

CRISPR-Cas9n-mediated *ELANE* promoter editing for gene therapy of severe congenital neutropenia

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Severe congenital neutropenia (CN) is an inherited pre-leukemia bone marrow failure syndrome commonly caused by autosomal-dominant *ELANE* mutations (*ELANE*-CN). *ELANE*-CN patients are treated with daily injections of recombinant human granulocyte colony-stimulating factor (rhG-CSF). However, some patients do not respond to rhG-CSF, and approximately 15% of *ELANE*-CN patients develop myelodysplasia or acute myeloid leukemia. Here, we report the development of a curative therapy for *ELANE*-CN through inhibition of *ELANE* mRNA expression by introducing two single-strand DNA breaks at the opposing DNA strands of the *ELANE* promoter TATA box using CRISPR-Cas9D10A nickases—termed *MILESTONE*. This editing effectively restored defective neutrophil differentiation of *ELANE*-CN CD34⁺ hematopoietic stem and progenitor cells (HSPCs) *in vitro* and *in vivo*, without affecting the functions of the edited neutrophils. CRISPResso analysis of the edited *ELANE*-CN CD34⁺ HSPCs revealed on-target efficiencies of over 90%. Simultaneously, GUIDE-seq, CAST-Seq, and rhAmpSeq indicated a safe off-target profile with no off-target sites or chromosomal translocations. Taken together, *ex vivo* gene editing of *ELANE*-CN HSPCs using *MILESTONE* in the setting of autologous stem cell transplantation could be a universal, safe, and efficient gene therapy approach for *ELANE*-CN patients.

INTRODUCTION

Patients with severe congenital neutropenia (CN), an inherited pre-leukemia bone marrow failure syndrome, suffer from severe bacterial infections that generally occur shortly after birth.¹ The reason for these infections is a myeloid differentiation defect of hematopoietic stem and progenitor cells (HSPCs) with an almost complete inability to form mature neutrophils. In addition to defective granulopoiesis, CN patients are at risk of developing hematological malignancies,

including myelodysplastic syndrome, acute myeloid leukemia (AML), and, in some rare cases, chronic myelomonocytic leukemia, acute lymphoblastic leukemia, or bi-phenotypic leukemia. Autosomal-dominant mutations in the *ELANE* gene encoding neutrophil elastase (NE) protein frequently cause CN.^{1–3}

Since its clinical use in 1987, rhG-CSF⁴ became the standard treatment option for CN patients. Although most *ELANE*-CN patients respond to daily treatment with subcutaneous injections of rhG-CSF, some do not, even at doses up to 50 µg/kg/d.⁵ Some patients continue suffering from frequent infections despite rhG-CSF therapy; in others, especially in puberty or adulthood, rhG-CSF causes severe bone pain, leading to discontinuation of treatment and a subsequent high risk of developing severe infections.^{1,5} The only potentially curative treatment available for CN is allogeneic hematopoietic stem cell (HSCs) transplantation, which, despite its benefits, still has a 3-year mortality rate of 17% and causes severe side effects in 21% of patients.⁶ Thus, there is an unmet need for an alternative curative treatment for CN patients.

Understanding the pathophysiology of defective granulopoiesis in CN patients downstream of *ELANE* mutations is essential for developing alternative therapies. NE is a proteolytic enzyme of the neutrophil serine protease family whose members also include the proteases cathepsin G

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(CG), proteinase 3 (PRTN3), azurocidin 1 (AZU1), and serine protease 57 (PRSS57, previously referred to as NSP4).^{7,8} These proteases, stored in cytoplasmic granules and secreted into extracellular and pericellular spaces upon cellular activation, are considered crucial components of bacterial defense.⁸ *ELANE* mutations in CN patients are distributed throughout all five exons and introns (but mainly intron 4) of the *ELANE* gene, affecting different functional domains of the NE protein.^{3,9} The ultimate mechanisms underlying the defective granulocytic differentiation of HSPCs with *ELANE* mutations are not yet fully understood. We and others have reported that the inhibition of HSPC proliferation and differentiation observed in CN patients harboring *ELANE* mutations is caused by an enhanced unfolded protein response (UPR) in the endoplasmic reticulum instigated by misfolded mutant NE protein.^{10–13} Therefore, we recently hypothesized that *ELANE*-CN could be treated and its associated “maturation arrest” corrected by CRISPR-Cas9-sgRNA-mediated *ELANE* knockout.¹⁴ Indeed, we reported that knocking out the *ELANE* gene in CN HSPCs successfully rescued defective granulopoiesis, an approach that was later validated by others.¹⁵ However, editing *ELANE*’s coding sequence (CDS) region with CRISPR-Cas9 could introduce new *ELANE* variants in the CDS—an unwanted outcome of on-target editing.

Therefore, to mitigate potential safety concerns, it is beneficial to establish a universal strategy for modulating *ELANE* mRNA expression without targeting the CDS region of *ELANE*. There are multiple possible approaches for safely inhibiting the expression of a gene of interest, including RNA interference (RNAi), CRISPR interference (CRISPRi), and CRISPR-Cas9-based editing of gene enhancer regulatory elements. Such approach, previously described for targeting the enhancer region of the *BCL11A* gene to inhibit suppression of γ -globin, has been approved as a treatment for sickle cell disease and transfusion-dependent β -thalassemia (TDT).^{16,17} Some studies have reported that introducing a double-strand break (DSB) using paired Cas9 nickases (Cas9n) greatly increases genome-editing precision, adding a safety factor.^{18–23} Using nickase-based gene editing for gene therapy has also been shown to enhance the safety profile 50 to 1,000-fold.²¹ Thus, replacing Cas9 with Cas9D10A/H840A nickases adds an extra level of safety in clinical gene therapy strategies. Although a dual-nickase strategy is generally thought to be less efficient, in the specific case of SpCas9D10A nickase (CRISPR-Cas9n), it has been shown that this approach can be as efficient or even more efficient in inducing double-stranded breaks than native Cas9, depending on the single guide RNA (sgRNA) design and the targeted genomic locus.²⁰

In addition to genomic safety, assessing the functional safety of gene-edited cells is essential for *ex vivo* HSPCs-modified therapies. Given that *ELANE* mutations are autosomal dominant, the inability of the *ELANE*-KO approach to discriminate between wild-type and mutated alleles leads to reduced levels of both mutated and wild-type NE. Nevertheless, we recently found that eliminating mutated *ELANE* completely restored granulopoiesis despite simultaneous suppression of wild-type *ELANE* *in vitro* and *in vivo*, causing no harmful effects on neutrophil differentiation or functions, including reactive oxygen species (ROS) production, chemotaxis, and phagocytosis of Alexa

594-conjugated *Staphylococcus aureus* BioParticles.¹⁴ *Elane*^{−/−} mice also have average neutrophil counts and no defects in neutrophil maturation.^{24–26} Moreover, neutrophils from patients with Papillon-Lefevre Syndrome (PLS), a rare autosomal-recessive syndrome caused by loss-of-function mutations in the *CTSC* (cathepsin C) gene locus (encoding dipeptidylpeptidase I [DPPI]) that leads to severe defects in neutrophil serine proteases, including NE, can effectively kill bacteria, such as *S. aureus* (gram positive) and *Escherichia coli* (gram negative).²⁷ These data suggest that, because of existing redundancies in the bactericidal mechanisms of neutrophils in humans, serine proteases are not essential for killing common bacteria, allowing us to conclude that therapeutic NE inhibition is safe and preserves neutrophil functions.

Here, we report the successful development of a universal and efficient approach for inhibiting *ELANE* mRNA expression by disrupting the regulatory region in the promoter upstream of the *ELANE* gene transcription start site (TSS). To establish this, we applied CRISPR-Cas9n and sgRNAs with excellent safety profiles, as assessed by GUIDE-seq, rhAmpSeq validation of off-targets, and CAST-seq of edited primary HSPCs. Editing *ELANE* in primary HSPCs of CN patients using this strategy led to successful granulocytic differentiation of edited cells *in vitro* and *in vivo*.

RESULTS

CRISPR-Cas9-based targeting of the *ELANE* TATA box inhibits *ELANE* transcription

CRISPR-Cas9-mediated gene editing of the *ELANE* CDS in CN patients’ HSPCs, either for knockout or mutation-correction purposes, can lead to the generation of *de novo* mutations in subpopulations of cells and could potentially raise long-term safety concerns regarding its clinical application in the setting of autologous transplantation of gene-edited HSPCs. In particular, we previously demonstrated the presence of small undesired in-frame deletions and insertions in gene-edited cells after targeting exon 2 of *ELANE*.¹⁴ Therefore, while the rationale underlying potential safety concerns of gene-editing approaches targeting the *ELANE* CDS will require further investigation in long-term *in vivo* studies, we here sought to circumvent this concern by designing an alternative CRISPR-Cas9-based genome-editing strategy that does not target the CDS of *ELANE* and so sidesteps these concerns.

