Harnessing DSB repair to promote efficient homology-dependent and -independent prime editing

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Prime editing recently emerged as a next-generation approach for precise genome editing. Here we exploit DNA double-strand break (DSB) repair to develop two strategies that install precise genomic insertions using an SpCas9 nuclease-based prime editor (PEn). We first demonstrate that PEn coupled to a regular prime editing guide RNA (pegRNA) efficiently promotes short genomic insertions through a homology-dependent DSB repair mechanism. While PEn editing leads to increased levels of by-products, it can rescue pegRNAs that perform poorly with a nickase-based prime editor. We also present a small molecule approach that yields increased product purity of PEn editing. Next, we develop a homology-independent PEn editing strategy, which installs genomic insertions at DSBs through the non-homologous end joining pathway (NHEJ). Lastly, we show that PEn-mediated insertions at DSBs prevent Cas9-induced large chromosomal deletions and provide evidence that continuous Cas9-mediated cutting is one of the mechanisms by which Cas9-induced large deletions arise. Altogether, this work expands the current prime editing toolbox by leveraging distinct DNA repair mechanisms including NHEJ, which represents the primary pathway of DSB repair in mammalian cells.
Proper utilization of cellular DNA repair mechanisms is instrumental to any successful genome editing strategy. The activity of different DNA repair pathways is highly dependent on tissue and cell type, chromatin context, and the DNA sequence of the target locus1–4.

Targeted DNA insertions represent a particularly challenging type of precise genome modification but have a considerable therapeutic potential. About 25% of ClinVar human pathogenic variants are deletions, the majority of which are <25 bp in length and thus potentially actionable by prime editing5. A common approach to introduce DNA insertions is to induce a targeted DNA double-strand break (DSB) using a site-specific nuclease combined with the delivery of a donor DNA repair template to stimulate homology-directed repair (HDR) at the targeted locus. A major disadvantage of this strategy is the limited activity of homologous recombination, which is restricted to S/G2 phases of the cell cycle and is generally absent in postmitotic cells6.

Unlike homologous recombination, DNA end joining repair mechanisms such as non-homologous end joining (NHEJ) or alternative end joining (a-EJ) pathways remain active throughout the cell cycle and act as the major pathways of DSB repair in mammalian cells7–9. While being typically considered error prone, NHEJ can repair DSBs with high fidelity10,11. In contrast, the homology-dependent a-EJ pathway leads to deletions, which are highly predictable12,13. The respective precision and predictability of NHEJ and a-EJ have been successfully exploited for precise genome modifications including DNA insertions14–17. Harnessing DNA end joining pathways represents a valuable addition to the prime editing toolbox, because most adult tissues are comprised of postmitotic cells unable to perform homologous recombination3,18.

The recently developed CRISPR-based prime editing can install a wide spectrum of genomic modifications including deletions, substitutions, and insertions without the need of a separate DNA template and without introducing DSBs5, therefore offering a major advantage over existing genome editing methods. The PE2 prime editor combines Cas9 (H840A) nickase with an engineered reverse transcriptase (RT) to install an edit encoded directly in the prime editing gRNA (pegRNA). The cascade of events leading to a successful prime editing outcome is comprised of (1) Cas9-mediated nicking of the target site, (2) hybridization of the pegRNA-encoded primer binding site (PBS) to the 3’ end of the nick, (3) pegRNA-templated extension of the primed 3’ end of the nick by RT resulting in a “flap” containing the desired edit, and (4) hybridization and ligation of the flap with the targeted locus. Inhibition of mismatch repair was recently shown to enhance prime editing efficiency19, but DNA repair mechanisms responsible for the upstream steps of successful incorporation of the 3’ flap remain to be described in detail and might not be universally available in different cellular and genomic contexts, potentially limiting the scope and efficiency of the nickase-based PE2s. Recent reports suggest a possible dependency of PE2 editing on cell cycle progression20,21. Thus, a prime editing strategy harnessing a wider spectrum of DNA repair pathways would be a valuable addition to the prime editing toolbox.

