In trans paired nicking triggers seamless genome editing without double-stranded DNA cutting

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Precise genome editing involves homologous recombination between donor DNA and chromosomal sequences subjected to double-stranded DNA breaks made by programmable nucleases. Ideally, genome editing should be efficient, specific, and accurate. However, besides constituting potential translocation-initiating lesions, double-stranded DNA breaks (targeted or otherwise) are mostly repaired through unpredictable and mutagenic non-homologous recombination processes. Here, we report that the coordinated formation of paired single-stranded DNA breaks, or nicks, at donor plasmids and chromosomal target sites by RNA-guided nucleases based on CRISPR-Cas9 components, triggers seamless homology-directed gene targeting of large genetic payloads in human cells, including pluripotent stem cells. Importantly, in addition to significantly reducing the mutagenicity of the genome modification procedure, this in trans paired nicking strategy achieves multiplexed, single-step, gene targeting, and yields higher frequencies of accurately edited cells when compared to the standard double-stranded DNA break-dependent approach.
Programmable nucleases, and in particular RNA-guided nucleases (RGNs), are rendering genome editing applicable to numerous basic and applied research settings. RGNs are ribonucleoprotein complexes formed by a guide RNA (gRNA) and a Cas9 protein with two nuclease domains, i.e., HNH and RuvC. RGNs cleave DNA complementary to the 5′ end of the gRNA when a contiguous protospacer adjacent motif (PAM) is present. The fact that target DNA cutting is ultimately dictated by simple RNA-DNA hybridization rules confers versatility to RGN technologies. A major drawback of conventional DNA editing stems, however, from the fact that double-stranded DNA break (DSB) repair in mammalian cells often takes place via mutagenic non-homologous end joining (NHEJ) instead of accurate homologous recombination (HR). As a result, allistic and non-allistic mutations, loss-of-heterozygosity, translocations, and other unwarranted genetic changes caused by on-target and off-target DSBs, are frequent. Moreover, NHEJ also contributes to random and imprecise chromosomal insertion of the donor DNA. As a whole, these unpredictable genome-modifying events complicate the interpretation of experimental results and reduce the safety profile of candidate genetic therapies. Despite this, in certain experimental settings, such as those amenable to cell isolation and screening, homology-independent chromosomal DNA insertion is a valuable genetic modification strategy owing to its efficiency and applicability to non-dividing target cells.

Following from the above, developing new genome-editing principles that favor not only efficient but also precise homology-directed gene targeting in detriment of mutagenic NHEJ are in demand. Indeed, emergent genome-editing research lines involve testing small RNAs, drugs, or viral proteins that steer DSB repair towards the HR pathway by inhibiting the competing NHEJ. Parallel research lines exploit sequence-specific and strand-specific programmable nucleases (“nickases”) for generating single-stranded DNA breaks (SSBs), or nicks, which are non-canonical NHEJ substrates. Besides bypassing DSB formation, “nickases” do not alter the regular cellular metabolism as small RNAs, drugs, and viral proteins do. However, genome editing based on “nickases” is inefficient and in fact, the investigation of site-specific SSBs as triggers for homology-directed targeting of large DNA segments (e.g., entire transcriptional units) has not been explored.

Here, we investigate the feasibility of exploiting nicking RGNs containing the RuvC Cas9 mutant Asp10Ala (Cas9ΔD10A) or the HNH Cas9 mutant His840Ala (Cas9ΔH840A) to trigger genome editing via the simultaneous formation of SSBs at endogenous and exogenous DNA. We report that this strategy based on coordinated in trans paired nicking can improve the three main parameters of DNA editing, i.e., efficiency, specificity, and fidelity, and achieves multiplexing homology-directed DNA addition of large genetic payloads.

