Molecular Therapy
Nucleic Acids
Original Article

PINE-TREE enables highly efficient genetic modification of human cell lines

Carlye Frisch,[1,3] William W. Kostes,[1,3] Brooke Galyon,[1] Brycelyn Whitman,[1] Stefan J. Tekel,[1] Kylie Standage-Beier,[1,2] Gayathri Srinivasan,[1] Xiao Wang,[1] and David A. Brafman[1]

[1]School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ 85287, USA; [2]Molecular and Cellular Biology Graduate Program, Arizona State University, Tempe, AZ 85287, USA

Prime editing technologies enable precise genome editing without the caveats of CRISPR nuclease-based methods. Nonetheless, current approaches to identify and isolate prime-edited cell populations are inefficient. Here, we established a fluorescence-based system, prime-induced nucleotide engineering using a transient reporter for editing enrichment (PINE-TREE), for real-time enrichment of prime-edited cell populations. We demonstrated the broad utility of PINE-TREE for highly efficient introduction of substitutions, insertions, and deletions at various genomic loci. Finally, we employ PINE-TREE to rapidly and efficiently generate clonal isogenic human pluripotent stem cell lines, a cell type recalcitrant to genome editing.

INTRODUCTION

The emergence of deaminase fused-Cas9 base editing technologies as well as prime editing technologies has enabled precise chromosomal editing without the need for potentially deleterious double-stranded breaks or inefficient homology-directed repair.[1–5] While current base editors are restricted to a limited number of base substitutions, prime editors (PEs) can direct all single-base substitutions as well as insertions and deletions.[5] Even so, existing methods to purify edited cell populations are limited and rely on downstream sequencing techniques. Recent reports have shown that PE efficiency is low in cells resistant to genome modification, such as human pluripotent stem cells (hPSCs).[6,7] Previously, we developed a series of methods that employ transient reporters for editing enrichment (TREE) to facilitate highly efficient (>80%) base editing of cells.[8–11] Briefly, these TREE-based methods employ a transient episomal fluorescent reporter that allows identification and flow cytometry-based isolation of cells that have had single-nucleotide changes at precise genomic locations. Here, we built upon this work to establish a new TREE-based method, prime-induced nucleotide engineering using a TREE (PINE-TREE), to detect and report prime editing activity with a cell. Moreover, we demonstrate, at several independent loci and across various types of genomic modifications (i.e., base substitutions, insertions, and deletions), that PINE-TREE allows real-time identification and purification of edited cell populations. We employ PINE-TREE to modify hPSCs, resulting in editing efficiencies significantly exceeding those using typical enrichment strategies. Finally, we demonstrate that PINE-TREE provides efficient generation of clonal isogenic hPSCs at loci that are difficult to edit using traditional reporter of transfection (RoT)-based PE enrichment techniques. Overall, PINE-TREE is a highly adaptable and easily implemented method that will greatly enhance the use of PE technologies for numerous *in vitro* applications.

RESULTS

Establishment and characterization of the PINE-TREE reporter system

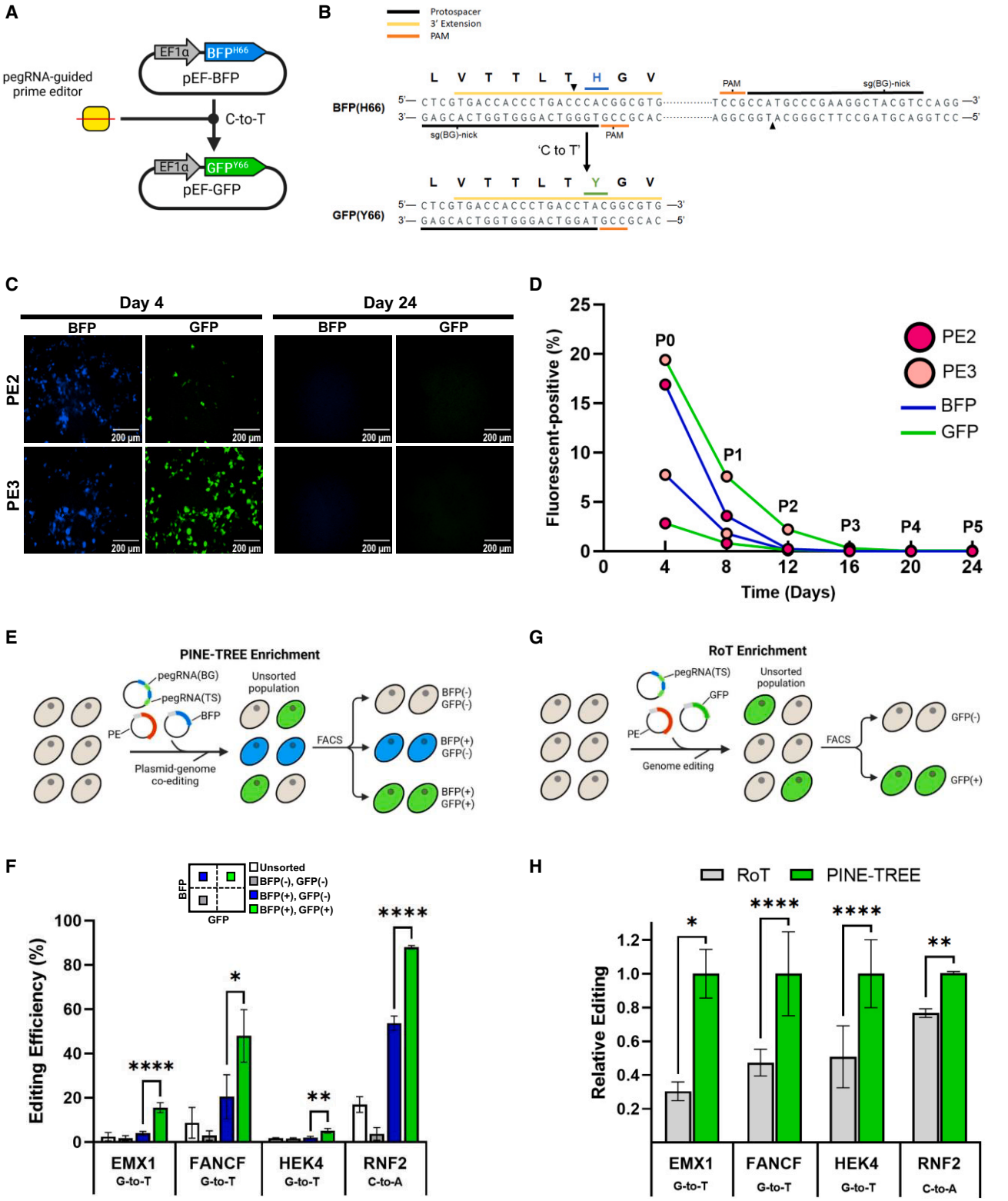
Prime editing has been employed in a variety of *in vitro* applications, including lineage tracing, transcriptional modulation, dissection of disease-relevant genetic variants, synthetic biology, and development of model cell lines.[12–16] Nonetheless, techniques to enhance isolation of edited cells are limited. Instead, most approaches employ transfection reports that do not report on editing activity within a cell. In addition, numerous factors can influence prime editing efficiency, including PE nuclear expression levels, Cas9 nuclease activity, endogenous levels of DNA mismatch repair, prime editing guide RNA (pegRNA) design, and pegRNA degradation.[17–19] To that end, we sought to build upon our previous TREE-driven base editing enrichment strategies[8–11] to develop a TREE-based reporter system (here referred to as PINE-TREE) that would report on PE activity and allow purification of prime-edited cell populations. Therefore, to develop a fluorescence-based assay to report on PE activity within a cell, we utilized a plasmid encoding a blue fluorescent protein (BFP) variant that converts to a green fluorescent protein (GFP) upon a PE-directed C-to-T nucleotide change (Figure 1A). Thus, co-transfection of cells with this BFP construct (pEF1α-BFP), a PE (pEF1α-PE2), and a pegRNA (pegRNA[BG]) targeting a C-to-T conversion in the "CAC" codon encodes for a histidine at the 66th amino acid will result in an amino acid change to a tyrosine encoded by a "TAC" codon (Figure 1B). This histidine-to-tyrosine change will result in a BFP-to-GFP conversion in cells in which the prime editing machinery is present and active. We also designed an additional construct (here

Received 16 January 2023; accepted 11 July 2023;
https://doi.org/10.1016/j.omtn.2023.07.007.

