ORIGINAL ARTICLE

# CRISPR-Cas9 Editing of the *HBG1* and *HBG2* Promoters to Treat Sickle Cell Disease

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## ABSTRACT

### BACKGROUND

Sickle cell disease is caused by a defect in the β-globin subunit of adult hemoglobin. Sickle hemoglobin polymerizes under hypoxic conditions, producing deformed red cells that hemolyze and cause vaso-occlusion that results in progressive organ damage and early death. Elevated fetal hemoglobin levels in red cells protect against complications of sickle cell disease. OTQ923, a clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9–edited CD34+ hematopoietic stem- and progenitor-cell (HSPC) product, has a targeted disruption of the *HBG1* and *HBG2* (γ-globin) gene promoters that increases fetal hemoglobin expression in red-cell progeny.

### METHODS

We performed a tiling CRISPR-Cas9 screen of the *HBG1* and *HBG2* promoters by electroporating CD34+ cells obtained from healthy donors with Cas9 complexed with one of 72 guide RNAs, and we assessed the fraction of fetal hemoglobin–immunostaining erythroblasts (F cells) in erythroid-differentiated progeny. The gRNA resulting in the highest level of F cells (gRNA-68) was selected for clinical development. We enrolled participants with severe sickle cell disease in a multicenter, phase 1–2 clinical study to assess the safety and adverse-effect profile of OTQ923.

### RESULTS

In preclinical experiments, CD34+ HSPCs (obtained from healthy donors and persons with sickle cell disease) edited with CRISPR-Cas9 and gRNA-68 had sustained on-target editing with no off-target mutations and produced high levels of fetal hemoglobin after in vitro differentiation or xenotransplantation into immunodeficient mice. In the study, three participants received autologous OTQ923 after myeloablative conditioning and were followed for 6 to 18 months. At the end of the follow-up period, all the participants had engraftment and stable induction of fetal hemoglobin (fetal hemoglobin as a percentage of total hemoglobin, 19.0 to 26.8%), with fetal hemoglobin broadly distributed in red cells (F cells as a percentage of red cells, 69.7 to 87.8%). Manifestations of sickle cell disease decreased during the follow-up period.

### CONCLUSIONS

CRISPR-Cas9 disruption of the *HBG1* and *HBG2* gene promoters was an effective strategy for induction of fetal hemoglobin. Infusion of autologous OTQ923 into three participants with severe sickle cell disease resulted in sustained induction of red-cell fetal hemoglobin and clinical improvement in disease severity. (Funded by Novartis Pharmaceuticals; ClinicalTrials.gov number, NCT04443907.)

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N Engl J Med 2023;389:820-32.
DOI: 10.1056/NEJMoa2215643

Sickle cell disease is an autosomal recessive disorder caused by mutations in the gene *HBB*, which encodes the β-globin subunit of adult hemoglobin ($\alpha_2\beta_2$).[1] The most common sickle cell disease mutation causes a homozygous p.Glu6Val substitution, resulting in the production of sickle hemoglobin ($\alpha_2\beta^S_2$).[2] Sickle hemoglobin polymerizes at low oxygen concentrations, causing red cells to become sickle-shaped, rigid, and fragile, leading to microvascular occlusion, hemolysis, and inflammation. Persons with sickle cell disease have chronic anemia, recurrent pain, progressive multiorgan damage, and an increased risk of early death.[2,3] Symptoms of sickle cell disease appear during infancy as γ-globin gene (*HBG1* and *HBG2*) transcription switches to β-globin (*HBB*), causing a shift from fetal hemoglobin ($\alpha_2\gamma_2$) to adult hemoglobin in red cells.

Medical therapies for sickle cell disease, including hydroxyurea, blood transfusions, and recently approved drugs (L-glutamine, crizanlizumab, and voxelotor), are only partially effective.[4-8] The only potentially curative option is allogeneic hematopoietic stem-cell transplantation (HSCT), ideally from a human leukocyte antigen (HLA)–matched donor; this procedure is available for fewer than 20% of patients.[9] Allogeneic HSCT is associated with immunologic complications, including graft rejection and graft-versus-host disease, which tend to be worse in patients who have received transplants from HLA-mismatched donors.[9]

Induction of fetal hemoglobin is a proven strategy for treating sickle cell disease.[2,10-12] A naturally occurring, benign genetic condition termed hereditary persistence of fetal hemoglobin, which results in pancellular elevation of fetal hemoglobin levels in postnatal red cells, ameliorates symptoms of coinherited sickle cell disease.[2,3,13] Five paralogous β-like globin genes are located in the β-globin gene cluster on human chromosome 11p. *HBE* encoding ε-globin, *HBG2* (Gγ) and *HBG1* (Aγ) encoding γ-globin, *HBD* encoding δ-globin, and *HBB* encoding β-globin are sequentially located on chromosome 11p in that order and are activated successively during human ontogeny from embryonic to adult life. Around the time of birth, the site of red-cell production shifts from the fetal liver to the bone marrow, and this transition is associated with a switch from γ-globin (*HBG1* and *HBG2*) production to β-globin (*HBB*) production.[2] Consequently, the level of fetal hemoglobin decreases and the level of adult hemoglobin (or sickle hemoglobin in persons with sickle cell disease) increases.