Results
Mutagenesis caused by cleaving Cas9 vs. nicking Cas9. We started by confirming that unwarranted, potentially adverse, genome-modifying events (i.e., target allele mutagenesis and chromosomal translocations) do occur more frequently in cells exposed to cleaving Cas9 than in those subjected to nicking Cas9 proteins. Firstly, we assessed the mutation rates resulting from RGN complexes consisting of cleaving (i.e., Cas9ΔD10A:gRNAΔX) or nicking Cas9 nucleases (i.e., Cas9ΔD10A:gRNAΔX or Cas9ΔH840A:gRNAΔX), where “X” symbolizes the target locus. The Cas9ΔD10A and Cas9ΔH840A proteins differ from wild-type Cas9 in that they have amino-acid substitutions disrupting the catalytic centers of their RuvC and HNH nuclease domains, respectively. As a result, RGN complexes with Cas9ΔD10A and Cas9ΔH840A induce sequence-specific and strand-specific breaks on opposite DNA chains, namely, on the chain complementary and non-complementary to the gRNA, respectively. The AAVS1 locus at 19q13.42 was selected for these experiments owing to its frequent use as a “safe harbor” for the targeted chromosomal insertion of exogenous DNA. This assessment is based on a series of studies showing that AAVS1 integrants are neither disturbed by, nor disturb the surrounding genomic environment, providing for long-term and stable transgene expression in different cell types. A target site genotyping assay in human embryonic kidney 293 T cells showed that Cas9ΔD10A:gRNAΔX complexes targeting the AAVS1 locus readily yielded substantially higher levels of DSBs than their Cas9ΔD10A,gRNAΔX counterparts (Supplementary Fig. 1a). To augment the stringency of the genotyping assay, we next carried out dose–response experiments in human cervix carcinoma HeLa cells using increasing amounts of adenoviral vectors encoding either Cas9 or Cas9ΔD10A, each mixed with a fixed amount of an adenoviral vector expressing a gRNA addressing each Cas9 protospacer in AAVS1. A direct relationship between the detection of small insertions and deletions (indels) and nuclease concentrations could be readily established after Cas9:gRNAΔX delivery, whereas this was much less so upon Cas9ΔD10A:gRNAΔX transfer (Supplementary Fig. 1b). These data directly correlated with the much higher frequencies of indel-derived EGFP disruption in EGFP+ H27 reporter cells triggered by cleaving Cas9:gRNAΔX when compared to those induced by nicking Cas9ΔD10A:gRNAΔX or by Cas9ΔH840A:gRNAΔX complexes (Supplementary Fig. 1c).

Secondly, we setup a PCR assay to compare the assembly of chromosomal translocations caused by the formation of DSBs vs. SSBs at two distinct loci. To this end, HeLa cells were transfected with plasmids coding for cleaving or nicking RGNs targeting DMD and AAVS1 sequences. Amplicons diagnostic for translocation events between DMD and AAVS1 were exclusively detected in cells exposed to the cleaving RGNs (Supplementary Fig. 1d). Sanger sequencing of individual amplicons established their origin at t(X;19)(p21;q13) (Supplementary Fig. 1e). Taken together, these experiments formally demonstrate that unwarranted, potentially adverse, genome-modifying events occur more frequently in cells receiving RGNs containing cleaving Cas9 than in those harboring nicking Cas9ΔD10A.

In trans paired nicking yields seamless DMD gene targeting. Next, we sought to investigate homology-directed gene targeting based on inducing DSBs vs. SSBs not only at acceptor chromosomal sequences but also at donor DNA templates. The DMD gene at Xp21.2 was chosen as target locus. By spanning over 2.4 Mb, DMD is the largest human protein-coding gene known. Of note, defective DMD alleles cause Duchenne muscular dystrophy (DMD), a progressive lethal neuromuscular disease affecting ∼1 in 3500–5000 boys. For these experiments, we generated plasmid pgRNAΔDMD, to address Cas9 proteins to DMD intron 43, and EGFP-encoding constructs pDonorΔDMD and pDonorΔDMD:TS to serve as exogenous HR substrates (Fig. 1a). Construct pDonorΔDMD:TS differs from pDonorΔDMD in that it has a target site (TS) for gRNAΔDMD next to its targeting module (Fig. 1a). Importantly, all transgene-containing donors used in the present study have autonomous transcription units, which in contrast to splice acceptor-containing gene trapping constructions, avoid biased selection of on-target integrants. Genome-editing experiments were initiated by exposing HeLa cells to pDonorΔDMD and cleaving Cas9:gRNAΔDMD complexes (standard setting) or to pDonorΔDMD:TS and nicking Cas9ΔD10A:gRNAΔDMD complexes (in trans paired nicking; NickΔ). After eliminating episomal DNA by sub-culturing, genetically modified cells were