Here we introduce Prime Editor nuclease (PEn), which combines RT and the wild-type SpCas9 nuclease and show that prime editing can be performed at DSBs by utilizing DNA end joining repair pathways. We present two PEn strategies to robustly install small insertions via distinct DNA repair mechanisms. The first strategy utilizes regular pegRNAs to promote small DNA insertions by a homology-dependent DSB repair mechanism. This strategy worked robustly across different genomic loci as well as with pegRNAs displaying inefficient editing when combined with PE2. The second strategy relies on a modified sgRNA design to install small insertions through precise NHEJ. We also present a small molecule approach to decrease unintended by-products of PEn editing. Finally, we show that unlike editing with Cas9 alone, PEn does not induce large unintended on-target deletions, likely because PEn-mediated insertions at DSBs prevent NHEJ-mediated restoration of the wild-type sequence at the target locus. This suggests that the futile cycle of nuclease-mediated cut and NHEJ-mediated precise repair may be a possible cause of DSBs genotoxicity associated with Cas9 treatment.

Results

SpCas9 nuclease-based prime editing. To test if a Cas9 nuclease-based prime editor can install small insertions at DSBs through a DNA end joining repair mechanism, we reverted the Cas9(H840A)-based PE2 into wtCas9–PE, designated here as PEn. We have constructed pegRNAs encoding small insertions of various sizes (6–18 bp) (Supplementary Data 1) against 10 genomic target sites and co-transfected HEK293T cells with a pegRNA and either PEn or PE2. NGS analysis of the editing outcomes at the targeted sites revealed successful intended insertions with varying frequencies and product purities for both PEn and PE2 (Fig. 1a). We classified the edited alleles into three categories: (1) all prime edits, representing any type of RT-templated insertions, (2) precise prime edits, that represent RT-templated insertions of intended size, and (3) other indels. As expected, PE2 editing resulted in high product purity, but also showed large site-to-site variability of insertion efficiency. PEn editing resulted in variable rates of precise prime edits but in general higher as compared to PE2. At some tested sites (HEK3, DPM2, AAVS1, EGFR) PE2 achieved similar or higher editing efficiency compared to PEn and clearly outperformed PE2 in terms of precise editing purity. However, PEn installed insertions efficiently even with pegRNAs that were suboptimal for PE2 in our hands (Fig. 1a, PCSK9, FANCF, TRBC, PDCD1, CTLA4). We observed a similar trend in HeLa and HCT116 cells, where we tested PE2-optimal (AAVS1) as well as suboptimal (CTLA4) pegRNAs (Supplementary Fig. 1). As expected, due to the use of wild-type Cas9 that cuts both DNA strands, we also observed variable levels of PEn-induced imprecise prime edits and indels. Alignments of PEn-edited reads revealed that most of the imprecise prime edits represent additional integrations matching RT templates (Fig. 1b).

Mechanism of PEn-based prime editing. We reasoned that the integrated RT-templated homology tails might be products of DSB repair mediated by non-homologous end joining (NHEJ), while the precise insertions could occur through a homology-dependent process (Fig. 1c). If true, the inhibition of NHEJ could shift the outcomes of PEn editing by decreasing the frequency of imprecise prime edits. To test this, we performed PEn editing in HEK293T cells treated with AZD7648, a small molecule inhibitor of DNA-PK, an essential mediator of NHEJ22. Indeed, upon DNA-PK inhibition, the additional RT template integrations were abolished, and the remaining prime edits represented almost exclusively insertions of intended sizes (Fig. 2a, b). At several loci, DNA-PK inhibitor treatment also led to an increase of total rates of correct insertions (Fig. 2a – DPM2, AAVS1, EGFR).

Having pinpointed the contribution of NHEJ to PEn editing, we then investigated the mechanisms responsible for the homology-dependent DSB repair resulting in precise insertions. We reasoned that the short homology tails used in our pegRNA designs could utilize the a-EJ pathway, which typically uses DSB-proximal homology regions ~2–20 bp in length8. To test this hypothesis, we performed PEn editing using the AAVS1 pegRNA with a 13 nt homology tail in HEK293T cells deficient in DNA Polymerase θ (encoded by the POLQ gene), a crucial mediator of DNA Polymerase θ.
First, to confirm a-EJ inhibition in POLQ/- background, we performed Cas9 editing of the AAVS1 locus in these cells with or without DNA-PK inhibition. As expected, no indels were detected at the targeted site upon DNA-PKI treatment of POLQ/- cells (Supplementary Fig. 2), suggesting both NHEJ and e-EJ pathways were disabled. Despite this, PEn-mediated editing still proceeded efficiently, suggesting a mechanism independent of a-EJ (Fig. 2c).

Altogether, our data reveal that the imprecise PEn prime edits are mediated by NHEJ. Accordingly, DNA-PK inhibition improves the purity of PEn editing and leads to increased efficiency in a locus-dependent fashion. Interestingly, PEn-mediated precise insertions appear to be independent of the Pol θ-mediated a-EJ pathway.