We established a CRISPR-Cas9n-based gene-editing approach for inhibiting NE expression that targets the non-coding region of *ELANE* and thus does not generate new, unwanted *ELANE* variants. To screen the efficiency of different guides and their combinations, we engineered a NE-reporter cell line using THP-1 AML cells²⁸ expressing high levels of NE. We used a split nano luciferase reporter system (see [materials and methods](#)). It consists of a large (18 kDa) protein (LgBiT) and a small (11 amino acid) protein termed HiBiT,²⁹ the latter of which can be used to tag endogenous proteins. Adding LgBiT protein and the luciferase substrate furimazine to lysed HiBiT reporter cells for a tagged protein makes it possible to quantitatively determine the expression of the tagged protein by measuring its luminescence signal.²⁹ The HiBiT tag was fused to the N terminus of the NE protein by CRISPR-Cas9-mediated

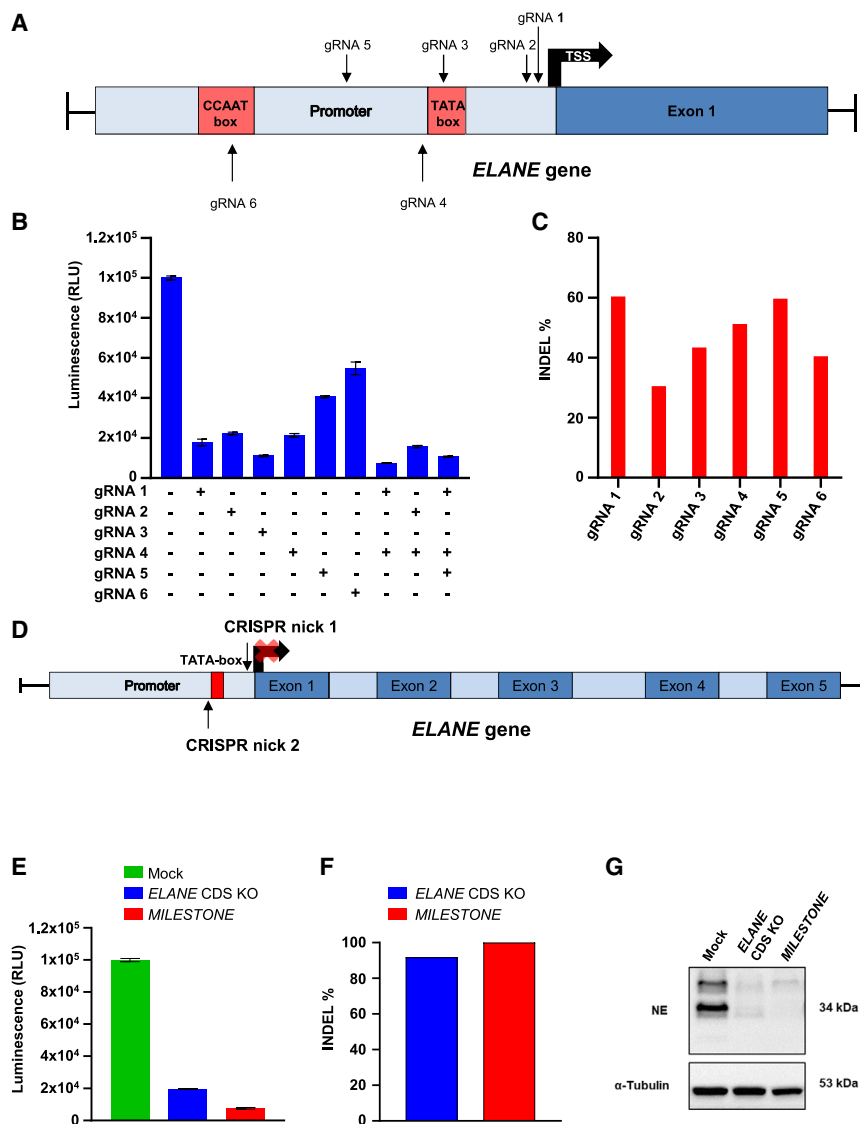


Figure 1. Development of a CRISPR-Cas9n-based gene-editing strategy to inhibit the *ELANE* transcription process by targeting its promoter's TATA box

(A) Schematic representation of the sgRNA selection. HiFi-Cas9 protein guided by sgRNAs targeting multiple areas of the *ELANE* gene promoter was used to find the region critical for repression of the *ELANE* mRNA transcription. CCAAT-box (–146 nucleotide from TSS) and TATA box –29 nucleotides from TSS were predicted using the eukaryotic promoter database (EPD) with p value <0.001. (B) Neutrophil elastase (NE)-HiBiT-tagged THP-1 single cell-derived cells were electroporated with the indicated sgRNAs. Seventy-two hours after electroporation, the bioluminescence signal of the HiBiT-tagged NE protein was assessed using a GloMax bioluminescent plate reader. Data represent means $\pm$ standard deviation (SD) from triplicates of a representative experiment. (C) On-target editing efficiency of each sgRNA 72 h post-electroporation of NE-HiBiT-tagged THP-1 cells of a representative experiment. (D) Schematic representation of the *MILESTONE* genome-editing strategy. Cas9D10A nickase guided by a pair of gRNAs targeting opposite strands of *ELANE* TATA box was used to introduce a double-strand break followed by small deletion to reduce the *ELANE* mRNA transcription. (E) NE-HiBiT-tagged THP1 cells were electroporated using *ELANE* CDS KO, *MILESTONE*, or Mock control. Seventy-two hours post-electroporation, the bioluminescence signal of the HiBiT-tagged NE protein was assessed using a GloMax bioluminescent plate reader. Data represent means $\pm$ SD from triplicates. (F) On-target editing efficiency of the *ELANE* CDS KO and *MILESTONE* 72 h post-electroporation of NE-HiBiT-tagged THP-1 cells. (G) Representative western blotting (WB) images of NE and α -tubulin protein expression are depicted. NE-HiBiT-tagged THP1 cells were electroporated using *ELANE* CDS KO or *MILESTONE*. Seventy-two hours post-electroporation, western blotting analysis of the NE protein was performed.

homology-directed repair (HDR), as described in [materials and methods](#). A pure single-cell-derived clone of the NE-reporter THP-1 cell line was successfully generated by limiting dilutions on the bulk-edited cell population. This was followed by screening luminescence levels of single-cell-derived populations after 3 weeks of culture by assessing luminescence signals, with verification by Sanger sequencing (Figures S1A–S1C). The TSS and promoter motifs of the *ELANE* gene were previously identified using the synergistic activation mediator (SAM) web tool (<http://sam.genome-engineering.org>)³⁰ and the eukaryotic promoter database (EPD).³¹ Combining these publicly available data, we designed six sgRNAs targeting nucleotides –2 to –146 bp relative to the *ELANE* TSS. We screened editing results obtained using sgRNAs 1–6 and the sgRNA combinations 1 + 4, 2 + 4, and 1 + 4 + 5 using HiFi Cas9-based gene editing (Figure 1A; Figure S1D). Although all selected

sgRNAs reduced NE expression, the combination of sgRNAs 1 and 4, targeting the predicted TATA box (Goldberg-Hogness box) at position –29 bp relative to the TSS, led to the most pronounced reduction in NE levels according to the measured luminescent signal (Figure 1B). sgRNAs on-target efficiencies assessed by applying the DECODR (Deconvolution of Complex DNA Repair) algorithm³² to Sanger sequencing traces of gene-edited THP-1 cells obtained 72 h post-electroporation were over 50% (Figure 1C). To implement an extra layer of safety in the *ELANE*-knockdown procedure, we used a double-nickase Cas9D10A strategy for sgRNA 1 (nick 1) and sgRNA 4 (nick 2), to target opposite strands of the *ELANE* promoter TATA box, thereby introducing a DSB that led to deletions that reduced the efficiency of *ELANE* mRNA transcription (Figure 1D). We termed the proposed strategy *MILESTONE*: Modifying *ELANE* Goldberg-Hogness box to inhibit expression.

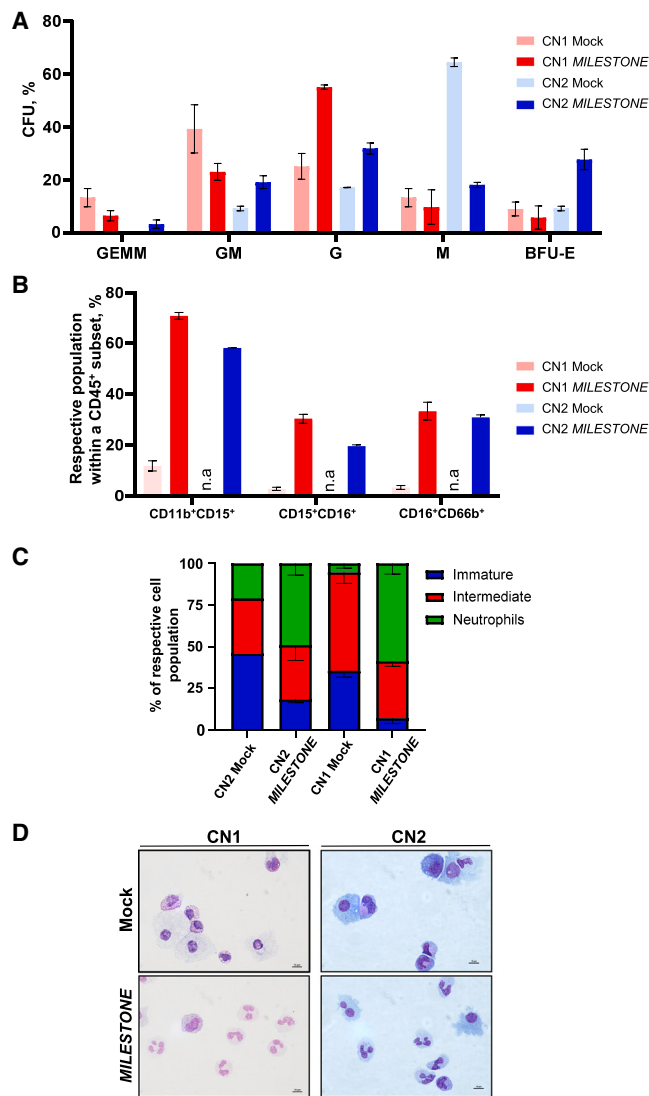


Figure 2. Restored granulocytic differentiation of MILESTONE-edited primary ELANE-CN HSPCs