The switch from γ-globin to β-globin is an intriguing model of the developmental regulation of gene expression and is clinically important because β-hemoglobinopathies can be treated by inhibiting this switch.[2] This perinatal switch from γ-globin to β-globin is mediated by transcriptional repressor proteins, BCL11A and LRF/ZBTB7A, that bind cognate cis-regulatory elements in the *HBG1* and *HBG2* promoters.[14] Inhibition of the binding of these repressors to their targets in adult red-cell precursors can reactivate expression of γ-globin and fetal hemoglobin.[14]

Transduction of hematopoietic stem cells (HSCs) obtained from participants with sickle cell disease with a lentiviral vector encoding an erythroid-expressed short hairpin RNA against *BCL11A*,[10] or targeted disruption of a *BCL11A* erythroid-specific enhancer by the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 nuclease system followed by autologous HSCT,[11] has resulted in elevated fetal hemoglobin levels and reduced symptoms of sickle cell disease. Additional strategies to increase fetal hemoglobin levels through genetic manipulation of HSCs also exist.[2,13] It is not yet known which strategies are the safest and most effective for clinical application.

OTQ923 is an autologous, ex vivo CRISPR-Cas9–edited, CD34+ HSC product that has a targeted disruption in the *HBG1* promoter, the *HBG2* promoter, or both caused by a ribonucleoprotein complex consisting of *Streptococcus pyogenes* Cas9 protein–single guide RNA (gRNA-68). This gRNA was discovered in an unbiased screening for cis-regulatory elements that repress *HBG1* and *HBG2* transcription. The gRNA-68 targets a site that is 246 bp upstream of the transcriptional start in each of the nearly identical tandem *HBG1* and *HBG2* genes (Fig. 1A), resulting in multiple editing outcomes, including an approximately 5-kb intergenic deletion that produces a single hybrid gene with the *HBG2* promoter sequence fused to the downstream *HBG1* (Fig. 1B). In preclinical studies, transfection of CD34+ HSCs with a gRNA-68–Cas9 ribonucleoprotein complex produced near-pancellular fetal hemoglobin induction in erythroid progeny, like that observed in hereditary persistence of fetal hemoglobin. Here, we de-

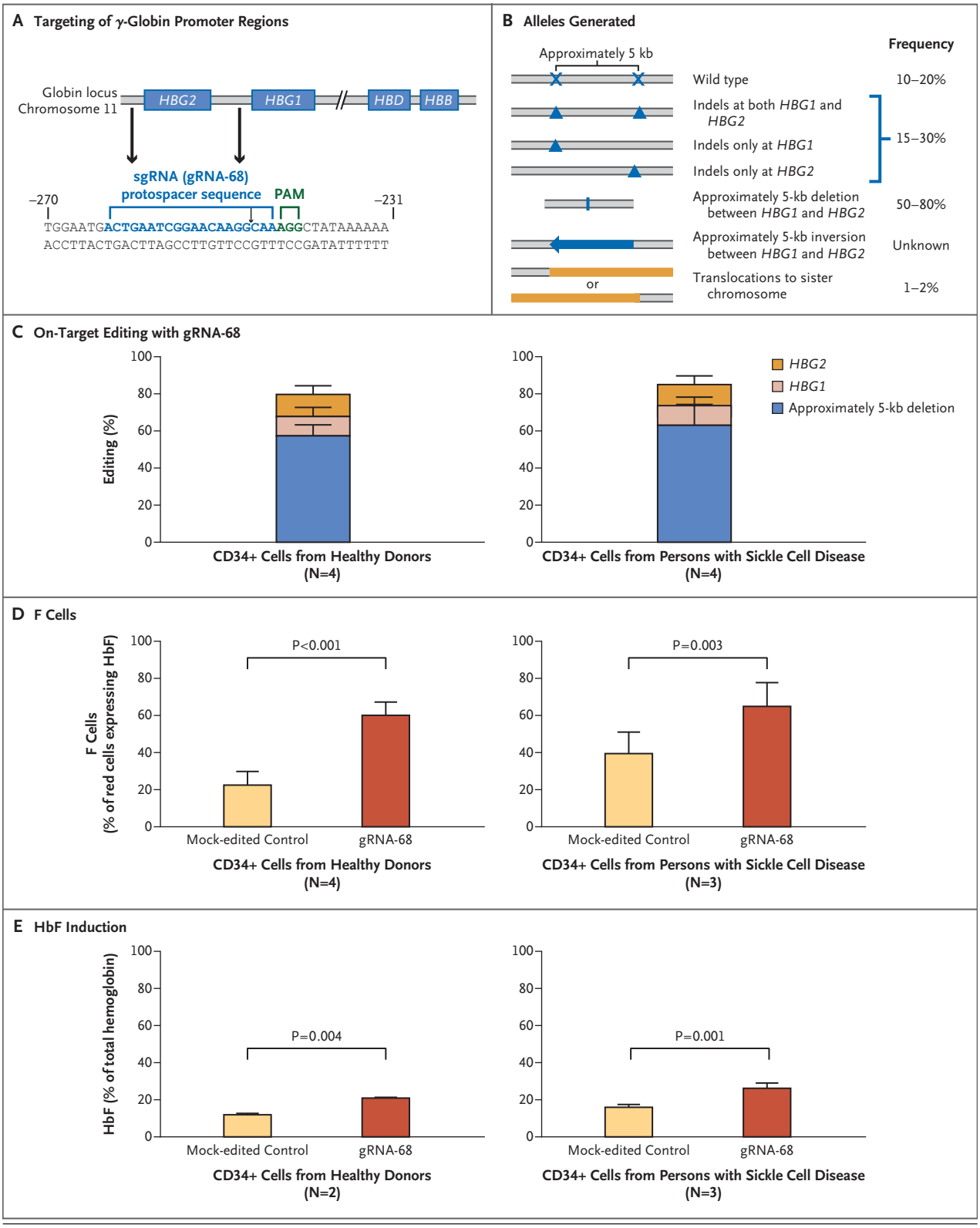

**Figure 1 (facing page). Molecular Approach and Preclinical Characterization of Guide RNA-68 (gRNA-68)–Edited Hematopoietic Stem Cells.**