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quantified through flow cytometry. This analysis revealed that the in trans paired nicking strategy led to significantly higher percentages of genetically modified cells when compared to those obtained through the standard approach (Fig. 1b). Similar results were obtained by using donor constructs whose DMD-targeting modules were flanked by the gRNA(DMD) TS in a direct or inverted repeat orientation (Supplementary Fig. 2). These data are consistent with earlier theoretical models and more recent experimental systems indicating a role for nicked HR partners as recombination-initiating substrates. Of note, although at this target sequence paired DSB formation (in trans paired breaking; DSB2) yielded the highest frequencies of EGFP+ cells, the resulting free-ended HR substrates are prone to aberrant concatemer assembly (see below). Indeed, it has been previously shown that the DSB2 strategy results in higher frequencies of random chromosomal insertions through illegitimate recombination processes when compared to those obtained by the standard DSB-dependent gene targeting approach. Conversely, consistent with previous studies, generating SSBS exclusively at chromosomal DNA yielded the lowest frequencies of stably transfected cells.

Subsequently, we compared in trans paired nicking with standard gene targeting in terms of their relative specificities and fidelities. The specificity is ascertained by detecting donor sequences at the target site; the fidelity is established by demonstrating that telomere-sided and centromere-sided junctions between donor and target DNA are formed through error-free HR (jT+ and jC+, respectively). Randomly selected EGFP+ HeLa clones (n = 98) were screened via PCR assays targeting both junctions (Fig. 1c and Supplementary Fig. 3). In the set of clones modified through the delivery of pDonor(DMD), pCas9 and pgRNA(DMD) (n = 51), the DMD-targeted frequency was 27.5% with 21.6% of these integrants being accurately targeted (jT+/jC+). Notably, in the set of clones modified via the transfer of pDonor(DMD)TS, pCas9D10A and pgRNA(DMD) (n = 47), these fractions were 93.6% and 42.6%, respectively (Fig. 1c). We conclude that, when compared to conventional DSB-induced gene targeting, in trans paired nicking was more efficient, specific, and accurate at the DMD locus.

In trans paired nicking yields seamless AAVS1 gene targeting. We next examined the performance of in trans paired nicking and standard gene targeting at AAVS1 (Fig. 2a). As aforementioned, this locus is commonly used as a “safe harbor” for the chromosomal insertion of exogenous DNA in human cells. These experiments were initiated by transfecting HeLa and 293T cells with pDonor.E51 or pDonor.E51TS each mixed with plasmids encoding either Cas9:gRNA51 or Cas9D10A:gRNA51 (Fig. 2a). The pDonor.E51TS construct has its targeting module flanked by two gRNA51 TS (Fig. 2a). The rationale for this donor design was provided by the experiments showing that such arrangement yields significantly higher frequencies of stably transfected cells when compared to isogenic templates containing a single gRNA51 TS (Supplementary Fig. 4). In agreement with the DMD-targeting experiments, when compared to experiments involving single DSBs (standard setting) or single SSBS, in trans paired nicking of AAVS1 and pDonor.E51TS led to significantly higher percentages of genetically modified cells (Fig. 2b). Similar results were gathered by using different gRNA and donor DNA reagents or the alternative nicking Cas9HE40A variant whose inactivated RuvC-disabled Cas9D10A counterpart (Supplementary Fig. 5). Importantly, amplitons
diagnostic for HR-derived integrants were readily retrieved not only from cells subjected to inaccurate DNA editing by paired DSB formation but also from cells exposed to the accurate in trans paired nicking procedure (Fig. 2c). Indeed, in striking contrast to inducing in trans paired DSBs (DSB2), generating in trans paired SSBs (Nick2), did not result in the assembly of disruptive donor DNA concatemers (Fig. 2c), presumably emerging through ligation of free-ended termini generated in cells by Cas9:gRNASt.

Finally, we probed an alternative in trans paired nicking gene targeting strategy in which two different gRNAs generate tandem SSBs within the interacting homologous sequences. This strategy, tandem paired nicking, yielded stable
transfection levels that were within the range of those achieved by using the standard, DSB-dependent, gene targeting procedure (Supplementary Fig. 6).