[3]These authors contributed equally

Correspondence: David Brafman, PhD, School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ 85287, USA.
E-mail: david.brafman@asu.edu

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).



(legend on next page)

referred to as pegRNA(BG)-nsgRNA(BG)) to be utilized with the PE3 system, which employs an additional nicking single guide RNA (nsgRNA) that nicks the non-edited strand near the pegRNA target, enhancing editing efficiency (nsgRNA(BG)).[5]

We first sought to validate PINE-TREE in a cell line that was relatively easy to transfect and has been shown previously to be amenable to prime editing: HEK293T cells. Thus, HEK293T cells were co-transfected with BFP, a PE, and a pegRNA in the context of the PE2 or PE3 system. Cells transfected with pEF1α-BFP or pEF1α-GFP only were used to establish the flow cytometry sorting gates to determine reporter-positive and -negative cell populations (Figure S1A). 72 h post transfection, fluorescence microscopy and flow cytometry analysis demonstrated that the BFP-targeting pegRNA resulted in generation of GFP-positive cells, implying that episomal C-to-T editing allowed BFP-to-GFP conversion (Figures S1B and S1C). As expected, HEK293T cells transfected with the PE3 system displayed higher percentages of GFP-positive cells compared with the PE2 system, which is consistent with previous reports[5,20] (Figures S1B and S1C). In addition, we wanted to confirm that the fluorescence associated with PINE-TREE reporting was transient in nature. In that regard, we measured the long-term fluorescence of GFP-positive cells after PINE-TREE-based editing using fluorescence microscopy (Figure 1C) and flow cytometry (Figure 1D). Collectively, this analysis revealed no long-term detectable GFP signal, demonstrating the transience of the PINE-TREE fluorescent signal.

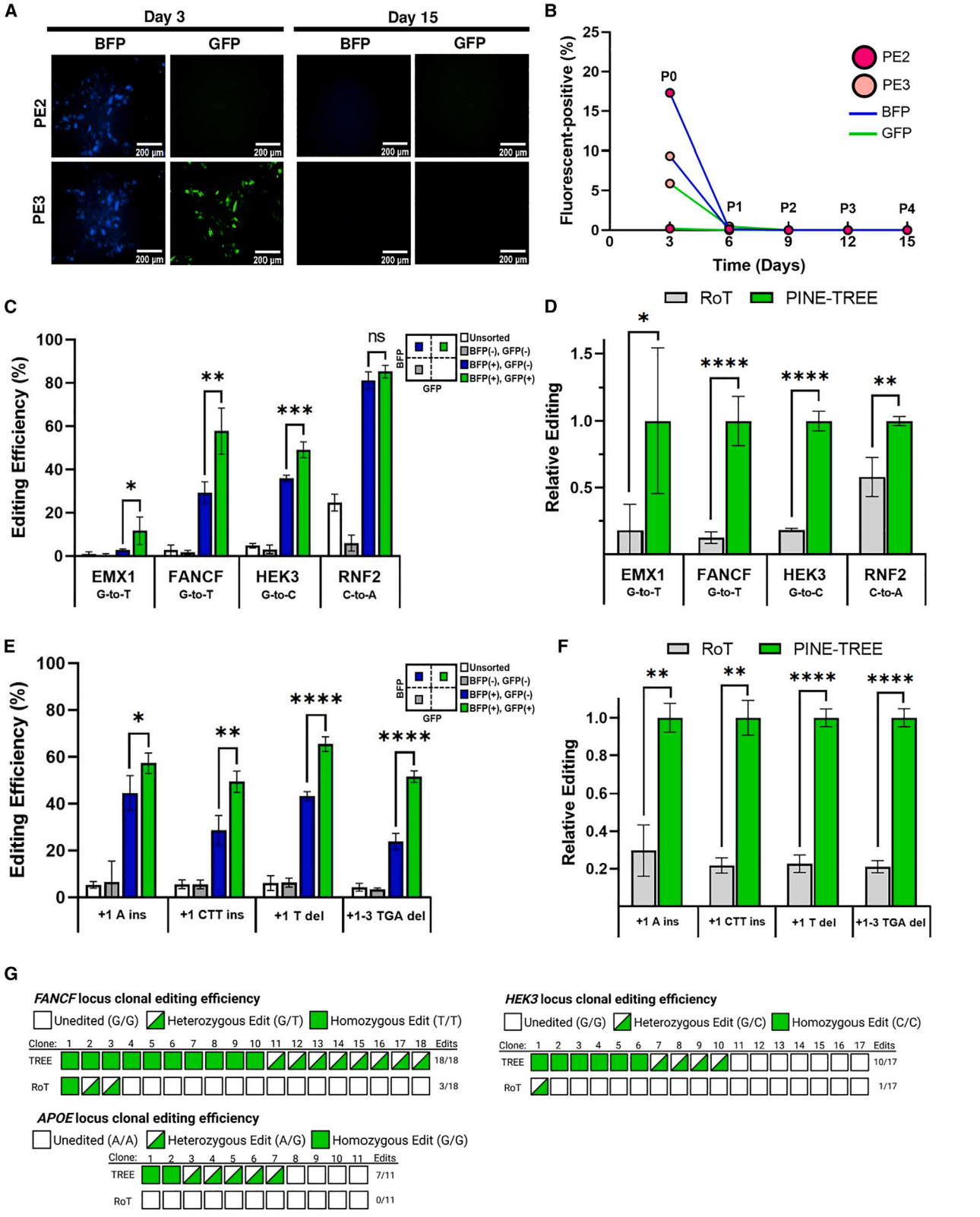
Next, we wanted to demonstrate that PINE-TREE could be utilized to enrich edited cell populations. Because we observed that the PE3 system allowed higher levels of BFP-to-GFP episomal editing, we proceeded with using this system in the context of PINE-TREE enrichment methods. Therefore, we designed a dual-targeting vector (PE-pegRNA(DT)-nsgRNA(DT)) that contained pegRNA(BG)-nsgRNA(BG) as well as a pegRNA for an endogenous target site (pegRNA(TS)) and an additional sgRNA that nicks the non-editing strand adjacent to the pegRNA(TS) target (sgRNA(TS)). The dual-targeting vector was constructed to enable cloning of any target sites via simple restriction enzyme cloning digestion and ligation of oligonucleotides[21] (Figure S2). To evaluate PINE-TREE enrichment of cells that have been edited at specific loci, we co-transfected HEK293T cells with pEF1α-BFP and pEF1α-PE2 as well as PE-pegRNA(DT)-nsgRNA(DT) vectors designed to introduce single-nucleotide mutations at various loci (*EMX1*, *FANCF*, *HEK4*, and *RNF2*). Flow cytometry was then used to purify fluorescent cell populations, and Sanger sequencing was performed on the targeted genomic loci in the isolated cell populations (Figure 1E). This analysis revealed that cells positive for the PINE-TREE reporter (i.e., BFP+GFP+) had a higher level of editing compared with double-negative cells (i.e., BFP−GFP−) (Figure 1F). Critically, cells only positive for the transfection marker but negative for the PINE-TREE reporter (i.e., BFP+GFP−) had significantly lower editing across all loci compared with the GFP+ cell population (Figure 1F). In addition, we wanted to compare the editing efficiency in PINE-TREE-enriched cells with that of cell populations that had been enriched using conventional reporters of transfection (Figure 1G). In this vein, we co-transfected HEK293T cells with a reporter plasmid (pEF1α-GFP), a PE (pEF1α-PE2), and a PE-pegRNA(TS)-nsgRNA(TS) at various genomic target sites. Flow cytometry was used to separate RoT reporter-positive cell populations, and Sanger sequencing was performed on the targeted genomic sites. This analysis demonstrated that, across all target sites, the BFP/GFP double-positive cells isolated using PINE-TREE had significantly higher levels of prime editing than reporter-positive cells enriched using RoT approaches (Figure 1H). Overall, these results demonstrate that PINE-TREE can not only be used to identify but also enrich for prime-edited cell populations across a variety of genomic target loci.