Panel A shows the targeting site of the ribonucleoprotein complex consisting of Cas9–gRNA-68. Genes in the β-like globin cluster on chromosome 11 are shown as boxes, with ribonucleoprotein targeting sites in the *HBG1* and *HBG2* promoters indicated. The sequence is numbered in accordance with the start of transcription at +1. The predicted cutting site is indicated by the arrow in the protospacer sequence. PAM denotes protospacer adjacent motif, and sgRNA single guide RNA. Panel B shows the editing outcomes as determined by means of quantitative polymerase chain reaction,[15] targeted next-generation sequencing, and the unique identifier tagmentation next-generation sequencing assay. Most edits generated a deletion of approximately 5 kb caused by simultaneous double-stranded DNA breaks at the two target sites, thereby generating a single functional *HBG2–HBG1* fusion gene with the *HBG2* promoter sequence fused to the downstream *HBG1* (48.8 to 87.4% alleles). Editing also resulted in small indels at either targeting site that accounted for 7.1 to 36.9% of alleles. No inversion of the approximately 5-kb fragment was detected. Panel C shows the distribution of editing outcomes in gRNA-68–edited cells generated from CD34+ cells obtained from four healthy donors and from four persons with sickle cell disease. Panel D shows the percentages of F cells in erythroblasts generated by in vitro differentiation of gRNA-68–edited cells (described in Panel C) or control cells obtained from four healthy donors and three persons with sickle cell disease and measured with the use of flow cytometry. Panel E shows the amount of fetal hemoglobin (HbF) protein estimated by high-performance liquid chromatography in erythroblasts generated by in vitro differentiation of gRNA-68–edited or control cells obtained from two healthy donors and from three persons with sickle cell disease. In Panels C, D, and E, bars indicate the mean and T bars the standard deviation.

scribe the outcomes in the first three participants with sickle cell disease who received OTQ923 infusions in a phase 1–2 clinical study.

## METHODS

### SCREENING TO DETECT REGULATORY REGIONS WITHIN THE *HBG1* AND *HBG2* PROMOTERS

To identify new DNA targets for genetic induction of fetal hemoglobin, we performed a CRISPR-Cas9 screen by electroporating CD34+ cells obtained from healthy donors with Cas9 complexed with one of 72 gRNAs tiled across the *HBG1* and *HBG2* promoters (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). The on-target editing efficiencies and the fraction of fetal hemoglobin–immunostaining erythroblasts (F cells) were determined after in vitro erythroid differentiation, and the gRNAs resulting in the highest levels of F cells were selected for further development. Edited human HSCs were xenotransplanted into sublethally irradiated mice to assess the durability of editing and the extent of multilineage reconstitution.

### STUDY OVERSIGHT

The study was designed by Novartis Pharmaceuticals (the sponsor) in collaboration with the authors. The protocol, available at NEJM.org, was approved by the institutional review boards or ethics committees at the participating sites, and the study conduct was monitored by an independent data and safety monitoring committee. All the participants provided written informed consent. Data collection and analysis were performed by Novartis Pharmaceuticals in collaboration with the authors. The first author wrote the first draft of the manuscript and subsequently revised it in consultation with the last two authors. Two professional medical writers who were paid by the sponsor provided editorial support with an earlier version of the manuscript. All the authors had full access to the study data and critically reviewed the manuscript. No one who is not an author contributed to the writing of the manuscript.

The investigators vouch for the accuracy and completeness of the data generated at their respective sites, and the investigators and Novartis Pharmaceuticals vouch for the fidelity of the study to the protocol. Confidentiality agreements were in place between the sponsor and each investigative site during the study.

### STUDY DESIGN AND ELIGIBILITY

In this ongoing study, participants were eligible for enrollment if they were between 18 and 40 years of age and had a confirmed diagnosis of sickle cell disease and at least one of the following indicators of disease severity: three episodes of vaso-occlusive crisis or two episodes of acute chest syndrome within the previous 2 years; recurrent priapism; a history of stroke; long-term,

regular receipt of transfusions; or red-cell alloimmunization. Detailed inclusion and exclusion criteria are provided in the protocol.

### OTQ923 Production and Infusion

The study participants received monthly red-cell exchange transfusions for at least 2 months before collection of CD34+ cells. CD34+ HSCs were mobilized with plerixafor,[16] collected by means of apheresis,[17] cryopreserved, and shipped to a central manufacturing facility (Fig. S1). After thawing, the apheresis product was enriched for CD34+ cells by means of immunomagnetic selection and electroporated with the CRISPR-Cas9–gRNA-68 ribonucleoprotein complex to produce OTQ923. Before infusion of OTQ923, the participants underwent conditioning with myeloablative busulfan that was pharmacokinetically targeted to a cumulative area-under-the-curve exposure goal of 80 to 100 mg×hour per liter (Table S2). Additional details regarding the preclinical characterization, manufacture, and infusion of OTQ923 are provided in the Methods section in the Supplementary Appendix.

### Assessments of Clinical Outcomes

Adverse events and transfusions of blood products were monitored to assess the safety and adverse-effect profile of OTQ923. The time to neutrophil engraftment (absolute neutrophil count, ≥500 cells per cubic millimeter for 3 consecutive days), time to platelet engraftment (platelet count, ≥50,000 cells per cubic millimeter without platelet transfusion in the preceding 7 days), and fetal hemoglobin expression, as measured with the use of flow cytometry (for F cells) and capillary electrophoresis (to determine the percentage of fetal hemoglobin), were determined after OTQ923 infusion. On-target editing was assessed longitudinally in peripheral-blood and bone marrow cells by means of next-generation sequencing to detect small insertions and deletions (indels) and by a droplet digital polymerase-chain-reaction (PCR) assay to quantify the approximately 5-kb deletion.