To gauge the specificity and fidelity resulting from in trans paired nicking vs. standard gene targeting at AAVS1, randomly selected EGFP+ clones (n = 275) were isolated from HeLa and 293 T cell populations and were screened through junction PCR (Fig. 2d and Supplementary Fig. 7). We observed that 63.9% and 66.7% of the HeLa and 293 T cells exposed to the standard setting underwent accurate homology-directed gene targeting (J+/JC+), respectively (Fig. 2d). In the remaining clones, illegitimate recombination led instead to off-target integrants (J+/JC−) and to on-target integrants lacking HR-derived junctions either from the centromeric or telomeric side (J+/JC+ or JT−/JC+, respectively). Remarkably, the fraction of properly targeted HeLa and 293 T cells subjected to in trans paired nicking was as high as 97.2 and 100%, respectively (Fig. 2d). Finally, Sanger sequencing established that precisely targeted integrants resulting from in trans paired nicking and conventional gene targeting were indistinguishable (Supplementary Fig. 8).

To complement the previous gene targeting experiments involving sizable and transcriptionally active donor constructs, we next asked whether short, transcriptionally inert donor constructs, can equally serve as in trans paired nicking substrates. To this end, AAVS1-targeting plasmids p.S.DonorS1 and pS.DonorS1.TS, resistant and susceptible to RGNs, respectively (Fig. 2e, left panel), were transfected into human cells together with constructs expressing Cas9:gRNA51 or Cas9D10A:gRNA51 (Fig. 2e, middle panel). HR engaging p.S.DonorS1 or pS.DonoST.S sequences should result in the targeted chromosomal insertion of 18-bp DNA fragments incorporating restriction enzyme polymorphisms (Fig. 2e, middle panel). Detection of these genome-editing events by restriction enzyme fragment length analysis (RFLA) revealed that in trans paired nicking is compatible with the use of short, transcriptionally inert, donor DNA templates (Fig. 2e, right panel).

Paired RGNs inducing offset nicks on opposite chromosomal DNA strands ensure that DSBs are mostly restricted to their bipartite target sequences owing to the coordinated and local formation of SSBs on both polynucleotide chains24, 25. The resulting gains in DNA cutting specificity render this dual RGN approach appealing, hereafter named in cis paired nicking for the sake of consistency. Hence, albeit dependent on two gRNAs and on the generation of mutagenic DSBs, we sought nonetheless to compare in cis with in trans paired nicking as stimuli for site-specific chromosomal DNA insertion (knock-in). Therefore, in addition to the four experimental conditions tested before (Fig. 2b), in these new experiments, we transfected human cells with pDonorS1 and pCAG.Cas9D10A mixed with constructs expressing two different AAVS1-specific gRNA pairs (i.e., gRNA51/gRNA51.S or gRNA51/gRNA51.S51), Consistent with the previous data (Fig. 2b), the in trans paired nicking setup yielded the highest frequencies of genetically modified cells. The in cis paired nicking strategy led, in turn, to frequencies of genetically modified cells that were in the range of those obtained by inducing DSBs or SSBs exclusively at the target site (Supplementary Fig. 9).

In trans paired nicking in pluripotent stem cells. Despite their patent scientific and biomedical importance, genetic manipulation of human pluripotent stem cells (PSCs) remains limited by the typically low efficiency, specificity, and accuracy of homology-directed gene targeting, even when using programmable nucleases (see e.g., ref.21). Therefore, we investigated the performance of in trans paired nicking in human induced PSCs (iPSCs; Supplementary Fig. 10) and human embryonic stem cells (ESCs)26. In addition to pDonorS1 and pDonorS1.TS (Supplementary Fig. 4a), we included in these experiments, p.Donor.EPS1 and p.Donor.EPS1.TS, encoding Puromycin®2A.EGFP instead of EGFP. The data generated with these new HR substrates in HeLa cells (Supplementary Fig. 11) were similar to those of previous experiments showing the superiority of in trans paired nicking over standard gene targeting in achieving efficient cell engineering at AAVS1 (Fig. 2b and Supplementary Figs. 4b–6 and 9). Importantly, this superiority was equally established in iPSCs and ESCs by using dual-color flow cytometry and colony-formation assays involving the detection of EGFP+/TRA-1-81+ cells (Fig. 3a, b) and puromycin-resistant colonies stained for alkaline phosphatase, respectively (Fig. 3c). In addition, when compared to in trans paired nicking, DSB-triggered AAVS1 targeting induced higher frequencies of apoptotic Annexin V+ cells in ESC cultures (Supplementary Fig. 12). These results are consistent with the well-established sensitivity of PSCs to DSBs27.