(A) Colony-forming unit (CFU) assay of gene-edited *ELANE*-CN CD34⁺ HSPCs ($n = 2$) on day 14 of differentiation (CFU-GEMM: granulocytes, erythrocytes, monocytes, megakaryocytes; CFU-GM: granulocytes, monocytes; CFU-G: granulocytes; CFU-M: monocytes; BFU E: burst-forming unit–erythroid). Data represent means $\pm$ SD from duplicates. (B) Granulocytic differentiation of MILESTONE-edited *ELANE*-CN CD34⁺ HSPCs ($n = 2$) was assessed by liquid culture differentiation after 14 days of culture analyzing neutrophilic surface marker expression by flow cytometry. Data represent means $\pm$ SD from duplicates. n.a. = not applicable. (C) May-Grunwald-Giemsa staining of *in vitro* differentiated cells from *ELANE*-CN CD34⁺ HSPCs was conducted on day 14, allowing morphologic discrimination of the cells. Data represent means $\pm$ standard deviation (SD) from duplicates except for the CN2 mock which, due to the severity of the phenotype, only yielded enough cells for one cytopsin. (D) Representative cytopsin images of May-Grunwald-Giemsa staining of *ELANE*-CN HSPCs derived from the *in vitro* neutrophil differentiation culture on day 14 are depicted.

We compared MILESTONE with a previously reported *ELANE* CDS knockout (CDS KO) strategy¹⁴ by measuring the luminescence signal of NE protein in NE-reporter THP-1 cells, used as a readout of NE protein levels 72 h post-electroporation. We found that MILESTONE efficiently decreased NE protein levels compared with Mock-electroporated cells (Figure 1E). The editing efficiencies of *ELANE* CDS KO and MILESTONE approaches were 91.8% and 100%, respectively, as assessed by applying the DECODR algorithm to Sanger sequencing traces of edited THP-1 cells obtained 72 h post-electroporation (Figure 1F). In a separate experiment, western blot analyses using an NE-specific antibody confirmed the reduction in NE levels after *ELANE* editing (Figure 1G).

Collectively, these findings demonstrate the successful development of a novel gene-editing strategy for knocking down *ELANE* mRNA expression. This strategy prevents the emergence of *de novo* *ELANE* mutations from INDELs or novel splice variants induced by INDELs in the *ELANE* CDS, as described by Tuladhar et al.³³

MILESTONE restores granulocytic differentiation of primary *ELANE*-CN CD34⁺ HSPCs

To further evaluate the clinical applicability of MILESTONE as a treatment option for *ELANE*-CN, we performed MILESTONE editing on primary bone marrow-derived CD34⁺ HSPCs from two *ELANE*-CN patients—one, CN1, harboring p.Ala57Val (GenBank: NM_001972.4) and the other, CN2, bearing p.Ala79_Arg81del (GenBank: NM_001972.4) *ELANE* mutations—and evaluated their granulocytic differentiation using colony-forming unit (CFU) and *in vitro* liquid culture neutrophil differentiation assays. CFU assays showed a remarkable increase in granulocytic colony-forming units (CFU-G) in the MILESTONE group compared with the Mock-electroporated control group (Figure 2A). In line with this, *in vitro* granulocytic liquid culture differentiation assays revealed that granulopoiesis was restored on day 14 of differentiation compared with Mock-electroporated cells, assessed based on the percentage of cells expressing the pan-granulocyte marker status, CD45⁺CD11b⁺CD15⁺, and neutrophils with the two neutrophil-specific marker combinations, CD45⁺CD15⁺CD16⁺ and CD45⁺CD16⁺CD66b⁺³⁴ (Figures 2B and S2A). The phenotype of the CN2 patient was extremely severe and resulted in very low numbers of cells in a control Mock group generated on day 14 of granulocytic differentiation, which were not sufficient for flow cytometry analysis. Remarkably, even these cells differentiated efficiently into neutrophils following *ELANE* editing with MILESTONE. A morphological examination of cytopsin preparations of cells of both CN patients on day 14 of culture confirmed restoration of granulopoiesis upon MILESTONE treatment compared with controls (Figures 2C and 2D). The editing efficiency of MILESTONE was greater than 90%, as assessed by applying the DECODR algorithm to Sanger sequencing data for cells obtained on day 14 of differentiation (Figures S3A and S3B). These latter results were further confirmed by next-generation sequencing of rhAMPseq-generated targeted PCR amplicons from gene-edited cells from CN1 patient on day 7 of differentiation, as analyzed using the CRISPResso tool³⁵ (Figures S4A–S4C). Taken together, the *in vitro* neutrophilic differentiation data of

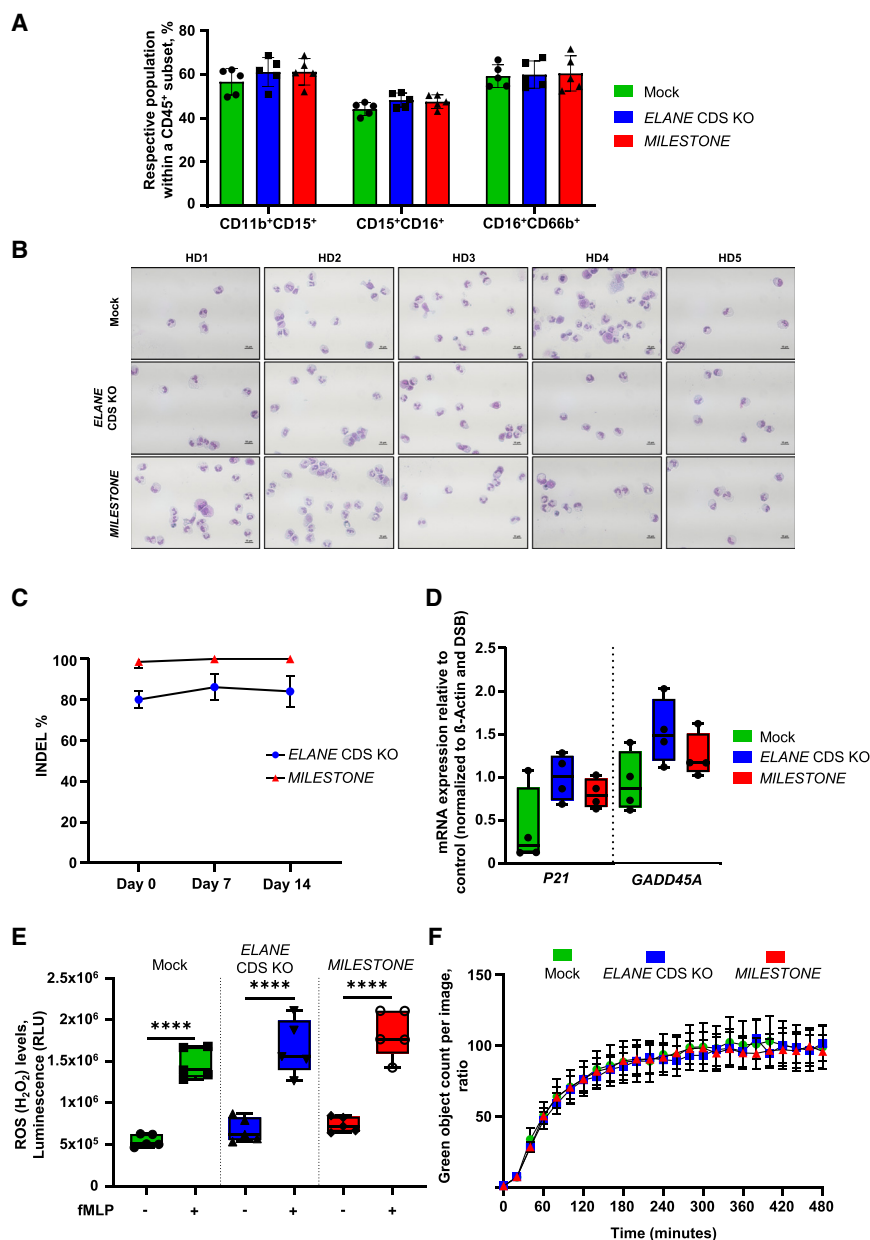


Figure 3. Healthy donors' primary HSPCs granulocytic differentiation remained unaffected upon applying MILESTONE

(A) Granulocytic differentiation of gene-edited CD34⁺ HSPCs of healthy donors was assessed by analyzing neutrophilic surface marker expression using flow cytometry. Data represent means $\pm$ SD from five biological replicates. (B) Representative cytopsin images of May-Grunwald-Giemsa staining of *in vitro* differentiated neutrophils from CD34⁺ HSPCs of healthy donors at day 14 are depicted. (C) On-target editing efficiency of the *ELANE* CDS KO, or *MILESTONE*-edited CD34⁺ HSPCs of healthy donors 4 days post-electroporation (day 0 of *in vitro* differentiation), 7 and 14 (*in vitro* differentiation end time point). Data represent means $\pm$ SD from five biological replicates. (D) qRT-PCR analysis of mRNA expression of *P21* and *GADD45A* in HSPCs collected 48 h post-electroporation of the Mock, *ELANE* CDS KO, or *MILESTONE*-edited healthy donors CD34⁺ HSPCs. Data were normalized to non-electroporated cells, β -actin, and double-strand breaks. Data represent means $\pm$ SD from four biological replicates. (E) Level of hydrogen peroxide (H₂O₂) reactive oxygen species (ROS), measured after stimulation of *in vitro* differentiated Mock, *ELANE* CDS KO, and *MILESTONE*-derived neutrophils (see A–C) with formylmethionine-leucyl-phenylalanine (fMLP) for 30 min. Data represent means $\pm$ SD from five biological replicates. **** p < 0.0001, unpaired *t* test. (F) Phagocytosis kinetic of pHrodo green *S. aureus* bioparticles of *in vitro* differentiated Mock, *ELANE* CDS KO, and *MILESTONE* neutrophils derived from CD34⁺ HSPCs of healthy donors at day 14 of differentiation, using IncuCyte ZOOM System. Data represent means $\pm$ SD from five biological replicates.