### Statistical Analysis

Data from preclinical experiments (mock vs. gRNA-68–edited HSCs) were compared with the use of the paired two-tailed t-test. Clinical data are reported descriptively. No statistical comparisons of the clinical data were performed given the small number of study participants.

## RESULTS

### Transcriptional Repressor Element Identified in *HBG1* and *HBG2* Promoters

On flow cytometry, the tiling CRISPR-Cas9 screen revealed several gRNAs that increased levels of F cells in erythroid progeny of edited CD34+ cells obtained from healthy donors (Fig. S2). Editing with gRNA-68 increased the percentage of F cells to levels equal to or higher than those achieved with other gRNAs, including a positive control targeting the *BCL11A* +58 erythroid-specific enhancer. The gRNA-68–targeted region included no known *HBG1* and *HBG2* regulatory elements.

### Preclinical Validation of OTQ923

The preclinical cell product was manufactured by editing CD34+ HSCs (obtained from healthy donors and from persons with sickle cell disease) with the use of gRNA-68. In HSCs obtained from healthy donors for the preclinical experiments, the mean (±SD) frequency of on-target *HBG1* and *HBG2* editing was 80.5±9.8% in four participants, resulting in 60.5±6.8% F cells in those four participants after in vitro erythroid differentiation, as compared with 22.9±3.5% F cells in mock-edited control samples (CD34+ cells electroporated with Cas9 protein only). In HSCs obtained from persons with sickle cell disease, the frequency of on-target *HBG1* and *HBG2* editing was 85.8±14.7% in four participants, resulting in 65.4±12.1% F cells in three participants, as compared with 39.6±12.4% F cells in mock-edited control samples (Fig. 1C and 1D).

We assessed induction of fetal hemoglobin by means of ion-exchange high-performance liquid chromatography in the hemolysates after in vitro erythroid differentiation of the HSCs. In HSCs obtained from healthy donors, the level of fetal hemoglobin protein after gRNA-68 editing was 20.9±0.2% as compared with 12.0±0.3% in mock-edited control samples (Fig. 1E). In HSCs obtained from persons with sickle cell disease, the level of fetal hemoglobin protein after gRNA-68 editing was 26.2±2.9% as compared with 16.1±2.3% in mock-edited control samples (Fig. 1E). The most common editing outcome was the approximately

5-kb deletion, as noted in previously published articles describing gRNA targeting the *HBG1* and *HBG2* promoter regions.[15,18]

After transplantation into immunodeficient nonobese diabetic mice with severe combined immunodeficiency (NOD SCID) and depletion of the interleukin-2 receptor γ (NOD SCID gamma mice), healthy donor–derived gRNA-68–edited CD34+ HSCs showed durable long-term engraftment and multilineage reconstitution similar to that of mock-edited control cells (Fig. S3). These findings indicated that genome editing with gRNA-68 did not impair the developmental potential of long-term repopulating HSCs. The indel patterns were consistent and reproducible across donors (Fig. S4), which suggests that the DNA-repair events were not random.

Potential off-target editing sites were identified with the use of computational methods and SITE-Seq,[19] which measures off-target indels caused by incubating purified DNA with ribonucleoprotein. Of the 279 unique potential off-target sites identified in these screening assays, none were detected in gRNA-68–edited CD34+ HSCs by means of PCR and next-generation sequencing analysis with a sensitivity of 1% at a median coverage of 34,840× (Tables S3 and S4). No abnormalities were identified in gRNA-68–edited CD34+ HSCs with a targeted next-generation sequencing cancer gene panel (Pan-Heme, version 1.0) (Tables S5 and S6).

#### PARTICIPANT OUTCOMES

As of March 27, 2023 (the data-cutoff date), 16 participants had consented to participate in the study. Of these, 3 had received OTQ923, a partial dose of OTQ923 had been manufactured but not yet infused in 4 participants, and the remaining 9 participants were found to be ineligible after signing consent forms, withdrew from the study, or had not started apheresis. Here, we report on the first 3 participants who received OTQ923.

Participant 1 was a 22-year-old man with the $\beta^S/\beta^S$ genotype. At study enrollment, he had been receiving hydroxyurea therapy and regular red-cell exchange transfusions for 6 years because of recurrent episodes of vaso-occlusive crisis (Table 1). He discontinued hydroxyurea 2 months before CD34+ cell mobilization while continuing to receive exchange transfusions. Before OTQ923 infusion, his total hemoglobin level was 10.0 g per deciliter, with 0.4% fetal hemoglobin and 4.0% F cells (Fig. 2A). His last red-cell transfusion was 26 days after OTQ923 infusion (Fig. 2B). Between 12 and 18 months after infusion, his total hemoglobin level was maintained at 10.3 to 11.9 g per deciliter, with 25.0 to 26.8% fetal hemoglobin and 78.1 to 87.8% F cells (Fig. 2A).

Participant 2 was a 21-year-old man with the $\beta^S/\beta^S$ genotype who had multiple episodes of vaso-occlusive crisis while receiving hydroxyurea (Table 1). Four months before CD34+ cell mobilization and collection, hydroxyurea was discontinued, and monthly exchange red-cell transfusions were initiated. At the screening evaluation, before the initiation of monthly red-cell transfusions, the participant's total hemoglobin level was 7.6 g per deciliter, with 4.2% fetal hemoglobin and 20.4% F cells (Fig. 2A). His last red-cell transfusion was 17 days after OTQ923 infusion (Fig. 2B). Between 6 and 12 months after infusion, his total hemoglobin level was maintained at 10.1 to 11.5 g per deciliter (except for a transient decrease to 8.6 g per deciliter after a total hip arthroplasty, described below), with 23.0 to 25.3% fetal hemoglobin and 80.4 to 86.8% F cells (Fig. 2A).