To determine the precision of genome editing in iPSCs subjected to in trans paired nicking vs. standard genome-editing protocols, puromycin-resistant clones (n = 80) were screened with a PCR assay specific for HR-derived junctions (Fig. 3d and Supplementary Fig. 13). The gene targeting specificity in iPSCs exposed to standard and in trans paired nicking using standard and in trans paired nicking strategies. a Diagram of standard and in trans paired nicking (Nick2) procedures. The former involve DSB formation only at the target sequence; the latter comprise SSB formation at target plus donor sequences. pDonorS1 and pDonorS1.TS have their transgenes framed by sequences homologous to AAVS1. pDonorS1.TS differs from pDonorS1 in that it has the gRNA51 target site (TS) bracketing its EGFP-encoding targeting module. Cas9gRNA51 and Cas9D10A:gRNA51 are cleaving and nicking RGNs, respectively. Open and solid magenta arrowheads, position of the phosphodiester bond cleavage induced by Cas9’s RuvC and HNH nuclease domains, respectively. Solid arrowhead, position of the SSB induced by Cas9D10A. Amplicons diagnostic for telomere-sided and centromere-sided transgenic-AAVS1 junctions (JT and JC, respectively, are depicted). b Quantification of stably transfected cells. Flow cytometry of long-term HeLa and 293 T cell cultures initially transfected with the indicated plasmids. The bars correspond to mean ± s.d. of six biological replicates from two independent experiments (three biological replicates per experiment). ** * P < 0.0001 (two-tailed t-tests). c Probing for wanted (gene targeting) and unwanted (concatemerization) genome-modifying events. Amplicons diagnostic for gene targeting (JC) and head-to-tail concatemers (JH-T) in 293 T cell populations transfected with the indicated constructs are presented. This assay was also run on EGFP-sorted cells (post-sorted). EGFP served as an internal control template. d Cumulative molecular characterization of integrants generated by the conventional and in trans paired nicking strategies. The frequencies of clones with random insertions (J+/JC−), HR-derived telomeric junctions (J+/JC−), HR-derived centromeric junctions (J−/JC+) and HR-derived telomeric and centromeric junctions (J+/JC+) are plotted. The respective PCR screening data are presented in Supplementary Fig. 7. e Homology-directed AAVS1 editing after inducing DSBs or SSBs. pS.DonorS1 and pS. DonorS1.TS have a restriction-fragment length polymorphism (RFLP) flanked by 300-bp AAVS1 sequences (“arms”). pDonorS1.TS has the gRNA51.S51 TS flanking its targeting module (open arrows). RFLA restriction-fragment length analysis; half arrows primers; PAM boxed sequence. RFLA products diagnostic for unedited and HR-edited AAVS1 alleles retrieved from HeLa cells transfected with the indicated plasmid combinations are identified by open and closed arrowheads, respectively.
nicking procedures was 65 and 93%, respectively (Fig. 3d and Supplementary Fig. 13). Contributing to the difficulty in isolating iPSC lines that undergo seamless genome editing is the fact that a sizable fraction of cells, in addition to the intended genetic modification at one of the target alleles, harbor mutations at the other allele. These mutations correspond to unpredictable indel footprints created after NHEJ-mediated repair of targeted DSBs. Hence, to further characterize the genetically modified iPSCs, nucleotide sequence analysis of target DNA was performed in individual iPSC clones subjected to standard and in trans paired nicking protocols. This analysis revealed the presence of a range of indel footprints exclusively in the iPSC lines generated by standard gene targeting (Fig. 3f). Indeed, the AAVS1 target site remained pristine in all of the randomly selected iPSC lines obtained after applying the in trans paired nicking protocols (Fig. 3f). These results are in agreement with our previous data.
(Supplementary Fig. 1) and the fact that, in contrast to DSBs, SSBS are not canonical substrates for NHEJ.