MILESTONE-edited cells from two *ELANE*-CN patients confirmed previous observation by our group¹⁴ and others¹⁵ that inhibition of *ELANE* expression in HSPCs from *ELANE*-CN patients effectively restores granulocytic differentiation.

MILESTONE DOES NOT AFFECT GRANULOCYTIC DIFFERENTIATION OF PRIMARY HSPCs FROM HEALTHY DONORS

To investigate whether *MILESTONE* has any adverse effects on granulopoiesis or neutrophil functions *in vitro*, we electroporated CD34⁺ HSPCs from five healthy donors with the CRISPR-Cas9 RNP complex of *ELANE* CDS KO or *MILESTONE* and processed

cells for *in vitro* neutrophilic differentiation 4 days later. We found that knockout or inhibition of *ELANE* expression did not impair neutrophil differentiation compared with Mock-electroporated cells (Figure 3A). May-Grunwald-Giemsa staining of neutrophils differentiated *in vitro* from CD34⁺ HSPCs of healthy donors at day 14, either with *ELANE* CDS knockout or *MILESTONE* editing, also confirmed this observation (Figure 3B). The editing efficiency of *ELANE* CDS KO averaged 80%, 86%, and 84% on days 0, 7, and 14 of differentiation, respectively. *MILESTONE*'s editing efficiency was greater than 99% at the start of *in vitro* differentiation and remained stable through day 14 (Figures 3C and S5A). Similar results were also obtained with targeted next-generation sequencing (NGS), which showed an editing efficiency for *MILESTONE* of 97% on day 0, as analyzed with the Cas-analyzer tool³⁶ (Figure S5B).

To evaluate TP53-triggered DNA damage responses upon genome editing, we measured the mRNA expression levels of the TP53 targets, *P21* and *GADD45A*, 48 h after electroporation. No significant

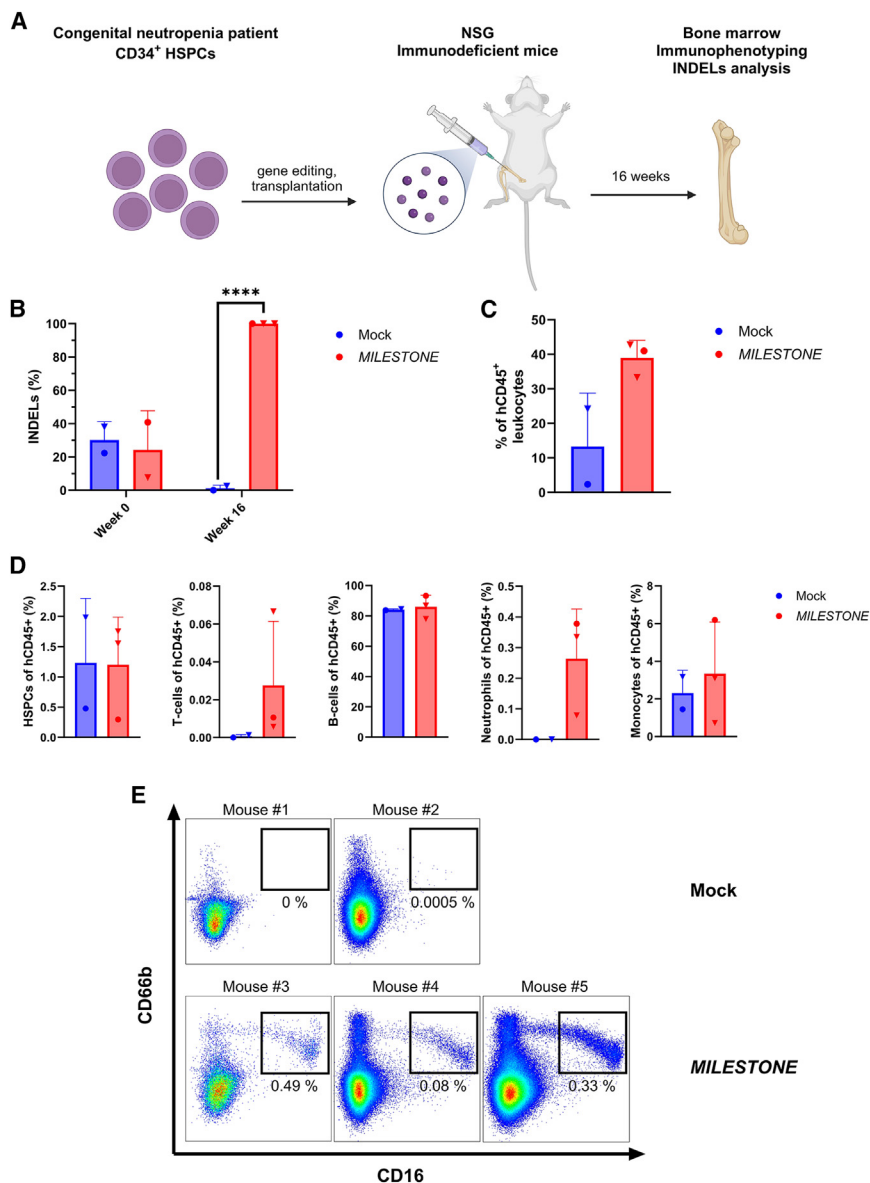


Figure 4. Xenotransplantation of MILESTONE-edited ELANE-CN HSPCs

(A) Schematic workflow of *in vivo* experiments. Depicted are genome editing, intra-femoral injection of edited cells, flow cytometry-based immunophenotyping, and INDELs analysis of bone marrow 16 weeks after transplantation. Created with BioRender.com. (B) INDELs frequencies observed 72 h post *ex vivo* editing (week 0) and at the experimental endpoint 16 weeks post-transplantation in engrafted human bone marrow cells (CN3). Data represent means $\pm$ SD from two independent experiments. **** $p < 0.0001$, unpaired t test. (C) Engraftment efficiency of human CD45⁺ cells 16 weeks post-transplantation. Data are shown as means $\pm$ SD, and dots represent individual animals. (D) Frequency of HSPCs (mCD45⁺hCD45⁺hCD19⁺hCD3⁺hCD33⁺hCD66b⁺hCD34⁺), T cells (mCD45⁺hCD45⁺hCD19⁺hCD3⁺), B cells (mCD45⁺hCD45⁺hCD19⁺hCD3⁺), neutrophils (mCD45⁺hCD45⁺hCD19⁺hCD3⁺hCD33⁺hCD66b⁺hCD16⁺), and monocytes (mCD45⁺hCD45⁺hCD19⁺hCD3⁺hCD33⁺hCD66b⁺hCD14⁺) shown as the percentage of total human CD45⁺ cells in the bone marrow. Data are shown as means $\pm$ SD, and dots represent individual animals. (E) Representative FACS images showing CD16⁺CD66b⁺ mature neutrophils (black gate) in Mock or MILESTONE recipient mice. The percentage of neutrophils in human CD45⁺ cells is shown below the gate. Symbols mark individual mice. Symbols with the same shape mark animals from the same experiment.

difference was observed for *ELANE* CDS KO or MILESTONE compared with control Mock-electroporated cells (Figure 3D).

We further analyzed the *in vitro* generated neutrophils for their functional capabilities. We first assessed ROS in formyl methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils by measuring H₂O₂ levels and found that both *ELANE* CDS KO and MILESTONE neutrophils were able to generate ROS at levels comparable to Mock-electroporated cells (Figure 3E). We next assessed phagocytosis using live-cell imaging of neutrophils incubated with pHrodo Green *S. aureus* bioparticles using the InCuCyte ZOOM system. We observed similar phagocytosis activity in Mock, *ELANE* CDS KO, or MILESTONE neutrophils (Figure 3F).

Mock

MILESTONE

Next, we evaluated whether MILESTONE gene-editing affected mRNA expression levels of *PRTN3* and *AZU1*, genes adjacent to the *ELANE* gene that encode two other serine proteases. To this end, we measured *ELANE*, *PRTN3*, and *AZU1* mRNA expression in neutrophils derived from liquid culture-differentiated MILESTONE-edited HSPCs from two healthy donors compared with that in the Mock group. Meanwhile, *ELANE* mRNA expression was markedly reduced in MILESTONE-edited neutrophils compared with control cells, and *PRTN3* and *AZU1* levels remained unaffected (Figures S6A–S6C).