PINE-TREE can be used for introduction of substitutions, deletions, and insertions into hPSCs

CRISPR nuclease-based approaches to genetically modify hPSCs utilize inefficient homology-directed repair (HDR) and require introduction of deleterious double-stranded breaks (DSBs).[22–28] Although prime editing-based approaches have been applied in the context of hPSCs, they require stable integration of an inducible prime editing cassette or employ iterative rounds of transfection to achieve modest levels of editing efficiency.[6,7] In addition, although reporter systems have been employed to increase prime editing levels, they have only been applied to a limited number of loci and have not been used in the context of clonal cell line generation.[29,30] Therefore, we wanted to establish that PINE-TREE could be used to enrich for prime editing-induced genomic modifications at various loci in hPSCs. First, we sought to establish the optimal prime editing transfection conditions

Figure 1. Establishment and validation of PINE-TREE-based enrichment of prime-edited cell in HEK293T cells

(A) Conversion of BFP to GFP allows detection of prime editing activity in cells. Targeting pEF1α-BFP with a PE results in a single-nucleotide C-to-T conversion, causing a change in the fluorescence emission spectra from BFP to GFP. (B) The mutant BFP in the pEF1α-BFP construct was engineered to convert to GFP upon a C-to-T conversion. The protospacer for the pegRNA, pegRNA(BG), is underlined in black, the protospacer adjacent motif (PAM) is underlined in red, and the 3′ extension sequence in the pegRNA is underlined in yellow. The nick site for the nicking sgRNA, nsgRNA(BG), is indicated by a black arrow. Targeting of the "CAC" codon in BFP with the pegRNA(BG)-nsgRNA(BG) will result in a C-to-T conversion to "TAC" and the associated amino acid change of histidine (blue) to tyrosine (green) at the 66th amino acid in BFP. (C and D) Fluorescence microscopy (C) and flow cytometry (D) analysis of HEK293T cells at various time points after transfection with pEF1α-BFP, pEF1α-PE2, and pegRNA(BG) (top panels, PE2 system) or pegRNA(BG)-nsgRNA(BG) (bottom panels, PE3 system). (E) Schematic for identification and enrichment of prime-edited cell populations using PINE-TREE. 72 h post transfection, flow cytometry is used to sort cell populations into reporter-positive and -negative cell fractions based on BFP and GFP expression levels. (F) Quantification of prime editing efficiency of single-nucleotide changes at various loci in unsorted (white bar), BFP−/GFP− (gray bar), BFP+/GFP− (blue bar), and BFP+/GFP+ (green bar) cell populations isolated using PINE-TREE-based enrichment strategies. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (G) Schematic for enrichment of prime-edited cell populations using reporter of transfection (RoT) approaches. (H) Quantification of relative prime editing in GFP+ cells isolated using RoT and BFP+/GFP+ cells isolated using PINE-TREE. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.



(legend on next page)

in hPSCs using the PINE-TREE reporter. Therefore, we co-transfected hPSCs with the PINE-TREE reporter (pEF1α-BFP), a PE (pEF1α-PE2), and a pegRNA(BG) using a range of total DNA amounts, plasmid ratios, and transfection volumes (Figure S3). Cells transfected with pEF1α-BFP or pEF1α-GFP only were used to establish the flow cytometry sorting gates to determine reporter-positive and -negative cell populations (Figure S4A). Flow cytometry analysis of BFP-to-GFP conversion revealed that the prime editing plasmid concentration and PE to pegRNA ratio could drastically affect prime editing efficiencies (Figure S3). After we established the optimal prime editing transfection conditions, we wanted to compare PE2 and PE3 editing efficiencies in hPSCs. Similar to our experiments with HEK293T cells, fluorescence microscopy and flow cytometry revealed a higher level of editing of the PINE-TREE reporter plasmid with the PE3 system than PE2 (Figures S4A and S4B). In addition, this analysis revealed that, compared with HEK293T cells, the relative percentage of cells displaying prime editing activity (i.e., GFP+) compared with transfected cells (i.e., BFP+) was significantly reduced, consistent with previous studies demonstrating that hPSCs are resistant to prime editing.[6,7] In addition, fluorescence imaging (Figure 2A) and flow cytometry (Figure 2B) confirmed no detectable BFP or GFP signal after 2 weeks of culture, demonstrating the transience of the PINE-TREE fluorescent reporters in hPSCs.

After establishing that PINE-TREE faithfully reports on active prime editing in hPSCs, we wanted to determine whether PINE-TREE-based methods could be used to enrich for cells with single-nucleotide edits at various loci in hPSCs. Therefore, we co-transfected hPSCs with pEF1α-BFP, pEF1α-PE2, and PE-pegRNA(DT)-nsgRNA(DT) vectors targeting single base-pair changes in *EMX1*, *FANCF*, *HEK3*, and *RNF2*. Consistent with editing in HEK293T cells, BFP/GFP double-positive cells displayed high levels of editing at the target loci that editing observed in unsorted, BFP−/GFP−, and BFP+/GFP− cell populations (Figure 2C). For comparison, we also employed a RoT-based strategy to enrich for editing at these same loci in hPSCs. Sanger sequencing was then performed on the targeted genomic sites in reporter-positive, reporter-negative, and unsorted cell populations enriched using PINE-TREE and RoT approaches. Overall, this analysis revealed that, across all targeted sites, BFP+/GFP+ cells enriched using PINE-TREE had a statistically significant higher level of editing compared with GFP+ cells isolated using traditional RoT-based enrichment strategies (Figure 2D). In fact, in the genomic sites (*EMX1*, *FANCF*, and *HEK3*), there was little to no detectable editing in the RoT reporter-positive cell populations. We also demonstrated that PINE-TREE could be used for enrichment of cells with desired single- and triple-nucleotide insertions and deletions at the *HEK3* locus. Specifically, this analysis demonstrated higher levels of these targeted insertions and deletions in BFP+/GFP+ cells compared with those observed in unsorted, BFP−/GFP−, and BFP+/GFP− cell populations. In fact, BFP+/GFP+ cells isolated using PINE-TREE had significantly higher levels of prime editing than GFP+ cells isolated using conventional RoT approaches (Figure 2F). Together, these data demonstrate that PINE-TREE can be used for highly efficient introduction of single base-pair substitutions as well as targeted insertions and deletions in hPSCs.