**PEN editing through NHEJ.** The observation that PEn-mediated imprecise edits were inserted via NHEJ prompted us to test whether a pegRNA design encoding the intended insertion, but no homology tail, could still perform precise primed insertions through NHEJ-mediated integration (Fig. 3a). This strategy could only be exploited to promote insertions at the cleavage site due to the end-to-end joining mechanism. To test this PRemediated INSertion strategy (PRINS), we removed the homology region from the RT template of AAVS1 pegRNA, resulting in a gRNA design with an extension containing only PBS and an intended insertion (Single PRimed INSertion gRNA, springRNA). PRINS editing of AAVS1 using springRNA was able to install the intended insertion in HEK293T cells and was completely abrogated by DNA-PK inhibition, confirming that NHEJ is responsible for the PRINS-mediated insertions (Fig. 3b). The imprecise insertions constituted either truncated inserts or inserts longer than the intended size due to integrations of the gRNA scaffold sequence of various lengths (Fig. 3c). We have further tested this approach using a panel of springRNAs against different targets in HeLa and HCT116 achieving variable but robust editing with up to 50% efficiencies (Fig. 3d, e). The unintended edits were sometimes prevalent such as in the case of CTLA4, where the top variant contained additional scaffold sequence (Fig. 3e and Supplementary Fig. 3). We have also tested all four 1nt insertions at the AAVS1 site and observed variable ratios of intended/scaffold-containing editing products, suggesting an effect of RT template and targeted DNA sequences on PRINS outcomes (Fig. 3d). Altogether, our results demonstrate that PEn can efficiently install precise insertions through NHEJ.

**Off-target analysis of PEn editing.** Integration of short double-stranded DNA fragments at DSBs has been exploited for Cas9 off-target detection25 and integration of single-stranded DNA fragments was shown to increase both on- and off-target editing by Cas924. Based on these studies, we reasoned that PEn might also show more pronounced off-target editing by actively modifying DSBs and in doing so preventing error-free DNA repair. To investigate PEn-mediated off-target editing, we have targeted three sites (FANCF, HEK3 and HEK4) with gRNAs that were previously profiled for off-target editing with both Cas9 and PE2.22. We used PEn and a matching PEn mutant (PEn-dRT) carrying previously reported RT-disabling mutations5 with either pegRNAs or springRNAs against the three targets in HEK293T cells and analyzed both on-target editing and a total of 11 off-target sites by deep amplicon sequencing (Fig. 4). Compared to PEn-dRT, PEn induced up to 2-fold higher total on-target editing. Similarly, we observed that PEn increased off-target editing across most target sites. The increase ranged from moderate at HEK4 off-targets (1.4–2.3-fold), to high at FANCF with off-target 1 reaching up to a 13-fold increase (Fig. 4). Examination of editing outcomes at these sites revealed that the increase was caused by RT-mediated insertions that constituted...
the majority of edits across PEn-edited sites (Supplementary Fig. 4). Thus, these results show that efficient priming can increase PEn-mediated off-target editing and highlight a need for stringent peg/springRNAs and/or high fidelity Cas9 enzymes to be used with PEn.

**PEn-mediated insertions at DSBs mitigate Cas9-induced large deletions.** Cas9 editing has been shown to frequently cause large deletions spanning kilobase-sized regions surrounding the Cas9 target site.\(^{25,26}\) This unintended consequence of Cas9 editing poses a potential roadblock for its therapeutic applications. As PEn generates DSBs, we wondered whether PEn editing also results in similar unwanted on-target editing. To test this, we used a diphtheria toxin (DT)-based selection system in HEK293T cells\(^{37}\) to assay for large deletions induced by Cas9, PE2, and PEn. In this system, the disruption of the HBEFG coding sequence generates cells resistant to DT treatment, while cells carrying an intact copy of the HBEFG coding sequence are efficiently killed by DT (Fig. 5a). To monitor large deletions induced by different editors, we targeted an intron of HBEFG with either Cas9, PE2 or PEn and subjected the edited cells to DT selection. The percentage of colonies surviving DT treatment normalized to the total HBEFG editing levels in each condition can be used to approximate the levels of large deletions in the cell population, as only cells carrying HBEFG deletions only resulted in basal levels of large deletions. Surprisingly, similar to PE2, PEn editing with pegRNA or springRNA led to minimal levels of large deletions compared to the expected Cas9 editing levels (Fig. 5b), despite efficient editing at the target site (Fig. S5a). We have analyzed large deletion patterns using PacBio long-read DNA sequencing\(^{36}\) of the edited HBEFG locus prior to DT selection. The alignment of HBEFG long reads confirmed the presence of large deletions in Cas9-edited sample but not in PE2 or PEn-edited samples (Fig. 5c).