MILESTONE editing enables granulocytic differentiation of primary ELANE-CN HSPCs in immune-deficient NSG mice

To assess the effect of MILESTONE editing on engraftment, proliferation, and granulocytic differentiation of edited HSPCs *in vivo*, we performed xenotransplantation studies in immune-deficient NSG (NOD SCID γ) mice.^{37,38} We analyzed their bone marrow 16 weeks post-transplantation (Figure 4A; Figure S7A). As a control, cells were edited with a sgRNA assembled with HiFi Cas9 targeting the safe harbor locus, AAVS1, within the *PPP1R12C* gene (Mock control). Two independent experiments were performed with primary

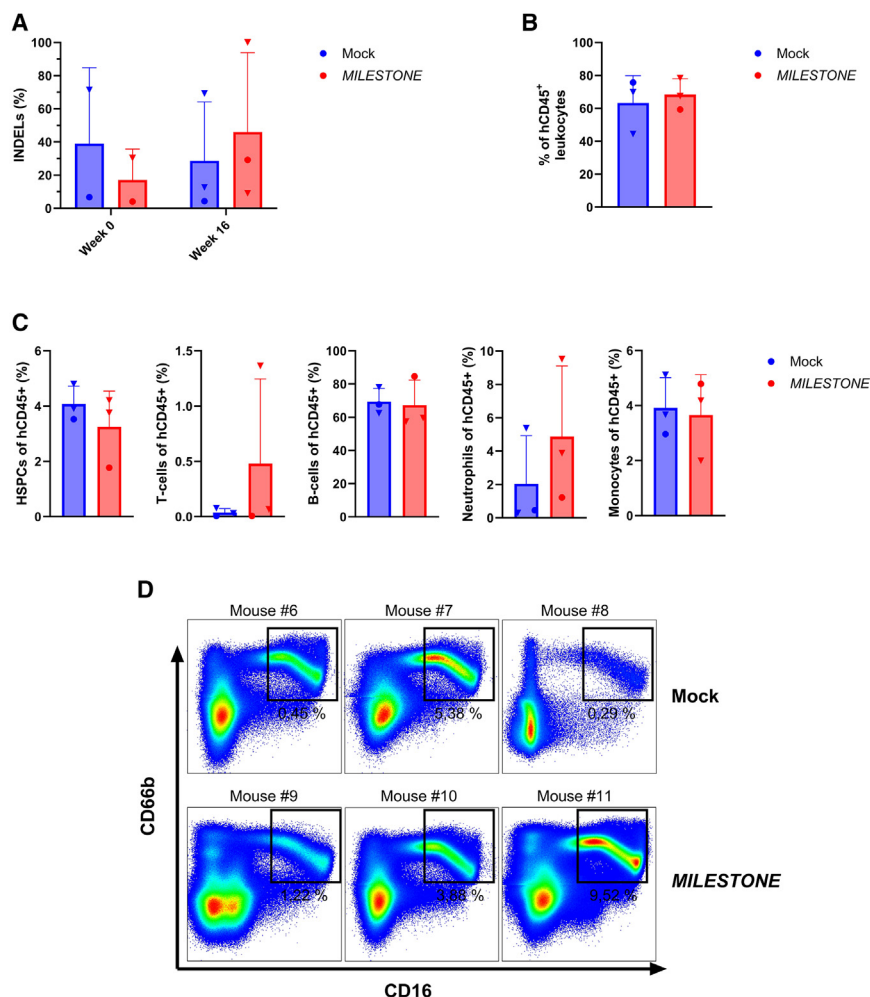


Figure 5. Xenotransplantation of MILESTONE-edited healthy donor primary HSPCs

(A) INDELs frequencies observed 72 h post *ex vivo* editing (week 0) and at experimental endpoint 16 weeks post-transplantation in engrafted human bone marrow cells (HD8 and HD9). Data represent means $\pm$ SD from two independent experiments. (B) Engraftment efficiency of human CD45⁺ cells 16 weeks post-transplantation. Data are shown as means $\pm$ SD, and dots represent individual animals. (C) Frequency of human HSPCs (CD45⁺CD19[−]CD3[−]CD33[−]CD66b[−]CD34⁺), T cells (CD45⁺CD19[−]CD3⁺), B cells (CD45⁺CD19⁺CD3[−]), neutrophils (CD45⁺CD19[−]CD3[−]CD33^{+/−}CD66b⁺CD16⁺), and monocytes (CD45⁺CD19[−]CD3[−]CD33⁺CD14⁺) shown as the percentage of total human CD45⁺ cells in bone marrow. Data are shown as means $\pm$ SD, and dots represent individual animals. (D) Flow cytometry analysis showing CD16⁺CD66b⁺ mature neutrophils (black gate) in Mock or MILESTONE recipient mice. The percentage of neutrophils in human CD45⁺ cells is shown below the gate. Symbols mark individual mice. Symbols with the same shape mark animals from the same experiment.

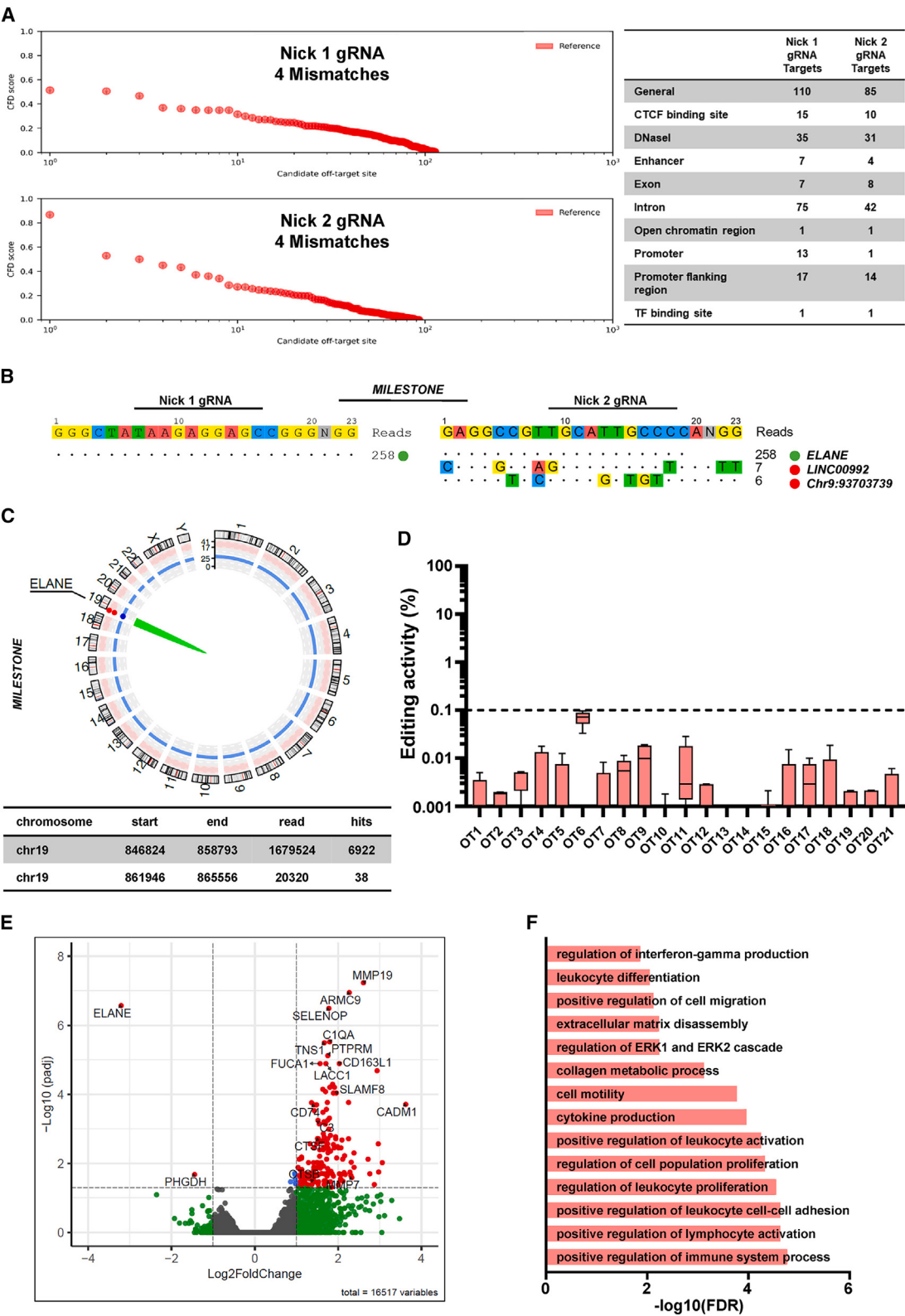
CD34⁺ HSPCs from one *ELANE*-CN patient (*ELANE* mutation GenBank: NM_001972.4, p.Val101Glu) and two healthy donors. *Ex vivo* gene-editing efficiency of the CN patient's HSPCs before transplantation was comparable between Mock (30.2%) and MILESTONE (24.3%) groups. However, editing in engrafted human leukocytes differed strongly and significantly ($p < 0.001$) between Mock- and MILESTONE-edited cells. Whereas Mock-edited cells engrafted poorly, with fewer than 5% of cells in the AAVS1 locus carrying INDELs, MILESTONE-edited cells showed a strong engraftment advantage, with 100% INDELs in all recipient mice (Figure 4B). We further found that engraftment of human CD45⁺ leukocytes was increased in MILESTONE-edited recipient mice compared with that in the control group (Figure 4C). In line with this superior engraftment, we found that the percentage of neutrophils was also markedly increased in MILESTONE-edited cells compared with that in the control group (Figures 4D and 4E). No significant differences were observed in other cellular compartments, including HSPCs, T cells, B cells, and monocytes (Figure 4D). *Ex vivo*, MILESTONE editing resulted in editing efficiencies in healthy donor HSPCs comparable to those in CN HSPCs. *In vivo*, Mock- and MILESTONE-edited healthy

donor HSPCs showed engraftment potentials that were similar to each other and slightly higher than MILESTONE-edited CN HSPCs (Figures 5A and 5B). Furthermore, MILESTONE editing did not severely alter the distribution of the engrafted immature and mature populations compared with that in the Mock control group (Figure 5C). A direct comparison of Mock- and MILESTONE-edited cells showed no differences in neutrophil maturation that exceeded inter-mouse differences (Figure 5D).