Highly efficient generation of clonal isogenic hPSC lines using PINE-TREE

Next, we wanted to compare the efficiency with which PINE-TREE and RoT-based methods could be employed to generate clonally edited isogenic hPSC lines at difficult-to-edit sites using conventional enrichment strategies. To this end, PINE-TREE and RoT approaches were used to target single-nucleotide changes at the *FANCF* and *HEK3* loci, which did not have extensive editing in unsorted cells. Reporter-positive cells were sorted into 96-well plates, expanded, and subjected to Sanger sequencing. Remarkably, analysis of 18 PINE-TREE-edited clones at the *FANCF* locus revealed that all clones had an edit at the target site and that greater than 50% of those edits were homozygous in nature (Figure 2G). By comparison, only 3 of the clones generated using an RoT approach had an edit at the target site, with only one of those clones displaying a homozygous modification (Figure 2G). Along similar lines, at the *HEK3* locus, only one RoT-generated clone displayed an edit, while over 50% of the clones isolated using PINE-TREE-based approaches displayed an edit at the target locus (Figure 2G). Importantly, we did not observe the presence of off-target modifications in any of the clones examined (Figure S5). We also employed PINE-TREE to edit the *APOE* locus, a genetic risk factor associated with increased likelihood of sporadic Alzheimer's disease (AD) onset.[31] Although PINE-TREE could be used to efficiently generate clonal cell populations modified at the *APOE* locus, RoT-based approaches did not result in generation of a single edited isogenic clone at the *APOE* target site (Figure 2H). Last, even though insertion or deletion (indel) formation is a caveat of all prime editing approaches, regardless of whether PINE-TREE-based approaches are employed, we analyzed repressive clones that had been edited at the *HEK3*, *FANCF*, and *APOE* loci. Importantly, this analysis revealed that indels, including pegRNA scaffold sequence insertions, were not observed in any of the clones at the target site (Figure S6).

Figure 2. PINE-TREE can be utilized for highly efficient prime editing of human pluripotent stem cells (hPSCs)

(A and B) Fluorescence microscopy (A) and flow cytometry analysis (B) of hPSCs cells at various time points after transfection with pEF1α-BFP, pEF1α-PE2, and pegRNA(BG) (top panels, PE2 system) or pegRNA(BG)-nsgRNA(BG) (bottom panels, PE3 system). (C) Quantification of prime editing efficiency of single-nucleotide changes at various genomic loci in unsorted (white bar), BFP−/GFP− (gray bar), BFP+/GFP− (blue bar), and BFP+/GFP+ (green bar) cell populations isolated using PINE-TREE-based enrichment strategies. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (D) Comparison of relative prime editing at single-nucleotide changes in GFP+ cells isolated using RoT and BFP+/GFP+ cells isolated using PINE-TREE. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (E) Quantification of prime editing efficiency of targeted small insertions and deletions at the *HEK3* loci in unsorted (white bar), BFP−/GFP− (gray bar), BFP+/GFP− (blue bar), and BFP+/GFP+ (green bar) cell populations isolated using PINE-TREE-based enrichment strategies. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (F) Quantification of relative prime editing of targeted small insertions and deletions in GFP+ cells isolated using RoT and BFP+/GFP+ cells using PINE-TREE. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (G) Analysis of clonal editing efficiency in hPSCs that were targeted at the *FANCF*, *HEK3*, and *APOE* loci using PINE-TREE- or RoT-based methods.

Because of the variability that has been reported between distinct hPSC lines,[32] we wanted to assess the robustness of PINE-TREE to efficiently generate clonal lines in additional hPSC lines (here referred to as hPSC lines 2 and 3). Therefore, we used PINE-TREE to target the *APOE* locus in additional hPSC lines. Analysis of single-cell clones revealed a similar level of efficiency at the *APOE* locus in hPSC line 2 (Figure S7). A chief advantage of using methods such as PINE-TREE that employ PEs as opposed to CRISPR nucleases is that PEs do not induce deleterious DSBs that result in insertion or deletion of DNA sequences, chromosomal translocations, apoptosis, and acquisition of potentially oncogenic mutations.[25–28] Nonetheless, we wanted to ensure that PINE-TREE did not result in generation of clonal cell populations with chromosomal aberrations or phenotypic abnormalities. Therefore, we performed karyotype analysis on two clones for hPSC lines 2 and 3 that had biallelic edits at the *APOE* locus (Figure S8A). This analysis revealed that all clones had a normal euploid karyotype. Additional characterization also revealed that PINE-TREE-edited clones displayed the characteristic hPSC morphology (Figure S8B), expression of the pluripotency markers OCT4, NANOG, and SOX2 (Figure S8C), and the ability to differentiate *in vitro* into cell types representative of the three main germ layers (Figure S8D).

Overall, these results demonstrate that PINE-TREE can not only be employed for efficient editing of hPSCs at various loci but can also enable generation of isogenic edited cell lines at genomic targets that are not easily modifiable with traditional RoT approaches.

DISCUSSION

In this study, we established that PINE-TREE is an effective method to efficiently enrich for prime-edited cell populations in multiple cell types and across multiple loci. Specifically, at several loci, PINE-TREE-based enrichment allowed a significant increase in editing efficiencies of single-nucleotide mutations, insertions, and deletions compared with a conventional RoT-based enrichment strategy. Additionally, we demonstrated that PINE-TREE could be used for highly efficient generation of clonal isogenic hPSCs, a cell type in which genomic modification, including use of primer editors, has been difficult to achieve.[6,33] In fact, to achieve editing efficiencies approaching those of PINE-TREE in hPSCs, other recently reported methods required stable integration of the PE into the genome or sequential, repeated transfection of editing components.[6,7] In addition, we demonstrate that PINE-TREE can allow highly efficient editing of hPSCs at loci that are difficult to edit using traditional RoT-based approaches. Importantly, characterization of clonal hPSCs generated using PINE-TREE demonstrated that these cells display a normal euploid karyotype and phenotypic characteristics typical of hPSCs.

Despite these features of PINE-TREE, it is important to note that several caveats and limitations exist with all prime-editing approaches. In particular, the main limitation that exists with many genome engineering approaches is the potential for off-target editing and formation of undesired indels.[34] Even though PEs have shown lower off-target editing efficiency and indel formation than CRISPR nucleases,[5] we wanted to ensure that PINE-TREE could results in clonal populations free from off-target modifications and indels. Indeed, analysis of clonal cell populations edited at multiple independent loci using PINE-TREE enrichment strategies did not reveal any editing at potential off-target sites or undesired indel formation. Along similar lines, this analysis did not demonstrate any pegRNA scaffold sequence incorporation at on- or off-target sites. As it relates to analysis of indel formation in bulk-sorted populations enriched using PINE-TREE, it is possible that Sanger sequencing is not sensitive enough to accurately detect low-frequency indels. Thus, moving forward, we recommend that next-generation sequencing approaches might need to be employed to quantify indel prevalence. In addition, it should also be noted that the PE3 system we employed in the context of PINE-TREE increases the frequency of undesired indels at the target site compared with PE2.[5] In the future, we envision that the recently developed PE4 and PE5 prime editing systems, which show improved edit-to-indel ratios compared with PE2 and PE3, can be used in conjunction with PINE-TREE.[35]

Another potential limitation of methods such as PINE-TREE that employ plasmid-based editing is potential integration of the plasmid DNA at the pegRNA cut site or randomly throughout the genome. However, because PEs do no introduce DSBs, the likelihood of integration of these plasmids into the genome would be exceedingly low. In this regard, we did not observe any integration of these PINE-TREE-associated plasmids at the target or off-target sites in our bulk-sorted and clonal cell populations. In addition, we demonstrate, in HEK293T cells and hPSCs, that the fluorescent signal is transient, suggesting that the PINE-TREE reporter plasmid (pEF1α-BFP) in particular does not integrate into the genome. In the future, low-frequency insertions of reporter plasmid DNA sequences at target sites can be detected using PCR-based methods followed by next-generation sequencing of the PCR products. As it relates to random integration of circular plasmid DNA, previous studies have shown that this is a rare occurrence, especially in hPSCs (<0.01%).[36–38] In the future, these low-frequency insertions at off-target sites or randomly throughout the genome will necessitate use of whole-genome sequencing.

