any detectable cleavage that differed from control untreated samples (Fig. 4). Taken together, these data demonstrate that we can successfully use an HDR-mediated CRISPR approach to correct the common

1.02 kb deletion mutation in iPSCs generated from patients with Batten disease.

Discussion

In this report, we employed plasmid-based CRISPR-Cas9 expression to correct genomically iPSCs derived from two patients carrying a common 1.02 kb mutation in Batten disease. We screened 24 guides for efficiency via T7E1 cleavage assay. The most efficient guide (sg1*CLN3*) demonstrated 8.8% cleavage in patient-derived iPSCs. Importantly, when we co-delivered a donor repair cassette and sg1*CLN3*-SaCas9-expressing plasmids to patient-derived iPSCs, we achieved wild-type *CLN3* transcript expression. These data represent important proof-of-concept in generating gene-corrected, patient-derived iPSCs for disease modeling and treatment of Batten disease.

As reported, we evaluated the activity of several guides paired with both SaCas9 and SpCas9. Of the 24 guides tested, only 11 demonstrated cleavage via T7E1 nuclease assays. Quantification data indicated that in this case, SaCas9 is more efficient than SpCas9. The 1.02 kb deletion locus carries large stretches of monomeric sequence, which may contribute to secondary structure formation. We hypothesize that the smaller SaCas9 nuclease may be less sterically hindered while navigating such sequences, and thus have greater access to target DNA than its larger SpCas9 counterpart. To target low-complexity regions of the genome more efficiently, use of even smaller Cas9 orthologues may be advantageous. The smallest Cas9 orthologue characterized to date is that from *Campylobacter jejuni* (CjCas9).²⁸ The protein is 984 amino acids (aa) in length, considerably shorter than SpCas9 (1,370 aa). The varying behavior of these Cas9 orthologues demonstrates the range of options available for targeting difficult loci.

FIG. 3. CRISPR-Cas9-HDR correction of compound heterozygous *CLN3* patient-specific iPSCs. **(A)** Schematic depicting wild-type *CLN3* alleles, those carrying the Leu101del3CTC, and the common 1.02 kb deletion mutation and the HDR donor construct containing wild-type *CLN3* sequence upstream (left arm) and downstream (right arm) of exons 7 and 8, an NcoI restriction site, and a floxed puromycin-resistance cassette. **(B)** NcoI digestion of genomic PCR products from unaffected iPSCs, *CLN3* compound heterozygous patient iPSCs, and 16 *CLN3* compound heterozygous patient iPSC clones treated with sg1*CLN3*, SaCas9, and the HDR donor cassette. Positively digested clones are labeled in red. **(C)** Genomic PCR of positively digested clones from **(B)**. **(D)** Genomic PCR of *CLN3* in unaffected (lane 1), *CLN3* compound heterozygous iPSCs (lane 2), and CRISPR-treated *CLN3* compound heterozygous from clone 16.20 before (lane 3; No Cre) and after (lane 4; +Cre) treatment with Cre recombinase. **(E)** RT-PCR analysis of *CLN3* in unaffected (lane 1), compound heterozygous iPSCs (lane 2), and CRISPR-treated compound heterozygous iPSCs from clone 16.20 before (lane 3) and after (lane 4) treatment with Cre recombinase. Diagrams below each gel image depict the location of primers. Amplification of *POLR2A* was used as a reference gene.

FIG. 4. Off-target activity of sg1*CLN3*. **(A)** Representative gel images of T7E1 assays for each off-target locus tested in clone 1 of CRISPR-treated *CLN3* Hom Δ iPSCs. **(B)** Representative gel images of T7E1 assays for each off-target locus tested in clone 16.20 of CRISPR-treated *CLN3* compound heterozygous iPSCs. **(C)** Ideogram of human genome indicating locations of predicted off-target loci. Arrowheads on top of chromosomes indicate the target is on the positive strand; arrowheads on the bottom of chromosomes indicate the target is on the reverse strand.

Genomic analysis of several CRISPR-corrected clones indicated that the majority of puromycin-resistant clones carried the donor cassette on at least one allele. However, none of these clones demonstrated wild-type *CLN3* transcript expression. When we delivered a plasmid expressing Cre recombinase to facilitate removal of the floxed puromycin cassette, *CLN3* transcript expression was restored in selected clones. In addition, these studies indicate that

it is possible to achieve biallelic correction of this large 1.02 kb genomic deletion. This will allow us to determine if a partial cellular phenotype is present in monoallelic-corrected cells. However, Batten disease is an autosomal recessive disorder, and we hypothesize that monoallelic correction will be sufficient for therapeutic purposes.

When we employed CRISPR-Cas9 correction in heterozygous patient iPSCs, we discovered that the allele

harboring the Leu101del3CTC mutation was preferentially targeted. Only after further expansion of one of the puromycin-resistant clones were we able to obtain a population in which the deletion mutation band was not observed in genomic DNA, and the *CLN3* transcript was restored, albeit at a relatively lower level. This result suggests that the purified clone represents a heterogeneous population of cells in which some cells still carry the selection cassette. We hypothesized that this was due to the fact that the HDR cassette has a greater degree of homology to the Leu101del3CTC allele (67%) than it does to the 1.02 kb deletion allele (37%). To address this issue, it may be possible to employ allele-specific targeting of the breakpoint sequence.^{22,29–31} Fortuitously, there is a suitable SpCas9 PAM site within 2 bp of the breakpoint that would facilitate guide sequence-mediated specificity. Future studies employing this guide with our HDR cassette may selectively target the deletion allele and improve efficiency of correction of the intended allele in compound heterozygous patient cells.