A comparison of neutrophil maturation between Mock-edited healthy cells and MILESTONE-edited CN HSPCs further highlighted the fact that the MILESTONE strategy fully restored granulocytic differentiation in CN HSPCs to the levels of healthy HSPCs *in vivo* (Figures 4E and 5D). These data further support our *in vitro* observations and are consistent with previously published observations by Rao et al.¹⁵ who reported successful restoration of *in vivo* neutrophilic differentiation upon *ELANE* KO in an *ELANE*-CN xenograft mouse model, that was established based on gene edited healthy donor HSPCs.

The safety profile of the MILESTONE approach

Currently, no algorithms are available for *in silico* off-target prediction of CRISPR-Cas9n double nickases. Therefore, we performed two independent *in silico* off-target predictions for MILESTONE guide RNAs, calculating up to four mismatches and annotating the predicted potential of off-target sites using CRISPRitz and CRISPRme tools^{39,40}; we identified 110 targets for nick 1 sgRNA and 85 targets for nick 2 sgRNA (Figure 6A). Since the simultaneous activity of both nickases on opposing strands of the genome is



(legend on next page)

necessary to introduce a DNA DSB, we used two genome-wide off-target profiling methods, GUIDE-Seq and CAST-Seq, in our study. With GUIDE-Seq, we profiled the *MILESTONE* approach in primary CD34⁺ HSPCs from one healthy donor. We observed two potential off-target sites for Nick2 sgRNA—LINC00992 (Long Intergenic Non-Protein Coding RNA 992) and Chr9:93703739, a non-coding intergenic region—which contained six or seven mismatches relative to the on-target site (Figure 6B). To determine whether genome editing triggered structural variations, we additionally performed a quantitative evaluation of chromosomal rearrangements in *MILESTONE*-edited primary CD34⁺ HSPCs using CAST-Seq,^{41,42} which detected on-target aberrations but no translocations (Figure 6C). We also performed *in silico* off-target prediction, GUIDE-seq, and CAST-seq for our previously reported *ELANE* CDS KO approach¹⁴ and identified seven potential off-target sites starting with four mismatches compared with the target site. In contrast, CAST-seq revealed large deletions/inversions at the on-target site but no translocations (Figures S8A–S8C).

For off-target site validation, we designed an rhAmpSeq panel that included all potential off-target sites identified by GUIDE-seq and CRISPRitz based on the CFD (cutting frequency determination) score for both *ELANE* CDS KO and *MILESTONE* (Table S1). Genomic DNA from five healthy donors 4 days post-nucleofection with *ELANE* CDS KO or *MILESTONE* gene-editing (Figures 3C and S5A) was used for rhAmpSeq library preparation and NGS. The analysis of sequencing data with CRISPECTOR⁴³ revealed INDELs above the noise threshold in GUIDE-seq-identified off-target site 5 (OT5) (Chr5:1762021-1762044) at an average of 0.8% in all five biological samples (Figure S8D). We found no INDELs in the *MILESTONE* off-target site that exceeded threshold levels, indicating a high specificity that was even higher than the already specific *ELANE* CDS KO approach (Figure 6D).

To further investigate the specificity of the *MILESTONE* approach in reducing *ELANE* mRNA expression levels, we performed RNA sequencing of neutrophils generated by *in vitro* liquid culture differentiation of *MILESTONE*- or Mock-edited primary bone marrow CD34⁺ HSPCs from two healthy donors (HD12 and HD13)

(Figures S9A–S9C). Differentially expressed genes (DEGs) between *MILESTONE*- and Mock-electroporated neutrophils were extracted by analyzing RNA sequencing results using the nf-core pipeline (Figures 6E; Table S2). This analysis revealed a more than 9-fold (log2-fold = 3.21) downregulation of *ELANE* mRNA expression, with an adjusted *p* value of 2.68×10^{-7} . Only one additional gene, *PHGDH* (phosphoglycerate dehydrogenase), was significantly downregulated (log2-fold 1.44, adjusted *p* value 2.10×10^{-2}). *PHGDH*, which is involved in L-serine synthesis, should be further investigated to determine whether its downregulation is an outcome of *MILESTONE* or indicates a feedback mechanism related to the downregulation of NE. We also evaluated whether the *MILESTONE* approach affected mRNA levels of the *ELANE*-neighboring genes, *AZU1*, *PRTN3*, *CFD*, and *MED16*, which lie within an approximately 68-kb genetic; we found that none of them was among identified DEGs (Table S2). Functional enrichment analysis of upregulated genes among the list of DEGs using the ShinyGO web server⁴⁴ (database version 0.80) identified “positive regulation of immune system process,” “positive regulation of leukocyte proliferation,” “cytokine production,” and “positive regulation of cell migration” as enriched biological processes activated upon inhibition of *ELANE* expression (Figures 6F; Table S3). The fact that only one gene, besides *ELANE*, is downregulated by less than 2 log2fold supports our hypothesis that *ELANE* is not an essential gene for activating transcriptional programs, including those critical for neutrophil development and activation.

DISCUSSION

This study presents a pioneering approach for suppressing *ELANE* expression without introducing novel variants in the CDS of the edited gene utilizing a straightforward and highly effective method termed *MILESTONE*. This method, based on a recent proof-of-principle—restoration of granulopoiesis in CN HSPCs through *ELANE* knockout targeting its CDS—that we and others recently described,^{14,15} successfully restored granulocytic differentiation of *ELANE*-CN HSPCs both *in vitro* and *in vivo*. The main advantage of *MILESTONE* editing is that it does not modify the *ELANE* CDS and thus avoids creating new mutant variants of *ELANE* and NE protein, which can be deleterious for HSPC functions. Additionally,

Figure 6. *MILESTONE* safety profile

(A) *In silico* off-target profiling of each gRNA, performed using CRISPRme tool to determine potential off-target sites for up to four mismatches with calculated cutting frequency determination (CFD) score of each possible off-target site according to the human reference genome assembly GRCh38. The genomic region functional annotation of off-target sites was annotated using the CRISPRitz tool. (B) Genome-wide profiling of off-target cleavage in *MILESTONE*-edited healthy donor CD34⁺ HSPCs (HD10) using GUIDE-seq. The *ELANE* promoter target sequence is shown in the top line, with cleaved sites underneath. Mismatches to the on-target site are highlighted in color. GUIDE-seq read counts are displayed to the right of each site. The on-target site is marked with a green circle, and off-target sites are marked with a red circle. All the genomic coordinates are based on human reference genome assembly GRCh37. (C) CAST-Seq for the quantitative evaluation of chromosomal rearrangements in *MILESTONE*-edited healthy donor CD34⁺ HSPCs (HD11). The Circos plot shows on-target site aberrations (ON, green). From the outer to the inner layer, black rectangles show the DNA location of the translocation sites. All the genomic coordinates are based on human reference genome assembly GRCh38. (D) Validation of potential off-target sites determined by rhAmpSeq in healthy donor CD34⁺ HSPCs (HD1-5). NGS data were analyzed using the CRISPECTOR pipeline. Data represent means $\pm$ SD from five independent experiments. (E) Volcano plot showing differentially expressed genes (DEGs) in *MILESTONE*-edited terminally differentiated neutrophils derived from healthy donor CD34⁺ HSPCs (*n* = 2). The x axis shows the log2 fold change (magnitude of change), and the y axis shows the $-\log_{10}$ adjusted *p* value (statistical significance). Colors represent the significance of the genes in terms of *p* value and log2 fold change. Selected differentially expressed genes are annotated. (F) Functional enrichment analysis using a list of significantly upregulated genes in *MILESTONE*-edited terminally differentiated neutrophils from healthy donor CD34⁺ HSPCs (*n* = 2). A complete list of pathways is provided in Table S3. Data are displayed as $-\log_{10}$ adjusted *p* value (FDR).

using the nickase variant of Cas9 nuclease reduces the probability of unintended double-strand breaks, which can lead to INDELs at off-target sites. Our novel approach for modifying the expression of a gene with a gain-of-function mutation through targeting the promoter region of the target gene using Cas9 nickases is not only uniquely applicable to *ELANE*; it can be applied to many other genetic syndromes caused by gain-of-function mutations in genes with redundant functions. Because *ELANE* mutations are autosomal dominant, neither CDS- nor promoter-based approaches discriminate between wild-type and mutated alleles of *ELANE*, simultaneously knocking out both forms of the NE protein. Despite strongly reducing NE levels, *MILESTONE* did not affect the functions of neutrophils differentiated from gene-edited cells, a finding in line with our previous observations on CN patients' *ELANE*-KO gene-edited HSPCs¹⁴ and reports on PLS patients²⁷ and *Elane*^{-/-} mice.^{24,26,45} Moreover, incomplete gene-editing efficiency and the consequent residual expression of *ELANE* mRNA in *MILESTONE*-edited cells *in vivo* will result in some neutrophils that express normal (unedited cells) in addition to those that express reduced (edited cells) levels of NE, thus sustaining bactericidal activity. Simultaneously, our data argue for the dose-dependent effects of mutated NE on granulocytic differentiation—even with remaining levels of mutated NE, HSPCs could efficiently differentiate into mature neutrophils. The high level of the *ELANE* gene editing achieved by *MILESTONE* suffices to reach the current estimate for a therapeutic threshold of *ELANE* knockout of 11%, as evaluated in healthy individuals with mosaic congenital neutropenia-causing *ELANE* mutations.¹⁵ Whether the remaining mutant *ELANE* may still contribute to leukemogenic transformation in CN patients remains to be investigated. Because *MILESTONE* successfully restored granulopoiesis, we assume that the probability of developing leukemia in *MILESTONE*-treated patients will be at least reduced because these patients will not require chronic therapy with rhG-CSF anymore, and the endogenous G-CSF levels are expected to normalize.