Participant 3 was a 24-year-old woman with the $\beta^S/\beta^S$ genotype who had had frequent episodes of vaso-occlusive crises resulting in hospital admission, as well as avascular necrosis of the hips and iron overload (Table 1). She discontinued hydroxyurea 8 months before her first CD34+ cell mobilization while continuing to receive monthly exchange transfusions. After initiation of exchange transfusions but before myeloablation, this participant's total hemoglobin level was 8.3 g per deciliter, with 1.4% fetal hemoglobin and 6.2% F cells. Her last red-cell transfusion was 19 days after OTQ923 infusion (Fig. 2B). Between 4 and 6 months after infusion, her total hemoglobin level was maintained at approximately 10.5 g per deciliter, with 19.0 to 23.4% fetal hemoglobin and 69.7 to 85.6% F cells (Fig. 2A).

The indels in unsorted nucleated peripheral-blood cells, sorted T cells, B cells, and myeloid cells or neutrophils, as well as bone marrow cells obtained from the participants after OTQ923 infusion, were diverse, with the most common one being the approximately 5-kb deletion (Fig. 2C and Fig. S5). The indel allele frequencies were consistent among the three participants and were simi-

**Table 1. Demographic Characteristics of the Participants and Outcomes.**

| Variable | Participant 1 | Participant 2 | Participant 3 |
|---|---|---|---|
| **Demographic characteristics and disease severity** | | | |
| Age at screening (yr) | 22 | 21 | 24 |
| Sex | Male | Male | Female |
| Sickle cell disease genotype | $\beta^S/\beta^S$ | $\beta^S/\beta^S$ | $\beta^S/\beta^S$ |
| Sickle cell disease–related symptoms before study enrollment | Six episodes of acute chest syndrome over the past 10 yr and a history of a silent cerebral infarction, retinopathy, and priapism | Four episodes of vaso-occlusive crisis, three episodes of acute chest syndrome, and a silent cerebral infarction during the preceding 20 yr | Twenty five episodes of vaso-occlusive pain in the 2 yr before enrollment |
| Treatment for sickle cell disease ongoing at study enrollment | Regular blood transfusions and hydroxyurea | Hydroxyurea | Regular blood transfusions and hydroxyurea |
| **Apheresis collection and OTQ923 manufacture** | | | |
| Mobilization cycles lasting 2–3 days each (no.) | 3 | 2 | 3 |
| Cell dose manufactured (million/kg) | 2.80, a combination of two manufacturing batches, each with 84% editing efficiency | 5.99, a combination of three batches with editing efficiencies of 78%, 75%, and 73%, respectively | 5.04, a combination of two batches with editing efficiencies of 87% and 82%, respectively |
| **Follow-up and outcomes** | | | |
| Neutrophil engraftment | Day 26 | Day 20 | Day 18 |
| Platelet engraftment | Day 44 | Day 29 | Day 29 |
| Adverse events since OTQ923 infusion (no.) | 36 | 16 | 45 |
| Adverse events considered by investigators to be related to OTQ923 (no.) | 0 | 0 | 0 |
| Follow-up since OTQ923 infusion (mo) | 18 | 12 | 6 |
| Sickle cell disease–related events since OTQ923 infusion* | One episode of vaso-occlusive crisis with acute chest syndrome occurred at 17 mo after infusion; recurrent intermittent priapism; no new stroke or silent cerebral infarction; continued mild hemolysis; worsening osteonecrosis of femur | One episode of vaso-occlusive crisis occurred at 12 mo after infusion; no acute chest syndrome, stroke, or priapism; continued mild hemolysis; persistent osteonecrosis of femoral head | One episode of vaso-occlusive crisis occurred at 9 mo after infusion†; continued mild hemolysis; persistent osteonecrosis of femoral head |

* The observation period for post-treatment sickle cell–related events starts on the day of the first OTQ923 infusion and ends on the day of last follow-up.
† This event happened after the data-cutoff date, and hence the rest of the follow-up is only up to 6 months.

lar to those in preclinical samples. Indel frequencies were also consistent between bone marrow cells that were representative of the HSCs and the sorted B cells and myeloid cells or neutrophils in each participant. Indel frequencies fluctuated in the T cells during the first few months after infusion.

The participants had several adverse events (Table S7), but all were considered by the investigators to be related to either myeloablative busulfan conditioning or underlying sickle cell disease. None of the adverse events were considered by the investigators to be related to OTQ923.

After undergoing OTQ923 infusion, all three participants had at least one sickle cell disease–related event. Participant 1 had a vaso-occlusive crisis associated with back and leg pain, which progressed to acute chest syndrome at 17 months after infusion. He subsequently started to have intermittent priapism associated with sexual activity; this condition was treated as needed with pseudoephedrine. Participant 2 had leg pain associated with a vaso-occlusive crisis at 12 months after infusion. Participants 1 and 2 were admitted to the hospital for the management of pain episodes. After the data-cutoff date, Participant 3 also had hip and lower-back pain associated with a vaso-occlusive crisis (9 months after infusion); the pain was managed conservatively in the outpatient setting. All three participants also continued to have focal intermittent episodic pain in their hips and lower legs; this pain was similar in quality to that from preexisting femoral osteonecrosis and perhaps subjectively worse in Participant 1. Of note, Participant 1 was in a motor vehicle accident approximately 18 months after infusion and sustained major injuries, including a liver laceration and a distal radial fracture, but the participant recovered promptly with conservative management, specifically without blood transfusions or triggering a vaso-occlusive crisis. Similarly, Participant 2 underwent a planned total hip arthroplasty 6 months after infusion and recovered promptly without any complications or periprocedural blood transfusions. Furthermore, in Participants 1 and 2, at 1 year after infusion, cardiac chamber sizes and cardiac function on echocardiography either remained the same or slightly improved as compared with their baseline values before the infusion. Pulmonary-function tests and renal function (estimated glomerular filtration rate) remained stable as well.