Finally, iPSC lines genetically engineered through standard and in trans paired nicking remained pluripotent (Fig. 3e and Supplementary Fig. 14). We conclude that, instead of generating DSBs, targeted DNA integration at the AAVS1 “safe harbor” in different cell types is best achieved via coordinated RGN-induced paired nicking of donor and acceptor DNA.

**Multiplexing gene targeting by in trans paired nicking.** To confirm that AAVS1-targeting donor DNA subjected to RGN nicking is a superior substrate for site-specific chromosomal DNA insertion, we setup competition experiments involving the co-targeting of two donors each encoding a different reporter, i.e., EGFP or mTurquoise2 (Fig. 4a). For these experiments, one of the two donors contained TS sequences, whereas the other did not (Fig. 4a). Flow cytometry showed that pDonorSS1.TS and pDonor.TurqS1.TS subjected to RGN-induced nicking led to 15-fold and 23-fold higher frequencies of genetically modified cells, respectively, when compared to their competitor, RGN-resistant, donor counterparts pDonorS1 and pDonor.TurqS1 (Fig. 4b, c).

Consistent with these results, homology-directed gene targeting in cells containing both RGN-resistant and RGN-susceptible donors involved primarily the latter substrates, independently of the product that they encoded (Fig. 4d).

Hitherto, multiplexing genome editing has primarily entailed NHEJ-based manipulations such as those involving RGN pairs for knocking-out two genes simultaneously or for creating chromosomal deletions. Such approaches are, however, not applicable for the targeted addition of new genetic information. For this purpose, multiplexing homology-directed DNA insertion based on different donor constructs can, in principle, be used instead. Unfortunately, HR-dependent chromosomal knock-in of two different donors in individual cells is a very rare event. Moreover, in addition to generating high frequencies of indel footprints, the necessary programmable nuclease pairs can induce loss-of-heterozygosity and/or translocations (Supplementary Fig. 1). Therefore, engineering cells with exogenous DNA inserted at two different loci or at two alleles of a single locus (bi-allelic targeting) is normally a complex and time-consuming procedure. Indeed, these procedures include constructing donors with positive/negative selection markers for isolating and screening the few cells that undergo seamless gene targeting, often followed by marker removal. This lengthy process is subsequently repeated on the selected cell clone(s) using this, time, a second donor construct.

We thus sought to capitalize on the higher efficiency, specificity and accuracy of in trans paired nicking over the conventional DSB-dependent strategy at AAVS1, for testing one-step co-targeting of different alleles. These multiplexing knock-in experiments were initiated by exposing HeLa cells to pDonorS1.TS, pDonor.TurqS1.TS, and nicking Cas9D10A:gRNA (Fig. 5a). Controls consisted of treating HeLa cells with pDonorS1, pDonor.TurqS1, and cleaving Cas9:gRNA (Fig. 5a). Remarkably, in comparison with the control setting, the multiplexing approach based on in trans paired nicking yielded one order of magnitude higher amounts of doubly-labeled EGFP+/mTurquoise2+ cells as measured by flow cytometry (Fig. 5b, c). These results directly correlated with the detection of HR-specific amplicons in parallel genomic DNA samples (Fig. 5d). After flow cytometry-assisted sorting of these EGFP+/mTurquoise2+ cells (Supplementary Fig. 15), single-cell clonal analysis (n = 35) revealed that 89% of them underwent AAVS1-targeting events, of which 94% were bi-allelic events involving both donor DNA templates (Supplementary Fig. 16a, b). An independent assay based on Southern blot analysis confirmed co-targeting of both expression units in individual cells without evidence for random chromosomal DNA insertion (Supplementary Fig. 16a, b). Taken together, these data show that simultaneous in trans paired nicking of independent donor substrates can provide for a simpler and faster strategy for achieving, in a seamless manner, multiplexed addition of foreign DNA into the genome of human cells.