To achieve clinical translation, gene-editing technology must demonstrate both safety and efficacy.^{23,46,47} Our comprehensive analysis of on-target and off-target activity employing rhAmpSeq, GUIDE-seq, and CAST-seq revealed a high on-target editing efficiency and superior off-target profile of *MILESTONE* editing. In contrast, this analysis identified INDELs in OT5 for the *ELANE* CDS KO guide. Together, these results underscore the enhanced safety profile of the *MILESTONE* approach. Multiple groups recently described unintended on-target aberrations after targeting different cell types with Cas nucleases.^{48–51} Given these new data, further validation of *MILESTONE* editing, for example, using drop-off ddPCR or single-cell RNA sequencing of edited HSPCs, as performed by Nahmad et al.,⁴⁹ will be required to preclude potential on-target aberrations, including large deletions and aneuploidy. Another safety concern highlighted by recent studies is that DNA double-strand breaks can induce DNA damage response through TP53 activation, leading to reduced proliferation, engraftment, and clonogenic capacity of edited HSPCs.^{52,53} Approaches for HSPC gene editing in an autologous transplantation setting must preserve the stemness features of gene-

edited HSPCs. We demonstrated no significant difference in expression levels of the p53 downstream targets, *P21* and *GADD45A*, between HSPCs edited with *MILESTONE*, *ELANE* CDS KO and Mock samples 48 h after electroporation, indicating that both approaches limit DNA damage to their intended on-target site, largely without affecting TP53-initiated responses.

Additionally, we found that efficient engraftment and multilineage potential of *MILESTONE*-edited HD and *ELANE*-CN HSPCs were preserved in immunodeficient NSG mice and were accompanied by granulocytic differentiation *in vivo*. The fact that primary *ELANE*-CN *MILESTONE*-edited HSPCs exhibited a significant engraftment advantage—with 100% engraftment of edited cells, even in cell populations that do not constitutively express NE (e.g., lymphocytes)—indicates that mutant NE has pathological effects on early self-renewing HSPCs, in addition to its impact on myeloid progenitors. This exciting observation warrants further investigation and provide new insights on the pathogenesis of *ELANE*-CN.

An essential feature of *MILESTONE* is its universality—a considerable advantage given that pathogenic *ELANE* mutations occur at any exon and even in some introns of the *ELANE* gene, and more than 120 *ELANE* mutations have been reported to date.^{3,9} In a recent study, Tran et al. demonstrated the successful correction of a single *ELANE* mutation at exon 4 (p. L172P) using AAV-based delivery of the repair template.⁵⁴ However, such *ELANE* mutation position-specific gene-editing strategies are extraordinarily costly and time-intensive. Moreover, recent reports suggest poor engraftment and reduced repopulating activity of HSPCs after gene editing using AAV-based constructs *in vivo*.^{55–61} In the *MILESTONE* approach, only two sgRNAs and Cas9D10A protein are sufficient to correct neutropenia in all *ELANE*-CN patients, independent of the position of the *ELANE* mutation. Moreover, no AAV-based delivery of an HDR template is required. In another study, researchers demonstrated the successful restoration of defective granulocytic differentiation through allele-specific editing of *ELANE* mutations in *ELANE*-CN HSPCs *in vitro*.⁶² This group developed a CRISPR-based strategy utilizing single-nucleotide polymorphisms (SNPs) identified in the human population (healthy or CN) with two sgRNAs. These sgRNAs independently introduce DSBs, facilitating the introduction of a monoallelic knockout. Although this method is promising, introducing two double-strand breaks may increase genotoxicity, chromosomal aberrations, and risk of off-target effects, especially if the extensively cut genomic region is greater than 3 kb in size. This approach may also affect the expression of the neighboring genes, e.g., *CFD*, located ~240 base pairs (bp) from one of the proposed cut sites. Notably, such allele-specific gene editing only applies to *ELANE*-CN patients carrying the obligatory SNPs; all other patients are precluded from exploiting this type of therapy.

Taken together, our findings establish *MILESTONE* as a universal, safe, and effective gene therapy approach that reduces *ELANE* mRNA expression level by using a CRISPR-Cas9n double-nickase based editing of the *ELANE* promoter without targeting the *ELANE* CDS. The *MILESTONE* approach rescues granulopoiesis in

ELANE-CN patients. It offers a potentially curative therapy in the setting of autologous transplantation of gene-edited HSPCs for these patients, replacing daily rhG-CSF therapy and potentially decreasing the risk of leukemia development. This approach is also useful in gene engineering for the knockdown of genes, especially for targeting genomic sequences highly similar to other loci or genes with multiple isoforms not targetable with one guide RNA. We also envision the potentially broad applicability of this approach in gene therapy applications for example inhibition of the expression of genes with gain-of-function mutations or cell engineering.

MATERIAL AND METHODS

Cell isolation and culture

Human CD34⁺ HSPCs were isolated from the bone marrow mononuclear cell fraction from three *ELANE*-CN patients and 13 healthy donors by Ficoll gradient centrifugation and magnetic bead separation using the Human CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, #130-046-703). CD34⁺ cells were cultured in a density of 2×10^5 cells/mL in StemSpan SFEM II stem cell expansion medium (Stemcell Technologies, #09605) supplemented with 1% penicillin/streptomycin, 1% L-Glutamine and a cytokine cocktail consisting of 20 ng/mL interleukin (IL)-3, 20 ng/mL IL-6, 20 ng/mL TPO, 50 ng/mL SCF, and 50 ng/mL FLT-3L (all cytokines were purchased from R&D Systems). Samples were collected during the routine annual follow-up recommendation by the Severe Chronic Neutropenia International Registry or prior bone marrow transplantation. Informed written consent was obtained from the study participants. The study was conducted according to Helsinki's declaration. Study approval was obtained from the Ethical Review Board of the Medical Faculty, University of Tübingen.

THP-1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), 2 mM L-glutamine, and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37°C and 5% CO₂.

Design of the sgRNAs and repair template for HiBiT tagging of *ELANE*

Specific single-guide RNA (sgRNA) for endogenous bioluminescence tagging of the *ELANE* gene (cut site: chr19 [5' TCGGCGGCCGA GGGTCATGG 3', +852,330: -852,330], NM_001972.3 Exon 1, NP_001963.1 p. M1) and repair template to knock in HiBiT tag (5'-GGCAATGCAACGGCCTCCAGCACAGGGCTATAAGAGG AGCCGGGCGGGCACGGAGGGGACAGACCCCGGAGCCCCA GCCCACCATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATT AGCACCTCGGCCCGGACTCGCGTGTCTTTTCTCGCCTGT GTCCTGCCGGCCTTGCTGCTGGGGGTGAGTTTTTGAGT -3') were designed using Benchling [Biology Software] retrieved from [ensemble.org](https://benchling.com) and ordered via Integrated DNA Technologies (IDT). Specific sgRNAs for targeting the non-coding regulatory regions of the *ELANE* gene (cut site: chr19 [5' GGGCTATAAGAGGA GCCGGG 3', +852,258] and [5' GAGGCCGTTGCATTGCCCCA 3', -852,243] were designed using SAM webtool (<http://sam.genome-engineering.org>)³⁰ and ordered via IDT.

CRISPR-Cas gRNA RNP mediated gene editing

According to the manufacturer's instructions, electroporation was carried out using the Amaxa nucleofection system (P3 primary kit, #V4XP-3024). Briefly, 1×10^6 THP-1 cells or human CD34⁺ HSPCs were electroporated with assembled gRNA (8 µg) and HiFiCas9 or Cas9D10A (15 µg) protein (IDT); 1 µM of ssODN was added to the electroporation mix in the case of the HiBiT knockin experiment. Single-cell HiBiT-tagged *ELANE* clones were selected by single-cell subculturing of edited THP-1 cells as described by Ran et al.⁶³ Briefly, gene-edited cells were diluted to 0.5 cells per 100 µL. The diluted cell suspension was distributed on two 96-well plates. Plates were incubated for 2 weeks with bi-weekly media changes. After 2 weeks, half the cells were lysed, and luminescence was measured, as described below. Cells with a strong luminescence signal were selected and expanded for subsequent experiments.

NE protein measurement through HiBiT-based luminescence

To measure HiBiT-based NE protein levels, we followed the manufacturer's instructions. Briefly, 1×10^5 cells were suspended in 100 µL of RPMI supplemented with 10% FBS per well of a white 96-well tissue culture plate. Next, cells were lysed by adding an equal volume of Nano-Glo HiBiT Lytic Reagent (Promega N3030) and incubated at room temperature for 30 min while shaking. Nano-Glo HiBiT Lytic reagent consists of Nano-Glo HiBiT Lytic Buffer, Nano-Glo HiBiT Lytic Substrate, and LgBiT Protein. Luminescence was then measured with a GloMax Multidetector System plate reader.