Although all the participants' absolute reticulocyte counts and the serum bilirubin levels in Participants 1 and 2 remained elevated, the lactate dehydrogenase levels in all the participants decreased over time (Table 2). Approximately 8 months after infusion of OTQ923, Participant 1 had an upper respiratory tract viral infection, possibly coronavirus disease 2019, which was associated with a decrease in the hemoglobin level from 11.0 g per deciliter the month before to 8.0 g per deciliter (most likely due to viral bone marrow suppression). The hemoglobin level subsequently recovered spontaneously to preinfection levels in a few weeks after the participant had complete recovery from his infection symptoms. Participant 2 also had a decrease in his hemoglobin level during month 7 evaluations related to surgical blood loss during total hip arthroplasty. His hemoglobin level also returned to the presurgery level at the next follow-up. Neither participant received a blood transfusion. In fact, Participant 2 had been undergoing monthly phlebotomy to reduce preexisting iron overload. Morphologic assessment of the bone marrow was performed in the first two participants right before myeloablation and at 1 year after cell infusion. The bone marrow evaluations that were performed before myeloablation showed cellular marrow with erythroid-predominant trilineage hematopoiesis. At 1 year after infusion, the bone marrow in both participants had cellular marrow with relatively balanced and orderly trilineage hematopoiesis, a normal myeloid–erythroid progeny ratio, and no evidence of increased blasts or dysplasia.

## DISCUSSION

Genetic modification of autologous HSCs has shown great promise for the treatment of sickle cell disease, although the best approaches are not yet established. The lentiviral vector–mediated addition of an anti-sickling $\beta$-like globin is effective,[20] although this approach does not eliminate $\beta^S$-globin expression and creates a potentially toxic excess of $\beta$-like globin chains which can lead to ineffective erythropoiesis and erythroid dysplasia.[21] In contrast, induction of endogenous $\gamma$-globin transcription concomitantly reduces $\beta^S$-globin, thereby maintaining the balance of $\alpha$-globin and $\beta$-like globin chains.[2] Disrupting the *BCL11A* erythroid-specific enhancer with