We hypothesized that Cas9-induced large deletions might be a result of cyclic targeted DNA cutting by Cas9 after precise DSB

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**Fig. 2** NHEJ mediates imprecise PEn editing. a Selected data from Fig. 1a with additional DNA-PK inhibitor treatments of PEn samples. Plots represent PEn or PE2 editing using 8 different pegRNAs targeting endogenous loci in HEK293T. PEn edited cells were additionally pre-treated with DNA-PK inhibitor (PEn+i) or DMSO (PEn). Plots show mean ± SD of n = 3 biologically independent replicates. “prime edits - all” and “prime edits - precise” categories are superimposed. P-values were determined using Student’s paired t test (two-tailed) \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.001\). Calculated P values: PEn vs. PEn+i (DPM2 = 0.00001, AAVS1 = 0.0273, TRAC = 0.0536, PCSK9 = 0.0168, TRBC = 0.1189, CTLA4 = 0.1029, PDCD1 = 0.0091, EGFR = 0.0282), PEn+i vs. PE2 (DPM2 = 0.3229, AAVS1 = 0.0222, TRAC = 0.0467, PCSK9 = 0.0149, TRBC = 0.0121, CTLA4 = 0.0020, PDCD1 = 0.0202, EGFR = 0.0342). b Representative alignment and allele frequencies of AAVS1 locus edited with PEn and the indicated RT template in HEK293T cells treated with DNA-PK inhibitor. For each category, the top 10 variants are shown with a minimum frequency of 0.1%. c NGS analysis of PEn editing outcomes of AAVS1 locus in wild-type and POLQ-/- and HEK293T cells with or without DNA-PK inhibitor treatment. The plot shows mean ± SD of n = 3 biologically independent replicates. Source data for Fig. 2a, c are provided as a Source Data file.
Fig. 3 PEned editing through NHEJ. a Model of PRimed INSertions (PRINS) – an NHEJ-mediated mode of PEned editing using springRNA. b NGS analysis of PRINS-mediated editing at AAVS1 in HEK293T cells with or without DNA-PK inhibitor. c Representative alignment and allele frequencies of AAVS1 locus edited with PRINS and the indicated RT template in HEK293T cells. For each category, the top 10 variants are shown with a minimum frequency of 0.1%. d NGS analysis of PRINS-mediated editing at indicated loci using a panel of springRNAs in HeLa cells. e NGS analysis of PRINS-mediated editing at indicated loci using a panel of springRNAs in HCT116 cells. f NGS analysis of PRINS-mediated 1 nt insertions at AAVS1 using springRNAs with four different RT-templates in HEK293T cells. All plots show mean ± SD of n = 3 biologically independent replicates. “prime edits – all” and “prime edits – precise” categories are superimposed. Source data for Fig. 3b, d-f are provided as a Source Data file.

| FANCF | target sequence/PBS | PAM | PEned-dRT springRNA | PEned-dRT pegRNA | PEned springRNA | PEned pegRNA | PE2 springRNA | PE2 pegRNA |
|-------|---------------------|-----|---------------------|------------------|----------------|--------------|--------------|------------|
| on-target | GGAATCCCTTCTCGAGGACC | TGG | 33.63 | 35.77 | 63.50 | 52.28 | 7.08 |
| off-target 1 | GGAACCCCACTGAGGACC | AGG | 1.50 | 1.34 | 19.60 | 12.12 | 0.03 |
| off-target 2 | GGAATCCCTCAGGACC | AGG | 0.16 | 0.12 | 0.30 | 0.20 | 0.01 |
| off-target 3 | GGAATCCCTCTAGGACC | AGG | 0.04 | 0.04 | 0.24 | 0.14 | 0.01 |
| off-target 4 | aAGGCCCAGGACC | aAGG | 0.07 | 0.04 | 0.35 | 0.24 | 0.01 |