Genomic correction of the 1.02 kb deletion and restoration of *CLN3* expression in patient-derived cells now affords us the ability to model and test treatments for Batten disease. Recently, Lojewski *et al.* evaluated the pathology of iPSCs from Batten disease patients (three homozygous for the 1.02 kb deletion and one carrying compound heterozygous disease-causing variants). Patient-derived iPSCs demonstrated enlarged LAMP1-positive degradative autophagic vacuoles compared to unaffected control iPSCs. Additionally, patient-derived iPSCs displayed lysosomal storage material abnormalities when compared to controls. iPSC-derived neuronal progenitor cell cultures (NPCs) from these patients revealed increased numbers of LAMP1-positive puncta versus control NPCs and abnormal lysosomal storage in these neurons. Furthermore, overexpression of full-length, wild-type *CLN3* partially corrected the storage defects in these cells.³² However, the lack of complete rescue in these studies may be due to inefficient transduction of the NPCs by the AAVrh.10 vector. We recently reported the use of iPSCs from patients with molecularly confirmed mutations in *CLN3* and clinical Batten disease to test the utility of an adeno-associated virus (AAV)-mediated gene augmentation approach to restore full-length *CLN3* to stem cell-derived retinal precursor cells.²³ Similarly to Lojewski *et al.*, we found molecular restoration of both protein and transcript in a polyclonal population of patient-derived iPSCs using AAV2 serotyped *CLN3*-expressing vector. Extending these studies to include CRISPR-Cas9 correction in iPSCs prior to differentiation would address the challenge of partial functional restoration because a pure population of corrected cells can be obtained

prior to differentiation using both positive and negative selection.

Currently, there is no cure or Food and Drug Administration–approved treatment for Batten disease. The creation of CRISPR-corrected patient iPSCs helps mitigate this dearth by providing investigators with genetically matched, isogenic control cell lines. These isogenic controls could be differentiated into neuronal cells and compared side-by-side with affected (i.e., uncorrected) cells for rigorous disease modeling or drug selection experiments. A similar approach has been utilized to investigate the pathophysiological mechanisms of disease in cells with mutations that cause Duchenne muscular dystrophy,³³ cystic fibrosis,³⁴ or beta thalassemia.^{35,36} Although mutations in *CLN3* cause neuronal cell death throughout the CNS, the light-sensing photoreceptor cells of the neural retina are the first to succumb, suggesting they are especially sensitive to *CLN3* dysfunction and that using them as a disease model may provide critical insight into how *CLN3* mutations specifically alter neuronal function, leading to cell death. Furthermore, differentiation of affected and CRISPR-corrected *CLN3* patient-derived iPSCs into photoreceptor cells would enable one to perform RNA sequencing experiments to characterize the global transcriptomic changes that occur in photoreceptors affected with Batten disease. Further, high-throughput drug screening could be employed to identify compounds capable of preventing death of photoreceptor cells and ultimately neurons throughout the CNS.

We have established an approach for molecular correction of the common 1.02 kb genomic deletion mutation in *CLN3*, which roughly 85% of affected patients carry on at least one allele. As the vision loss observed in Batten disease is rapid, for patients who have already experienced significant photoreceptor cell loss, gene replacement therapy may not be a viable option. For those cases, delivery of CRISPR-corrected, autologous iPSC-derived photoreceptor cells would be an ideal therapeutic approach. One concern about the use of CRISPR-Cas9-mediated genomic correction is that of deleterious off-target modifications in areas of the genome other than target gene locus, especially within loci of known cancer genes, exons, or intronic regions involved in gene splicing. In this study, assessment of the top algorithm-determined sites displayed no off-target modification by our *CLN3*-specific guide RNA. There are several bioinformatic-based approaches for assessment of CRISPR-mediated off-target cleavage such as those included with the design programs Optimized CRISPR Design Tool, Benchling, and CRISPOR.^{14,37–39} For true global assessment of guide specificity, it is likely that

whole-genome sequencing and thorough downstream bioinformatics analysis combined with *in situ* assessment^{40–42} will be required prior to regulatory approval of CRISPR-corrected patient cells for cell-based therapy. However, the generation of gene-corrected, patient-derived iPSCs represents a powerful step toward treatment of the vision loss and thus extension of quality of life in individuals with Batten disease.

Conclusion

In this report, we show a strategy for CRISPR-Cas9-mediated correction of the most common mutation observed in *CLN3* that causes the fatal neurodegenerative disorder, Batten disease. Using a sgRNA and SaCas9, we achieved successful homology-dependent repair of two independent patient iPSC lines: one from a patient homozygous for the common 1.02 kb deletion and one from a patient that is a compound heterozygote. Following positive selection with puromycin and subsequent Cre-mediated removal of the floxed puromycin-resistance cassette, we observed molecular correction at the genomic DNA and mRNA levels in each iPSC line. These genomically corrected, patient-derived iPSCs will be important for rigorous disease modeling studies to elucidate the pathophysiological mechanisms that cause neuronal cell death in Batten disease.

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Author Disclosure Statement

The authors have no competing financial interests to disclose.

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