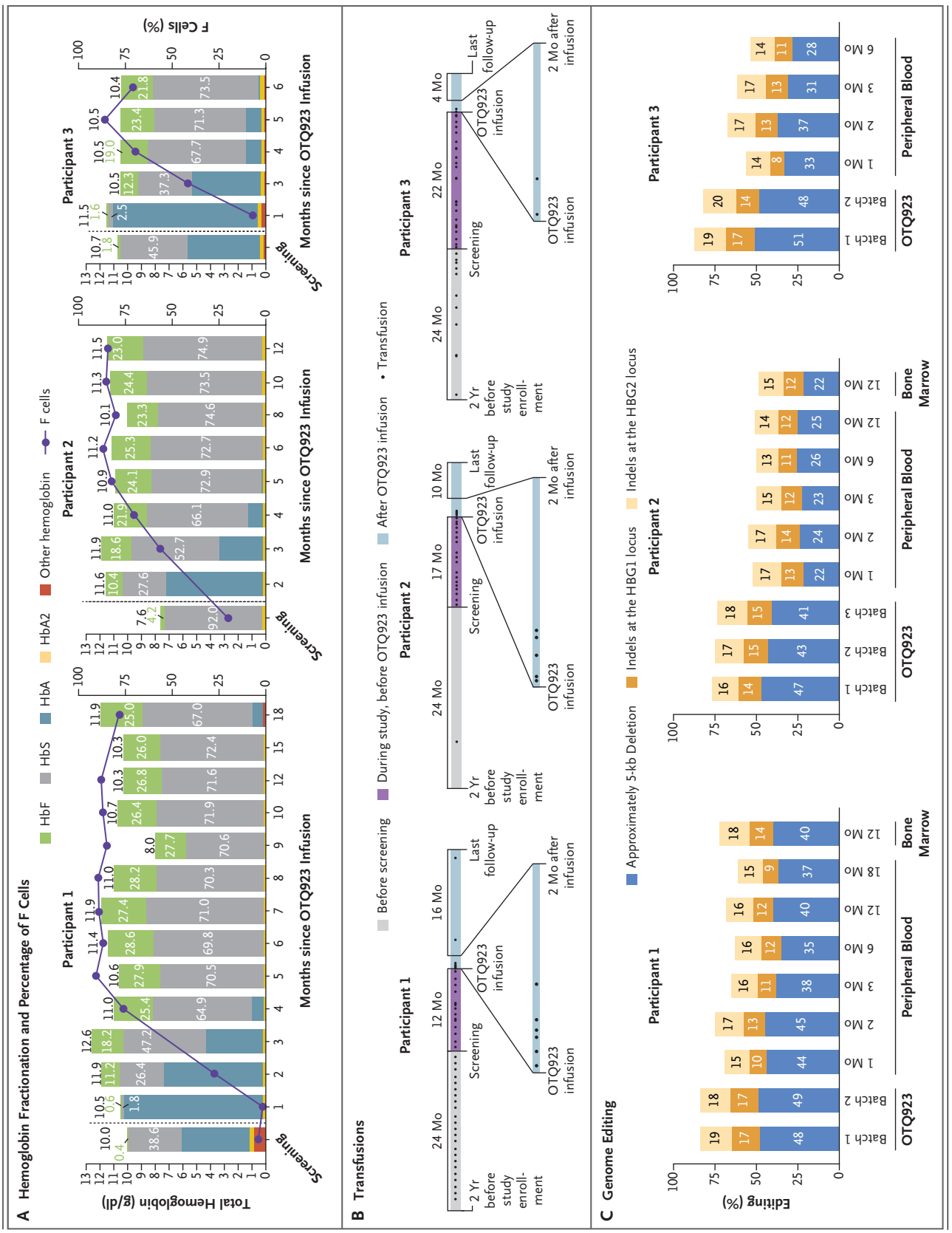

**Figure 2 (facing page). Total Hemoglobin and Its Fractions, Percentage of F Cells, and Tracking of Edited Alleles in Three Participants.**

Panel A shows the total hemoglobin level and hemoglobin fractionation over time in three participants. Participant 1 had a transient decrease in the hemoglobin level from 11.0 to 8.0 g per deciliter between months 8 and 9, coincident with an intercurrent viral infection and associated bone marrow suppression, which recovered spontaneously at the subsequent time point. The blue line represents the percentage of F cells over time after OTQ923 infusion and indicates near-pancellular fetal hemoglobin expression. "Other hemoglobin" refers to other minor hemoglobin subtypes that are not differentiated by capillary electrophoresis. HbA denotes adult hemoglobin, HbA2 hemoglobin A2, and HbS sickle hemoglobin. Panel B shows the number of blood transfusions received by the participants over time. Panel C shows the percentage of allelic editing in OTQ923 (two batches), nucleated peripheral-blood cells (≤18 months after OTQ923 infusion), or bone marrow (12 months after OTQ923 infusion) obtained from Participant 1; the percentage of allelic editing in OTQ923 (three batches), nucleated peripheral-blood cells (≤12 months after OTQ923 infusion), or bone marrow (12 months after OTQ923 infusion) obtained from Participant 2; and the percentage of allelic editing in OTQ923 (two batches) or nucleated peripheral-blood cells (≤6 months after OTQ923 infusion) obtained from Participant 3. In Panel B, each black dot represents a transfusion. In Panel C, the light and dark orange bars represent the percentages of indels detected by next-generation sequencing. The blue bars represent the approximately 5-kb deletion that was detected in the infused product by quantitative polymerase chain reaction (PCR) or by droplet digital PCR.

CRISPR-Cas9 can increase fetal hemoglobin levels and alleviate symptoms of sickle cell disease.[11] However, disrupting a repressor element in the *HBG1* and *HBG2* promoters is a more targeted approach than eliminating erythroid *BCL11A* expression entirely, because the latter may impair erythropoiesis.[22,23] Through a CRISPR-Cas9 screen, we identified a repressor element in *HBG1* and *HBG2* promoters. In our preclinical testing, on disruption, this repressor element caused F-cell induction with an efficacy similar to that of disruption of the *BCL11A* erythroid-specific enhancer.

Three participants with severe sickle cell disease who received a single infusion of OTQ923 had sustained increases in total hemoglobin, fetal hemoglobin, and F-cell levels, and the occurrence of vaso-occlusive crises decreased without any detected off-target effects. Engraftment of the genetically modified cells was observed in the time frame expected for genetically modified CD34+ selected grafts (18 to 26 days),[10,11,20] with no skewing in the differentiation of edited HSCs to myeloid or B-cell lineages. T cells had variable editing in the first few months, consistent with expansion of unedited T cells or those derived from the edited HSCs after infusion. Although all three participants had at least one episode of vaso-occlusive crisis after infusion, the frequency of such episodes was very limited. A similar incidence of episodes of vaso-occlusive crisis has been noted in another ongoing study with similar induction of fetal hemoglobin.[10]

In Participants 1 and 2, cardiac, pulmonary, and renal function appeared to be stable or had improved at 12 months after infusion. This preservation of organ function — at least in the short term — raises hope that sickle cell disease–induced organ dysfunction may be prevented by this therapy. However, osteonecrosis persisted and perhaps even worsened after treatment. Although exposure to busulfan could have contributed to worsening bone health,[24,25] continued sickle cell disease–related damage cannot be ruled out. The short-term safety profile of OTQ923 therapy was as expected after myeloablative busulfan and autologous HSCT.

Despite improved hematologic levels and a reduction in the incidence of symptoms of sickle cell disease, all the participants had ongoing mild hemolysis, findings that indicate that their red-cell fetal hemoglobin levels were insufficient to inhibit sickle hemoglobin polymerization completely. We observed marked improvement in some hematologic indexes, such as the total hemoglobin level and red-cell count. However, stress erythropoiesis ensued, although it was better than before therapy, as indicated by a normalized myeloid–erythroid progeny ratio noted on bone marrow assessments. Hemolysis appeared to be somewhat improved in all the participants, as suggested by improved biochemical markers of hemolysis such as lactate dehydrogenase and serum bilirubin, but it was still persistent as evidenced by an elevated reticulocyte count, findings that are similar to those in other ongoing clinical studies.[10,20] Transient viral bone marrow suppression in Participant 1 with a resulting decrease in the hemoglobin level is further evidence of continued hemolysis. However, the bone marrow assessments showing balanced and orderly trilineage hematopoiesis without any evidence of dysplasia, along with an