Western blotting

A total of 1×10^6 cells were lysed in 200 µL 3X Laemmli buffer, and protein was denatured for 10 min at 95°C. Five microliters of cell lysate in Laemmli buffer were loaded per lane. Proteins were separated on a 12% polyacrylamide gel and transferred on a nitrocellulose membrane (GE Healthcare) (1 h, 100V, 4°C). The membrane was blocked for 1 h in 5% BSA/TBST and incubated with primary anti-NE (Santa Cruz, #sc-9520) or α -Tubulin (Cell Signaling, #4970) antibody overnight at 4°C. After that, membranes were washed and incubated with a secondary HRP-conjugated antibody (Santa Cruz, #sc-2004) for 1 h at room temperature. Pierce ECL solution (Thermo Fisher) and Amersham Hyperfilms were used to detect the chemiluminescence signal of proteins.

CFU assay

CD34⁺ cells were resuspended in IMDM supplemented with 2% FBS (Stemcell Technologies, #07700) and enriched Methocult (Stemcell Technologies, #H4435). The cell suspension was plated on 3.5-cm dishes (3×10^3 cells/dish) for 14 days at 37°C and 5% CO₂.

Liquid culture differentiation of CD34⁺ cells

CD34⁺ cells were seeded at 2×10^5 cells/mL density. Cells were incubated for 7 days in RPMI 1640 GlutaMAX supplemented with 10% FBS, 1% penicillin/streptomycin, 5 ng/mL SCF, 5 ng/mL IL-3, 5 ng/mL GM-CSF, and 1 ng/mL G-CSF. The medium was exchanged every second day. On day 7, cells were plated in RPMI 1640 GlutaMAX supplemented with 10% FBS, 1% penicillin/streptomycin, and 1 ng/mL G-CSF. On day 14, cells were analyzed by flow cytometry using the

following mouse anti-human antibodies: CD34 (BD, #348811), CD33 (BioLegend, #303416), CD45 (BioLegend, #304036), CD11b (BD, #557754), CD15 (BD, #555402), CD66b (BioLegend, #305114), and CD16 (BD, #561248). The morphology of the cells was investigated on cytospin slides by May-Grunwald-Giemsa staining.

Assessing genome-editing efficiency

Genomic DNA was isolated using the QuickExtract DNA extraction kit (Lucigen, #QE09050). PCR was carried out using the primeSTAR max Polymerase Kit (Takara, #R045B) and gene-specific primers (Table S4). The PCR products were purified by ExoSAP, which is a master mix of one part of Exonuclease I 20 U/μl (Thermo Fisher Scientific, #EN0581) and two parts of FastAP thermosensitive alkaline phosphatase 1 U/μl (Thermo Fisher Scientific, #EF0651). Micro-synth performed Sanger sequencing of purified PCR products. CRISPR-Cas9-based genome editing was analyzed using the Deconvolution of Complex DNA Repair (DECODR) web tools.

In vitro ROS assay

Granulocytes from day 14 of liquid culture differentiation were cultured in RPMI 1640 medium supplemented with 0.5% BSA at the density of 5×10^4 per well of 96-well white-walled plate with or without fMLP (Sigma, #F3506) at a final concentration of 10 nM and incubated for 30 min at 37°C, 5% CO₂. According to the manufacturer's protocol, the hydrogen peroxide (H₂O₂) level, an ROS, was measured by ROS-Glo H₂O₂ Assay kit (Promega, #G8820) on a GloMax Multidetector System plate reader.

Assessment of phagocytosis kinetics using the IncuCyte ZOOM system

Granulocytes from day 14 of liquid culture differentiation were cultured in RPMI 1640 medium supplemented with 0.5% BSA and pHrodo Green *S. aureus* bioparticles (#4620 Essen Bio) according to the manufacturer's protocol in IncuCyte ZOOM system (Essen Bio) at 37°C, 5% CO₂. Briefly, 10^4 cells were seeded in 90 μL of medium, and 10 μg of Bioparticles were added to a final volume of 100 μL. The cells were monitored for 6 h. The analysis was conducted in IncuCyte S3 Software.

Xenotransplantation of HSPCs in NSG mice

HSPCs were thawed and cultured for 14–16 h before electroporation. Following electroporation, cells were cultured for an additional 6 h before transplantation. Bulk electroporated cells were distributed among different mice. Mock cells were electroporated with sgRNA targeting the AAVS1 safe harbor site (5 CTCCCTCCCAGGATCC TCTC 3').⁶⁴ Eight- to 16-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Charles River Laboratory # 001976) were sublethally (170 cGy) irradiated 4 h prior to transplantation. HSPCs were transplanted intra-femorally. To this end, 1×10^5 HSPCs were injected per mouse in the right femur. Mice were euthanized 16 weeks post-transplantation. Bone marrow cells were isolated and immunophenotyped by flow cytometry on a FACS ARIA. The following antibodies were used for staining: 7AAD (BD # 559925), anti-mouse CD45 PerCP (BioLegend # 103130), anti-human CD45 BV510

(BioLegend # 304036), anti-human CD19 BV421 (BioLegend # 302234), anti-human CD3 BV711 (BioLegend # 300464), anti-human CD14 BV650 (BioLegend # 301836), anti-human CD16 APC (BioLegend # 302012), anti-human CD33 APC-Cy7 (BioLegend # 303442), anti-human CD66b FITC (BioLegend #305104), and anti-human CD34 Pe-Cy7 (BD #348811). Genome-editing efficiencies were determined as described in the “assessing genome-editing efficiency” section above. Mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Tübingen according to German state and federal regulations, protocol M 23/21G.

Quantitative real-time PCR

RNA was isolated using RNeasy Micro Kit (Qiagen, #74004), and cDNA was prepared from 1 μg of total RNA with Omniscript RT Kit (Qiagen, # 205111). qPCR was performed using SYBR Green 3 qPCR master mix (Roche, # 04887352001) and Light Cycler 480 (Roche). Target genes were normalized to *ACTB* or *GAPDH* genes. Primer sequences are presented in Table S5.

GUIDE-seq

MILESTONE was applied on primary healthy donor CD34⁺ HSPCs along with annealed dsODN (5Phos/GTTTAATTGAGTTGTCA TATGTTAATAACGGT*A*T and 5Phos/ATACCGTTATTAACA TATGACAACCTCAATTAA*A*C, Phos represents a 5' phosphorylation and * indicates a phosphorothioate linkage). Four days post-electroporation, genomic DNA was isolated with the QIAamp DNA Mini Kit (Qiagen, # 51304) according to the manufacturer's protocol. NGS library preparation was performed as described by Malinin et al.⁶⁵ and sequenced by NovoGene. NGS files were analyzed using the pipeline provided by Tsai lab in Git Hub (<https://github.com/tsailabSJ/guideseq>).

CAST-seq

MILESTONE was applied on the primary healthy donor CD34⁺ HSPCs. According to the manufacturer's protocol, genomic DNA was isolated 4 days after electroporation using QIAamp DNA Micro Kit (Qiagen, #56304). CAST-seq library preparation for NGS and data analysis was performed, as Turchiano et al. explained,⁴¹ with an improved bioinformatics pipeline.⁴²

rhAmpSeq

Targeted amplicon sequencing was performed using IDT rhAmpSeq CRISPR analysis system. Briefly, the rhAMP primers to amplify multiple *ELANE* regions (promoter region, exon 2, exon 3, exon 4, and exon 5) or potential off-target sites (Table S1) were designed using the IDT rhAMP primer design software and ordered via IDT. Library preparation was performed according to the manufacturer's protocol, and Novogene performed NGS. CRISPRESSO package³⁵ and CAS-analyzer³⁶ were used to analyze the on-target data. CRISPECTOR⁴³ tool was used to analyze the off-target data.

RNA-seq

MILESTONE was applied on primary healthy donor CD34⁺ HSPCs, followed by granulocytic liquid culture differentiation for 14 days.

According to the manufacturer's protocol, the RNA was extracted using an RNeasy Micro Kit (Qiagen, #74004). Strand-specific paired-end RNA-seq was performed by Novogene. The nf-core RNAseq pipeline⁶⁶ - a framework for community-curated bioinformatics pipelines - was used to extract the gene-level count matrix for each sample. Differential analysis of count data was performed using the DESeq2 package and the iDEP web tool to obtain the differentially expressed genes (DEGs) (Table S2). The volcano plot of DEGs was generated using the Enhance volcano plot R-package.

Statistical analysis

Differences in mean values between groups were analyzed using two-sided, unpaired Student's *t* tests in GraphPad Prism software.

DATA AND CODE AVAILABILITY

For original data, please get in touch with Julia.skokowa@med.uni-tuebingen.de.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.ymthe.2024.03.037>.

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AUTHOR CONTRIBUTIONS

M.N., P.M., and J.S. made initial observations and designed and supervised the experiments; M.N., M.R., and J.S. interpreted the results and wrote the manuscript. M.N. performed CRISPRITZ and CRISPRme *in silico* analysis, rhAmpSeq off-target validation, RNA-seq and analyzed the data; M.N., M.R., and P.M. performed experiments with primary HSPCs with the help from B.D. and N.B.B.; M.R. and M.N. conducted GUIDE-seq and analyzed data; M.M.K., T.C., and M.N. performed CAST-seq; M.N. performed endogenous tagging of *ELANE* in THP1 with Y.X. assistance; M.R. designed and performed *in vivo* experiments and analyzed data. P.A.-T. assisted in *in vivo* experiments and wrote the manuscript. K.W. and C.Z. provided patients' material; K.W., M.K., C.Z., C.L., and T.C. gave insightful comments.

DECLARATION OF INTERESTS

M.N., P.M., and J.S. have filed a patent on the invention described in this study. T.C. is an advisor to Cimeio Therapeutics and Excision BioTherapeutics and holds a patent on CAST-Seq.

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