| HEK3 | target sequence/PBS | PAM | PEned-dRT springRNA | PEned-dRT pegRNA | PEned springRNA | PEned pegRNA | PE2 springRNA | PE2 pegRNA |
|------|---------------------|-----|---------------------|------------------|----------------|--------------|--------------|------------|
| on-target | GGCGCTGACGTGACGACCC | TGG | 89.96 | 84.00 | 94.81 | 91.10 | 43.42 |
| off-target 1 | caCGCAGTGGCACAGGAGG | TGG | 0.59 | 0.39 | 2.28 | 0.74 | 0.00 |
| off-target 2 | GACGACACCGGACAGGAGG | GGG | 1.54 | 1.10 | 1.72 | 2.46 | 0.02 |
| off-target 3 | aACGCTGGACGACGAGG | aGGG | 0.35 | 0.28 | 0.47 | 0.69 | 0.15 |
| off-target 4 | aAGCAGACGTGGACGAGG | aGGG | 0.01 | 0.01 | 0.02 | 0.02 | 0.00 |

| HEK4 | target sequence/PBS | PAM | PEned-dRT springRNA | PEned-dRT pegRNA | PEned springRNA | PEned pegRNA | PE2 springRNA | PE2 pegRNA |
|------|---------------------|-----|---------------------|------------------|----------------|--------------|--------------|------------|
| on-target | GGCACCTCGCGCTGGAGGTTGG | GGG | 80.30 | 80.82 | 85.35 | 92.02 | 30.68 |
| off-target 1 | tGCCACCTCGCGGCAGAGAGG | TGG | 48.13 | 49.13 | 63.42 | 64.48 | 0.04 |
| off-target 2 | GCCCTGCGCTGCCAGGAGG | TGG | 33.74 | 34.37 | 47.23 | 44.86 | 0.64 |
| off-target 3 | GCCCTGCGCTGCCAGGAGG | TGG | 24.58 | 15.59 | 44.49 | 36.60 | 0.14 |

Fig. 4 Off-target analysis of PEned editing. NGS analysis of editing outcomes at three on-target and eleven off-target sites with indicated editors and peg/springRNAs. Editing levels are shown as percentages of modified reads in each sample. The values represent the average of n = 3 biologically independent replicates. Mismatches to the on-target gRNA sequence are highlighted in red, the PBS region is highlighted in blue. Source data for Fig. 5 are provided as a Source Data file.
re-ligation by NHEJ. Since PEn does not rely on random indel generation by endogenous DSB repair system, it could efficiently disrupt this cycle by destroying the gRNA binding site upon successful RT-templated DNA insertion. To test this model, we have designed a springRNA encoding an insertion that reconstitutes the HBEGF gRNA binding site (PAMins), potentially allowing multiple rounds of PEn-mediated cutting. Indeed, we have observed ~8-fold higher rates of DT-sensitive clones after PAMins springRNA editing relative to editing with a pegRNA encoding a random non-PAM insertion (Fig. 5d). Long-read sequencing of these two samples confirmed the more pronounced presence of large deletions upon PAMins editing (Fig. 5e). We have also performed long-read sequencing analysis post DT-selection to examine large deletion patterns in more detail.
Fig. 5 Large on-target deletion induction by Cas9, PE2, or PEn editing. a Diphertheria toxin (DT) selection-driven assay to detect on-target large deletions induced by different genome editing systems. b Relative rates of surviving colonies after DT selection of cells edited by indicated genome editors. Data normalized to Cas9. The plot shows the mean of $n = 2$ biologically independent replicates. c Alignment of long HBEGF reads from samples targeted with Cas9, PE2, or PEn and harvested before DT selection. Red lines denote Cas9 cleavage site. Scalebar 5000 bp. Panels on the right show window of 100 bp around the cleavage site. d Relative comparison of the rates of surviving colonies after DT selection of cells edited with PEn and either springRNA with a random insert of PAM-reconstituting springRNA. Data normalized to PEn + springRNA. The plot shows the mean of $n = 2$ biologically independent replicates. e Alignment of long HBEGF reads from samples targeted with PEn and either springRNA with a random non-PAM insert of PAM-reconstituting springRNA (PAMins) harvested before DT selection. The y-axis is set from minimal to maximal read depth for each sample. Source data for Fig. 5b, d are provided as a Source Data file.