**Table 2. Markers Suggestive of Ongoing Hemolysis and Hematopoietic Recovery in the Study Participants after OTQ923 Infusion.**

| Marker | Normal Range | Participant 1 | | | | | Participant 2 | | | | Participant 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Screening | 3 Mo | 6 Mo | 12 Mo | 18 Mo | Screening | 3 Mo | 6 Mo | 12 Mo | Screening | 3 Mo | 6 Mo |
| Absolute reticulocyte count (million/mm$^3$) | 0.021–0.085 | 0.3364 | 0.4025 | 0.2419 | 0.2721 | 0.1943 | 0.3034 | 0.3478 | 0.3951 | 0.3802 | 0.3444 | 0.1841 | 0.2321 |
| Lactate dehydrogenase level (U/liter) | 94–260 | 345 | 265 | 263 | 217 | 292* | 397 | 387 | 284 | 243 | 359 | 341 | 325 |
| Total serum bilirubin level ($\mu$mol/liter) | 3–21 | 26 | 24 | 48 | 43 | 56* | 60 | 32 | 65 | 72 | 67 | 31 | 31 |

* These laboratory tests were performed a few weeks after the participant had a motor vehicle accident and sustained a liver laceration. Hence their clinical significance should be interpreted in conjunction with the participant's clinical status.

increase in the total hemoglobin level from baseline in all three participants, support improved hematopoiesis after the infusion of genetically modified cells. This increase in the total hemoglobin level has led to clinical improvement in all three recipients. Although the degree of fetal hemoglobin induction seen in these participants may be enough for attenuation of sickle cell disease, it is not sufficient for complete amelioration of disease.

Changing the manufacturing process may help to enhance fetal hemoglobin expression by increasing the on-target editing frequency of repopulating HSCs. First, we observed a decrease in the frequency of edited alleles in the reconstituted peripheral-blood cells and bone marrow in the participants as compared with the infused product. Thus, an opportunity exists to improve the editing of the long-term repopulating HSCs, which would then lead to persistence of a higher degree of editing in the reconstituted blood cells, resulting in a higher fetal hemoglobin level. Second, it is also possible that the *HBG1* and *HBG2* promoter site targeted by gRNA-68 is not a very potent repressor of *HBG1* and *HBG2* transcription in vivo, and hence disrupting this site causes only partial or incomplete derepression of fetal hemoglobin production. At the same time, perhaps the indels produced by gRNA-68 may be disrupting the regulatory element only partially. Ongoing studies to better characterize the putative negative regulatory motif targeted by gRNA-68 may facilitate its more precise disruption to further improve fetal hemoglobin induction. Third, we used an aryl hydrocarbon receptor antagonist in this study to expand gene-edited HSCs; this use could be further explored. Finally, combinatorial approaches targeting multiple regulatory elements in tandem could also improve fetal hemoglobin induction,[26] although this approach has yet to be evaluated with Cas9-directed editing. A potential downside of combinatorial approaches is the risk of a more potent DNA damage response by induction of multiple double-strand DNA breaks.

Previous studies of autologous HSCT for sickle cell disease used freshly collected cells to manufacture the cellular drug product.[10,11,20] In contrast, in the current study, we used a cryopreserved apheresis product to facilitate future access to OTQ923 therapy for the majority of patients with sickle cell disease who reside in low-resource settings.

We found that Cas9 disruption of a negative

regulatory region in the *HBG1* and *HBG2* promoters of autologous HSCs obtained from participants with sickle cell disease resulted in induction of red-cell fetal hemoglobin and a partial correction of sickle cell disease. Busulfan was used as a myeloablative agent in this study, as has been the case in most other ongoing studies.[10,11,20] However, given the associated toxicity profile of busulfan, alternatives such as reduced-intensity melphalan,[27] alternative reduced-toxicity alkylating agents such as treosulfan,[28] and nongenotoxic antibody–drug conjugates[29] should be evaluated in future clinical trials. Although the long-term durability of the response and the safety of this genetically modified product continue to be evaluated, our data suggest that this approach offers a safe and potentially disease-attenuating option for patients with severe sickle cell disease.

Supported by Novartis Pharmaceuticals.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

We thank the participants who participated in the study, their families, and the clinical associates and personnel at each site who assisted with the study, especially Stacy High, Sanjay Verma, Pam Young, and Christopher Omahen; our Novartis colleagues for their assistance, including Craig Mickanin, Carsten Russ, Konrad Mueller, Karin Abitorabi, Sven Schuierer, Shuai Chen, Qi Zhang, Madhura Panditrao, Alberto Del Rio-Espinola, Daher Ibrahim Aibo, Azeddine Elhajouji, Andrea Knight, Jennifer Snead, Christian Schmedt, Michael Patel, Imelda Schulhmann, Thomas Peters, Jean Regard, Marjolaine Phan, Filippo Vegni, Clement Purcell, Lantz Mackey, Aidan Condon, Liu Yiang, Karolina Kulec, Victor Lin, Ayala Tovy, Roman Sloutsky, Sejong Chun, Cameron Trenor, Lloyd Klickstein, Evan Beckman, Tewis Bouwmeester, Alice Shaw, David Steensma, Glenn Dranoff, and James Bradner; our colleagues at Intellia Therapeutics — Bo Han, Nishit Patel, and Dan O'Connell — for their contributions to the off-target characterization of guide RNA-68; Manoshi Nath, and Zoe Crossman for editorial assistance with an earlier version of the manuscript; Keith A. Laycock, an employee of St. Jude Children's Research Hospital, for scientific editing of an earlier version of the manuscript; Mitchell J. Weiss, for critical review of an earlier version of the manuscript and several constructive comments; and Jonathan Yen, Parul Rai, Gabriela Maron, and Amittha Wickrema, for their input.

## APPENDIX

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