Previously, PEs have been used in various organisms (e.g., animal, plant, prokaryotic), cell lines (e.g., immortalized, primary) and animals (e.g., mouse, zebrafish) in numerous applications, such as generating animal models, *in vivo* editing, dissecting genotype-to-phenotype relationships in disease-related pathological variants, and dissecting fundamental signaling pathways.[39–44] To that end, we envision that PINE-TREE can be utilized in many of these applications. First, we envision that the fluorescent reporter system associated with PINE-TREE could be utilized to rapidly optimize numerous parameters that influence prime editing efficiency, including PE plasmid concentration, PE-to-pegRNA ratios, and pegRNA design. In fact, as we show in this study, we employ this system to optimal prime editing transfection conditions. In the future, a similar PINE-TREE-driven approach can be used to optimize prime editing conditions or evaluate next-generation PEs.[45] Second, we anticipate that the same flow cytometry-based enrichment strategies

that were employed in this study with HEK293T cells and hPSCs could be employed with additional cell populations. In particular, CRISPR-based HDR is not achievable in post-mitotic cells; thus, PINE-TREE could be employed to enrich for editing in somatic or primary cells types in which CRISPR nuclease approaches cannot be utilized. Third, CRISPR-based methods suffer numerous technical limitations related to engineering of animal models of pathological disease variants, in particular those associated with single-nucleotide variation.[40] Although PEs have been utilized to generate such animal models, such methods suffer from a similar low efficiency that plagues *in vitro* applications. To that end, PINE-TREE could be used to increase the efficiency with which PEs can be used to generate animal models of disease. Fourth, we speculate that PINE-TREE can be utilized to modulate target gene expression through rapid generation of gene knockout lines, allowing dissection of fundamental signaling pathways. To that end, we have previously described use of base editors in the context of TREE-based strategies to generate knockout lines without the need for DSBs through introduction of premature stop codons or mutation of the start codons.[46] However, the number of genes that could be targeted by these approaches was limited by PAM accessibility, bystander editing, and other parameters that influence all base editing approaches. Therefore, PINE-TREE based strategies could be employed to generate cell populations with targeted gene knockouts at any loci. Finally, *in vivo* prime editing has great potential for treatment of inherited diseases through gene therapy.[44] In this regard, PINE-TREE can allow the real-time tracking of edited cell populations and preclinical assessment of potential *in vivo* editing strategies.

In this study, we established that PINE-TREE is an effective method to efficiently enrich for prime-edited cell populations in multiple cell types and across multiple loci. Specifically, at several loci, PINE-TREE-based enrichment allowed a significant increase in editing efficiency of single-nucleotide mutations, insertions, and deletions compared with a conventional RoT-based enrichment strategy. Additionally, we demonstrated that PINE-TREE could be used for highly efficient generation of clonal isogenic hPSCs, a cell type in which genomic modification, including use of primer editors, has been difficult to achieve.[6,33] In fact, to achieve editing efficiencies approaching those of PINE-TREE in hPSCs, other recently reported methods required stable integration of the PE into the genome or sequential, repeated transfection of editing components.[6,7] In addition, we demonstrate that PINE-TREE can allow highly efficient editing of hPSCs at loci that are difficult to edit using traditional RoT-based approaches. Importantly, characterization of clonal hPSCs generated using PINE-TREE demonstrated that these cells display a normal euploid karyotype and phenotypic characteristics typical of hPSCs.

Overall, PINE-TREE is an easily adoptable method that will significantly enhance use of prime editing approaches. In particular, because of the high editing efficiency, PINE-TREE does not require laborious screening of large numbers of clones with the targeted mutation. As we show, clonal lines can be identified, expanded, and characterized over the course of a few weeks. In addition, the high editing efficiency offered by PINE-TREE allows biallelic modification of hPSCs without the need for multiple transfections or re-targeting. Last, PINE-TREE utilizes readily available chemical transfection reagents and does not require complex cloning of large homology-based constructs or use of special cell transfection technologies. Finally, we employed our previously published design tool[21] to create pegRNA and sgRNA vectors to allow simple cloning of new target sites through use of restriction enzyme digestion and ligation of target oligonucleotides. In summary, we anticipate that these features of PINE-TREE will allow facile and broad implementation by biomedical researchers.

MATERIALS AND METHODS

Plasmid construction

Unless otherwise noted, all molecular cloning polymerase chain reactions (PCRs) were performed using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) using the manufacturer's recommended protocols. All restriction enzyme digestions were performed according to the manufacturer's instructions with high-fidelity enzymes (New England Biolabs). Ligation reactions were performed with T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions. PCR primers and oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). All PCR products and intermediate plasmid products were confirmed via Sanger sequencing (Genewiz).

For construction of PE-pegRNA(BG)-nsgRNA(BG), pegRNA oligos were synthesized as pairs of oligonucleotides.[21] Subsequently, 5′ phosphates were added to each oligonucleotide pair by incubating 1 μg oligonucleotide in 50-μL reactions containing 1× T4 DNA ligase buffer (New England Biolabs) and 10 units of T4 polynucleotide kinase (New England Biolabs) at 37°C overnight. Oligonucleotides were then duplexed by heating the kinase reactions to 90°C on an aluminum heating block for 5 min, followed by slowly returning the reaction to room temperature over 1 h. Following duplexing, pegRNA(BG) was cloned into a pHSG0-1C3 vector containing a U6 promoter[21] (Addgene, 164423). Next, pegRNA(BG) and the U6 promoter were PCR amplified with primers adding EcoRI/XbaI. PCR products were then digested with the respective restriction enzymes and ligated into the EcoRI/XbaI-digested pUC19 vector (Addgene, 50005). Following ligation and plasmid purification, this entire process was then repeated for nsgRNA(BG). In pHSG0-1C3, nsgRNA(BG) and the U6 promoter were PCR amplified with primers adding XbaI/PstI. Purified PCR products were then digested with XbaI and PstI restriction enzymes and ligated into the XbaI/PstI digested PE-pegRNA(BG) vector. The resultant vector contained pegRNA(BG) and nsgRNA(BG) expression cassettes was titled PE-pegRNA(BG)-nsgRNA(BG). To add pegRNA and nsgRNA expression cassettes, pairs of pegRNA and nsgRNA were PCR amplified with primers that added HindIII/SapI or SapI/HindIII restriction enzyme digestion sites. These products were then digested with HindIII/SapI and ligated into HindIII-digested and dephosphorylated PE-pegRNA(BG)-nsgRNA(BG).