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was generated by introducing reported mutations in RT (M3-deadRT M-MLV RT(R110S, K103L, D200N, T330P, L603W)) and pgRNA constructs were generated by customizing gag, pol, rna, and env sequences of the migh-Eagle’s modified hmt (Intronogen) supplemented with 10% fetal bovine serum. All cell lines were authenticated and regularly tested for mycoplasma. For gene editing experiments, cells were transfected using FuGENE HD reagent (Promega) as per manufacturer’s instructions. For 96-well plate format, cells were seeded 24 h prior to transfection at 20,000 (HEK293T) or 10,000 (HeLa, HT116) cells per well. Cells were transfected with 110 ng of plasmid DNA per well (55 ng of pgRNA/pgRNA + 55 ng of PEn/PE2/Cas9). FuGENE HD ratio used for all transfections was 3:1. For larger wells, cell seeding numbers and transfected DNA amounts were scaled up accordingly. Cells were harvested for gene editing analysis after 72 h. In DNA-PKsi experiments, AZD7698 (Med-Chem) was dissolved in DMSO and added to the growth medium 5 h prior to transfection to the final concentration of 1 µM.

Genomic DNA extraction and sequencing analysis. Cells were harvested using Quick Extract solution (Lucigen) according to manufacturer’s instructions. Amplicons were generated using Phusion Flash High-Fidelity PCR Mastermix (F548, Thermo Scientific) in a 15 µL reaction, containing 1.5 µL of genomic DNA extract and 0.5 µM of target-specific primers with NGS adapters (primers #1-50, as listed in the Supplementary Data 1). Applied PCR cycling conditions: 98 °C for 3 min, 30x (98 °C for 10 s, 60 °C for 5 s, 72 °C for 5 s). PCR products were purified using HighPrep PCR Clean-up System (MagBio Genomics). Size, purity, and concentration of amplicons were determined using a fragment analyzer (Agilent). Amplicons were subjected to the second round of PCR to add unique Illumina indexes. Indexing PCR was performed using KAPA HiFi HotStart Ready Mix (Roche). 1 ng of PCR template and 0.5 µM of indexed primers in the total reaction volume of 25 µL. PCR cycling conditions: 72 °C for 3 min, 98 °C for 30 s, 10x (98 °C for 10 s, 63 °C for 30 s, 72 °C for 30 s, 72 °C for 3 min) for 30 cycles. Indexed amplicons were purified using HighPrep PCR Clean-up System (MagBio Genomics) and analyzed using a fragment analyzer (Agilent). Samples were quantified using Qubit 4 Flurometer (Life Technologies) and subjected to sequencing using Illumina sequencing kit according to manufacturer’s instructions. For off-target analysis, amplicons were generated using QS Hot Start High-Fidelity 2x Master Mix (M04942, NEB). Amplicons for long-read sequencing were generated with QS High-Fidelity polymerase (M04925, NEB) using primers #51-52 (Supplementary Data 1) and the following PCR protocol: 98 °C 30 s 30x (98 °C 10 s 70 °C 10 s 72 °C 6 min) 72 °C 6 min.

Bioinformatic analysis. Demultiplexing of the NGS sequencing data was performed using bc2fastq software. The fastq files were analyzed using CRISPRESSO2 in the prime editing mode with the quantification window of 5 starting from the 3’ end of intended insertions. Detailed parameters are listed in the Supplementary Data 1. Prime edited overwrite sequences were used for each site. To generate the representative alignments, the window was extended to 30 to visualize homology arm integrations of different lengths. Histograms in Fig. 2a were generated using CRISPRESSO2. Barplots were generated using GraphPad Prism 9. To assess the rate of large deletions induced by genome editing, HEK293T cells were transfected with different combinations of PEn/PE2/Cas9 and gRNA/springRNA/pgRNA followed by a survival assay based on DT selection. In the survival assay, transfected cells (>50% confluence) were treated with DT (Sigma-Aldrich) at 20 ng/mL. Cell viability was measured using the AlamarBlue cell viability reagent (ThermoFisher) before and after DT selection. The ratio of cell viability before/after the selection was calculated to indicate the rate of large deletions. Genomic DNA was harvested from each sample before and after DT selection and indel rates for each sample were analyzed by NGS.
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Author contributions
M.P. and M.M. conceptualized the study. M.P., N.A., S.L., and S.W. performed most of the experimental work with help from P.H., D.D., J.B., S.v.d.P., P.M.-G., S.S., G.S., M.F., and M.M. M.F. performed bioinformatical analyses. M.P. prepared the manuscript with input from all authors. M.M. supervised the study.

Competing interests
M.P., N.A., S.L., S.W., P.H., D.D., J.B., S.v.d.P., P.M.-G., S.S., G.S., M.F., and M.M. are employees and shareholders of AstraZeneca. B.B. is a former employee of AstraZeneca. M.M. is listed as inventor in an AstraZeneca patent application (WO2021204877A2) related to this work.

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