All pegRNA and nsgRNAs were synthesized as pairs of oligonucleotides as listed in Table S1.

HEK293T cell culture

HEK293T (ATCC) cells were cultured on plates coated with poly-L-ornithine (4 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) in the following medium: 1× high-glucose DMEM, 10% (v/v) fetal bovine serum, and 1% (v/v) L-glutamine penicillin/streptomycin. The culture medium was changed every other day, and cells were passaged with Accutase every 4 days.

hPSC culture

HPSCs[47] (Table S2) were maintained in mTeSR™ Plus medium (STEMCELL Technologies, catalog number 100-0276) on plates coated with feeder-free Cultrex basement membrane extract (R&D Systems). Passaging was performed every 3 days using Accutase (Life Technologies) in mTeSR™ Plus medium supplemented with 5 μM Y-27632 (Tocris Bioscience).

HEK293T cell transfection

For PINE-TREE-based transfection, HEK293T cells were transfected in 24-well tissue culture plates 24 h after seeding, when cells were around 40% confluent. 300 ng PE (pEF1α-PE2), 100 ng pegRNA expression plasmid (PE-pegRNA(DT)-nsgRNA(DT)), and 100 ng pEF1α-BFP were transfected per well using 1 μL Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific) and 0.75 μL P3000 reagent (Thermo Fisher Scientific). For RoT transfection, 300 ng PE (pEF1α-PE2), 100 ng pegRNA expression plasmid (PE-pegRNA(DT)-nsgRNA(DT)), and 10 ng pEF1α-GFP were transfected per well using 1 μL Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) and 0.75 μL P3000 reagent (Thermo Fisher Scientific). The medium was changed 24 h post-transfection. Cells were dissociated using Accutase 72 h post transfection and passed through a 0.40-μm filter before sorting.

hPSC transfection and clonal isolation

HPSCs were passaged onto Cultrex BME-coated 12-well plates with mTeSR™ Plus supplemented with 5 μM Y-27632. The medium was changed, and transfection was performed 24 h after passage at 60% confluency. For PINE-TREE transfection, 750 ng PE (pEF1α-PE2), 125 ng pegRNA expression plasmid (PE-pegRNA(DT)-nsgRNA (DT)), and 125 ng pEF1α-BFP were transfected per well using 5 μL Lipofectamine™ Stem Transfection Reagent (Life Technologies). For RoT transfection, 750 ng PE (pEF1α-PE2), 125 ng pegRNA expression plasmid (PE-pegRNA(DT)-nsgRNA (DT)), and 125 ng pEF1α-GFP were transfected per well using 5 μL Lipofectamine™ Stem Transfection Reagent. The medium was changed 24 h post transfection. Cells were dissociated using Accutase 48 h post transfection and passed through a 0.40-μm filter. Single GFP+ hPSCs were sorted by fluorescence-activated cell sorting (FACS) into 96-well Cultrex-coated plates in mTeSR™ Plus supplemented with CloneR™ (STEMCELL Technologies), and plates were immediately centrifuged at 200 × *g* for 3 min and incubated at 37°C. The medium was changed 24 h post sorting with fresh mTeSR Plus supplemented with CloneR™. 96 h post sorting, the medium was changed to mTeSR™ Plus without supplement, and clonal hPSC colonies were expanded with fresh medium changes every 2 days until ready for subculture.

Tri-lineage differentiation of hPSCs

Cells were dissociated with Accutase and plated on ultra-low attachment plates in mTeSR™ Plus medium to allow formation of embryoid bodies. The following day, the medium was changed to differentiation medium (DM; DMEM/F12, 20% fetal bovine serum [FBS], 1% penicillin/streptomycin [Pen/Strep]). After 5 days in suspension, embryoid bodies (EBs) were plated on Matrigel-coated plates and continued to be cultured in DM. After 21 days in DM, cells were fixed for immunofluorescence analysis of germ layer markers.

Genotyping and sequence analysis

Bulk-sorted cells and clones were amplified with the primers listed in Table S3 to determine genotype following prime editing. Genomic DNA was prepared from expanded clones using the DNeasy Kit (QIAGEN), and PCR products were generated with Phusion high-fidelity polymerase (New England Biolabs). Amplicons were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions prior to Sanger sequencing (Genewiz). For bulk-sorted cells, cells were directly added to a 50-μL master mix consisting of 1× Phire Hot Start II DNA polymerase (Thermo Fisher Scientific), 1 μM forward primer, and 1 μM reverse primer. PCR was performed using the conditions specified in Tables S4 and S5. All product sizes were confirmed on a 1% agarose gel prior to Sanger sequencing.

Karyotype analysis

For each clonal line, karyotype analysis was performed by Cell Line Genetics on 20 metaphase cells using standard procedures for G-banding.

Quantification of prime editing

Sanger sequencing of PCRs from genomic DNA from either sorted HEK293T cells or hPSCs was analyzed using EditR.[48] For reverse sequencing reactions, the "sgRNA sequence" was the same as the 3′ extension sequence. For forward sequencing reads, the "sgRNA sequence" was the reverse complement of the 3′ extension sequence. The 5′ and 3′ start are the corresponding nucleotide numbers (starting at 1 for the first nucleotide of the sequencing read) 100 bp upstream and downstream of the protospacer, respectively.

Immunofluorescence

Cultures were washed twice with PBS prior to fixation. Cultures were fixed for 15 min at room temperature (RT) with BD Cytofix fixation buffer (BD Biosciences). The cultures were washed twice with PBS and permeabilized with BD Phosflow Perm Buffer III (BD Biosciences) for 30 min at 4°C. Next, cultures were washed twice with PBS. Primary antibodies were incubated overnight at 4°C and washed twice with PBS at RT. Secondary antibodies were incubated at RT for 1 h. Nucleic acids were stained for DNA with Hoechst 33342 (2 μg/mL, Thermo Fisher Scientific) for 10 min at RT and washed twice with PBS. Antibodies and concentrations used are listed in Table S6.

Fluorescence microscopy

All immunofluorescent imaging was performed on a Nikon Ti-Eclipse inverted microscope with a light-emitting diode-based

Lumencor SOLA SE Light Engine using a Semrock band-pass filter. GFP and BFP were visualized on a Nikon Ti2 inverted microscope. GFP was visualized with excitation at 472 nm and emission at 520 nm. BFP was visualized with the DAPI fluorescence channel with excitation at 395 nm and emission at 460 nm.

Flow cytometry

Cells were dissociated with Accutase for 5 min at 37°C, triturated, and passed through a 40-μm cell strainer. Cells were resuspended in PBS at a maximum concentration of 5×10^6 cells per 100 μL. Flow cytometry analysis was performed on an Attune NxT (Thermo Fisher Scientific). Flow cytometry files were analyzed using FlowJo (FlowJo, Ashland, OR, USA). Cells transfected with the pEF1α-BFP (125 ng) or pEF1α-GFP (125 ng) plasmid only were used to establish sorting gates.

Off-target analysis

Analysis was performed for the top five off-target loci for *HEK3* and *FANCF* spacer sequences predicted *in silico* via CCTop using default parameters for *Streptococcus pyogenes* Cas9 against human genome reference sequence hg38.[49] Analysis of prime editing at these off-target sites was performed in a manner similar to that at on-target sites. The PCR primers used to analyze these off-target sites are presented in Table S6.

Statistical analysis

Unless otherwise noted, all data are displayed as mean ± standard deviation (SD). For all pairwise comparisons, Student's t test was used.

DATA AND CODE AVAILABILITY

All reasonable requests for data can be made to the corresponding author at david.brafman@asu.edu.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.07.007.

ACKNOWLEDGMENTS

Funding for this work was provided by the National Institutes of Health (R01GM121698 to D.A.B., R21AG056706 to D.A.B., R21AG079279 to D.A.B., R21AG075612 to D.A.B., R01GM106081 to X.W., and R01EB031893 to X.W.) and the Alzheimer's Association. We would like to thank the ASU Flow Cytometry core for assistance with flow cytometry-related experiments.

AUTHOR CONTRIBUTIONS

C.F., W.W.K., S.J.T., K.S.-B., X.W., and D.A.B. designed the experiments and wrote the manuscript. X.W. and D.A.B. supervised the research. C.F., W.W.K., B.G., B.W., and G.S. performed the experiments and analyzed the data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Rees, H.A., and Liu, D.R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. Nat. Rev. Genet. *19*, 770–788. https://doi.org/10.1038/s41576-018-0059-1.

2. Moon, S.B., Kim, D.Y., Ko, J.H., and Kim, Y.S. (2019). Recent advances in the CRISPR genome editing tool set. Exp. Mol. Med. *51*, 1–11. https://doi.org/10.1038/s12276-019-0339-7.

3. Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I., and Liu, D.R. (2017). Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature *551*, 464–471. https://doi.org/10.1038/nature24644.

4. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature *533*, 420–424. https://doi.org/10.1038/nature17946.

5. Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M., Chen, P.J., Wilson, C., Newby, G.A., Raguram, A., and Liu, D.R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. Nature *576*, 149–157. https://doi.org/10.1038/s41586-019-1711-4.

6. Habib, O., Habib, G., Hwang, G.H., and Bae, S. (2022). Comprehensive analysis of prime editing outcomes in human embryonic stem cells. Nucleic Acids Res. *50*, 1187–1197. https://doi.org/10.1093/nar/gkab1295.

7. Li, H., Busquets, O., Verma, Y., Syed, K.M., Kutnowski, N., Pangilinan, G.R., Gilbert, L.A., Bateup, H.S., Rio, D.C., Hockemeyer, D., and Soldner, F. (2022). Highly efficient generation of isogenic pluripotent stem cell models using prime editing. Elife *11*, e79208. https://doi.org/10.7554/eLife.79208.

8. Brookhouser, N., Tekel, S.J., Standage-Beier, K., Nguyen, T., Schwarz, G., Wang, X., and Brafman, D.A. (2020). BIG-TREE: Base-Edited Isogenic hPSC Line Generation Using a Transient Reporter for Editing Enrichment. Stem Cell Rep. *14*, 184–191. https://doi.org/10.1016/j.stemcr.2019.12.013.

9. Brookhouser, N., Nguyen, T., Tekel, S.J., Standage-Beier, K., Wang, X., and Brafman, D.A. (2020). A Cas9-mediated adenosine transient reporter enables enrichment of ABE-targeted cells. BMC Biol. *18*, 193. https://doi.org/10.1186/s12915-020-00929-7.

10. Tekel, S.J., Standage-Beier, K., Brookhouser, N., Wang, X., and Brafman, D.A. (2021). Cytosine and Adenosine Base Editing in Human Pluripotent Stem Cells (hPSCs) Using Transient Reporters for Editing Enrichment (TREE). Nat. Protoc. *47*, In Press.

11. Standage-Beier, K., Tekel, S.J., Brookhouser, N., Schwarz, G., Nguyen, T., Wang, X., and Brafman, D.A. (2019). A transient reporter for editing enrichment (TREE) in human cells. Nucleic Acids Res. *47*, e120. https://doi.org/10.1093/nar/gkz713.

12. Tong, Y., Jørgensen, T.S., Whitford, C.M., Weber, T., and Lee, S.Y. (2021). A versatile genetic engineering toolkit for E. coli based on CRISPR-prime editing. Nat. Commun. *12*, 5206. https://doi.org/10.1038/s41467-021-25541-3.

13. Lu, C., Kuang, J., Shao, T., Xie, S., Li, M., Zhu, L., and Zhu, L. (2022). Prime Editing: An All-Rounder for Genome Editing. Int. J. Mol. Sci. *23*, 9862. https://doi.org/10.3390/ijms23179862.

14. Doman, J.L., Sousa, A.A., Randolph, P.B., Chen, P.J., and Liu, D.R. (2022). Designing and executing prime editing experiments in mammalian cells. Nat. Protoc. *17*, 2431–2468. https://doi.org/10.1038/s41596-022-00724-4.

15. Erwood, S., Bily, T.M.I., Lequyer, J., Yan, J., Gulati, N., Brewer, R.A., Zhou, L., Pelletier, L., Ivakine, E.A., and Cohn, R.D. (2022). Saturation variant interpretation using CRISPR prime editing. Nat. Biotechnol. *40*, 885–895. https://doi.org/10.1038/s41587-021-01201-1.

16. Choi, J., Chen, W., Minkina, A., Chardon, F.M., Suiter, C.C., Regalado, S.G., Domcke, S., Hamazaki, N., Lee, C., Martin, B., et al. (2022). A time-resolved, multi-symbol molecular recorder via sequential genome editing. Nature *608*, 98–107. https://doi.org/10.1038/s41586-022-04922-8.

17. Kim, H.K., Yu, G., Park, J., Min, S., Lee, S., Yoon, S., and Kim, H.H. (2021). Predicting the efficiency of prime editing guide RNAs in human cells. Nat. Biotechnol. *39*, 198–206. https://doi.org/10.1038/s41587-020-0677-y.

18. Chen, P.J., Hussmann, J.A., Yan, J., Knipping, F., Ravisankar, P., Chen, P.F., Chen, C., Nelson, J.W., Newby, G.A., Sahin, M., et al. (2021). Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. Cell *184*, 5635–5652.e29. https://doi.org/10.1016/j.cell.2021.09.018.

19. Nelson, J.W., Randolph, P.B., Shen, S.P., Everette, K.A., Chen, P.J., Anzalone, A.V., An, M., Newby, G.A., Chen, J.C., Hsu, A., and Liu, D.R. (2022). Engineered pegRNAs improve prime editing efficiency. Nat. Biotechnol. *40*, 402–410. https://doi.org/10.1038/s41587-021-01039-7.

20. Tao, J., Wang, Q., Mendez-Dorantes, C., Burns, K.H., and Chiarle, R. (2022). Frequency and mechanisms of LINE-1 retrotransposon insertions at CRISPR/Cas9 sites. Nat. Commun. *13*, 3685. https://doi.org/10.1038/s41467-022-31322-3.

21. Standage-Beier, K., Tekel, S.J., Brafman, D.A., and Wang, X. (2021). Prime Editing Guide RNA Design Automation Using PINE-CONE. ACS Synth. Biol. *10*, 422–427. https://doi.org/10.1021/acssynbio.0c00445.

22. Paquet, D., Kwart, D., Chen, A., Sproul, A., Jacob, S., Teo, S., Olsen, K.M., Gregg, A., Noggle, S., and Tessier-Lavigne, M. (2016). Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature *533*, 125–129. https://doi.org/10.1038/nature17664.

23. Kwart, D., Paquet, D., Teo, S., and Tessier-Lavigne, M. (2017). Precise and efficient scarless genome editing in stem cells using CORRECT. Nat. Protoc. *12*, 329–354. https://doi.org/10.1038/nprot.2016.171.

24. Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S., and Qi, L.S. (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell *16*, 142–147. https://doi.org/10.1016/j.stem.2015.01.003.

25. Chapman, J.R., Taylor, M.R.G., and Boulton, S.J. (2012). Playing the end game: DNA double-strand break repair pathway choice. Mol. Cell *47*, 497–510. https://doi.org/10.1016/j.molcel.2012.07.029.

26. Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., and Taipale, J. (2018). CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. Nat. Med. *24*, 927–930. https://doi.org/10.1038/s41591-018-0049-z.

27. Ihry, R.J., Salick, M.R., Ho, D.J., Sondey, M., Kommineni, S., Paula, S., Raymond, J., Henry, B., Frias, E., Wang, Q., et al. (2019). Genome-Scale CRISPR Screens Identify Human Pluripotency-Specific Genes. Cell Rep. *27*, 616–630.e6. https://doi.org/10.1016/j.celrep.2019.03.043.

28. Kosicki, M., Tomberg, K., and Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat. Biotechnol. *36*, 765–771. https://doi.org/10.1038/nbt.4192.

29. Simon, D.A., Tálas, A., Kulcsár, P.I., Biczók, Z., Krausz, S.L., Várady, G., and Welker, E. (2022). PEAR, a flexible fluorescent reporter for the identification and enrichment of successfully prime edited cells. Elife *11*, e69504. https://doi.org/10.7554/eLife.69504.

30. Schene, I.F., Joore, I.P., Baijens, J.H.L., Stevelink, R., Kok, G., Shehata, S., Ilcken, E.F., Nieuwenhuis, E.C.M., Bolhuis, D.P., van Rees, R.C.M., et al. (2022). Mutation-specific reporter for optimization and enrichment of prime editing. Nat. Commun. *13*, 1028. https://doi.org/10.1038/s41467-022-28656-3.

31. Raman, S., Brookhouser, N., and Brafman, D.A. (2020). Using Human Induced Pluripotent Stem Cells (hiPSCs) to Investigate the Mechanisms by Which Apolipoprotein E (APOE) Contributes to Alzheimer's Disease (AD) Risk. Neurobiol. Dis.104788 https://doi.org/10.1016/j.nbd.2020.104788.

32. Ortmann, D., and Vallier, L. (2017). Variability of human pluripotent stem cell lines. Curr. Opin. Genet. Dev. *46*, 179–185. https://doi.org/10.1016/j.gde.2017.07.004.

33. Hockemeyer, D., and Jaenisch, R. (2016). Induced Pluripotent Stem Cells Meet Genome Editing. Cell Stem Cell *18*, 573–586. https://doi.org/10.1016/j.stem.2016.04.013.

34. Tsai, S.Q., Zheng, Z., Nguyen, N.T., Liebers, M., Topkar, V.V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A.J., Le, L.P., et al. (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat. Biotechnol. *33*, 187–197. https://doi.org/10.1038/nbt.3117.

35. Ferreira da Silva, J., Oliveira, G.P., Arasa-Verge, E.A., Kagiou, C., Moretton, A., Timelthaler, G., Jiricny, J., and Loizou, J.I. (2022). Prime editing efficiency and fidelity are enhanced in the absence of mismatch repair. Nat. Commun. *13*, 760. https://doi.org/10.1038/s41467-022-28442-1.

36. Eiges, R., Schuldiner, M., Drukker, M., Yanuka, O., Itskovitz-Eldor, J., and Benvenisty, N. (2001). Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Curr. Biol. *11*, 514–518. https://doi.org/10.1016/s0960-9822(01)00144-0.

37. Moore, J.C., Atze, K., Yeung, P.L., Toro-Ramos, A.J., Camarillo, C., Thompson, K., Ricupero, C.L., Brenneman, M.A., Cohen, R.I., and Hart, R.P. (2010). Efficient, high-throughput transfection of human embryonic stem cells. Stem Cell Res. Ther. *1*, 23. https://doi.org/10.1186/scrt23.

38. Haridhasapavalan, K.K., Borgohain, M.P., Dey, C., Saha, B., Narayan, G., Kumar, S., and Thummer, R.P. (2019). An insight into non-integrative gene delivery approaches to generate transgene-free induced pluripotent stem cells. Gene *686*, 146–159. https://doi.org/10.1016/j.gene.2018.11.069.

39. Schene, I.F., Joore, I.P., Oka, R., Mokry, M., van Vugt, A.H.M., van Boxtel, R., van der Doef, H.P.J., van der Laan, L.J.W., Verstegen, M.M.A., van Hasselt, P.M., et al. (2020). Prime editing for functional repair in patient-derived disease models. Nat. Commun. *11*, 5352. https://doi.org/10.1038/s41467-020-19136-7.

40. Caso, F., and Davies, B. (2022). Base editing and prime editing in laboratory animals. Lab. Anim. *56*, 35–49. https://doi.org/10.1177/00236772219938 95.

41. Happi Mbakam, C., Rousseau, J., Tremblay, G., Yameogo, P., and Tremblay, J.P. (2022). Prime Editing Permits the Introduction of Specific Mutations in the Gene Responsible for Duchenne Muscular Dystrophy. Int. J. Mol. Sci. *23*, 6160. https://doi.org/10.3390/ijms23116160.

42. Hillary, V.E., and Ceasar, S.A. (2022). Prime editing in plants and mammalian cells: Mechanism, achievements, limitations, and future prospects. Bioessays *44*, e2200032. https://doi.org/10.1002/bies.202200032.

43. Ahmad, N., Awan, M.J.A., and Mansoor, S. (2023). Improving editing efficiency of prime editor in plants. Trends Plant Sci. *28*, 1–3. https://doi.org/10.1016/j.tplants.2022.09.001.

44. Zhao, Z., Shang, P., Mohanraju, P., and Geijsen, N. (2023). Prime editing: advances and therapeutic applications. Trends Biotechnol. *41*, 1000–1012. https://doi.org/10.1016/j.tibtech.2023.03.004.

45. Mathis, N., Allam, A., Kissling, L., Marquart, K.F., Schmidheini, L., Solari, C., Balázs, Z., Krauthammer, M., and Schwank, G. (2023). Predicting prime editing efficiency and product purity by deep learning. Nat. Biotechnol. https://doi.org/10.1038/s41587-022-01613-7.

46. Tekel, S.J., Brookhouser, N., Standage-Beier, K., Wang, X., and Brafman, D.A. (2021). Cytosine and adenosine base editing in human pluripotent stem cells using transient reporters for editing enrichment. Nat. Protoc. *16*, 3596–3624. https://doi.org/10.1038/s41596-021-00552-y.

47. Brookhouser, N., Raman, S., Frisch, C., Srinivasan, G., and Brafman, D.A. (2021). APOE2 mitigates disease-related phenotypes in an isogenic hiPSC-based model of Alzheimer's disease. Mol. Psychiatr. *26*, 5715–5732. https://doi.org/10.1038/s41380-021-01076-3.

48. Kluesner, M.G., Nedveck, D.A., Lahr, W.S., Garbe, J.R., Abrahante, J.E., Webber, B.R., and Moriarity, B.S. (2018). EditR: A Method to Quantify Base Editing from Sanger Sequencing. CRISPR J *1*, 239–250. https://doi.org/10.1089/crispr.2018.0014.

49. Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., and Mateo, J.L. (2015). CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool. PLoS One *10*, e0124633. https://doi.org/10.1371/journal.pone.0124633.