In trans paired nicking yields seamless gene editing at CCR5. The product of the C–C motif chemokine receptor 5 gene CCR5, located at 3p21.31, serves as an HIV-1 co-receptor on macrophages and T cells. Crucially, individuals homozygous for a 32-bp deletion disrupting CCR5 function (CCR5Δ32) are healthy and refractory to R5-tropic HIV-1 infection. Hence, this locus is an appealing target for testing HIV therapies based on viral co-receptor knockout and site-specific “stacking” of restriction factor gene. In addition, similarly to AAVS1, CCR5 is frequently used as a generic “safe harbor” for the targeted chromosomal insertion of foreign DNA in human cells. Thus, we next sought to compare DSB-dependent vs. SSB-dependent genome-editing approaches at CCR5 after delivering RGNs together with CCR5-targeting constructs pS.DonorR5 or pS.DonorR5.TS marked with restriction enzyme polymorphisms (Fig. 6a). In these experiments, RFLA and mismatch-sensing T7 endonuclease 1 (T7EI) genotyping assays were deployed for assessing genomic changes through HR and/or NHEJ (Fig. 6b). Human cells treated with in trans paired nicking (Nick3) and in trans paired breaking (DSB2) protocols readily yielded noticeable HR-specific RFLA products (Fig. 6c, top panel). A preponderance of T7EI-digested products, diagnostic for the cumulative build-up of NHEJ and HR events, was detected in cells subjected to DSB-inducing protocols (Fig. 6c, top panel).
Fig. 4 Competition for gene targeting between donor DNA resistant and sensitive to RGN-induced nicking. a Schematics of the experimental design. HeLa cells were co-transfected with the indicated donor templates together with plasmids encoding nicking Cas9D10A:gRNA51. b Quantification of stably transfected cell populations. The frequencies of genetically modified cells were determined at 27 days post-transfection by EGFP-directed and mTurquoise2-directed flow cytometry. The ratios between the frequencies of the various gene-modified subpopulations are presented. c Flow cytometry dot plots corresponding to the end-point of the experiments. Mock-transfected cultures served to set the thresholds for background fluorescence (negative control). d Gene targeting in cells containing donor DNA resistant and susceptible to RGN nicking. Amplicons diagnostic for homology-directed gene targeting involving EGFP-encoding and mTurquoise2-encoding donor templates are indicated. HPRT1 provided for an internal control target sequence.
**Fig. 5** Multiplexing homology-directed DNA addition. a Diagram of the experimental design. HeLa cells were co-transfected with the indicated donor constructs together with plasmids encoding either cleaving Cas9:gRNA<sup>S1</sup> or nicking Cas<sup>D10A</sup>:gRNA<sup>S1</sup> complexes. b Quantification of stably transfected cell populations. The frequencies of genetically modified cells were determined at 27 days post-transfection by EGFP-directed and mTurquoise2-directed flow cytometry. The ratios between the frequencies of the double-positive cell populations generated by standard and in trans paired nicking multiplexing, are presented. Numerals between brackets correspond to the fraction of each gene-modified subpopulation. c Flow cytometry dot plots corresponding to the end-point of the experiments. Parallel cultures transfected with a single donor construct mixed with plasmids expressing Cas9:gRNA<sup>S1</sup> or Cas<sup>D10A</sup>:gRNA<sup>S1</sup> served as controls for setting the thresholds for EGFP and mTurquoise2 detection. d Gene co-targeting in cells containing a mixture of two donors resistant or susceptible to RGN nicking. PCR products specific for homology-directed gene targeting involving EGFP-encoding and mTurquoise2-encoding donor templates are indicated. HPRT1 provided for an internal control target sequence.
middle panel). This outcome is consistent with the prevalence of the former over the latter pathway during the repair of DSBs in mammalian cells\(^5\). Of note, T7EI-digested products corresponding to the in trans paired nicking protocol mostly represent HR events as nicking complexes at CCR5 (single nick) led to the lowest signals in both genotyping assays (Fig. 6c). In a follow-up experiment, in addition to the four experimental conditions tested earlier (Fig. 6c), we included in cis paired nicking at CCR5 by transfecting HeLa cells with pS.Donor\(^{R5}\) and pCas9\(^{D10A}\) mixed with plasmids expressing the gRNA pair gRNA\(^{R5.1}/gRNA\(^{R5.2}\). In agreement with the previous data (Fig. 2c), in trans paired nicking induced robust accumulation of HR-specific RFLP products. Importantly, cells exposed to DSB-inducing single and dual RGN complexes had a higher proportion of disrupted CCR5 alleles...
when compared to those subjected to the SSB-inducing Cas9D10A:gRNA R5.1 complex (Fig. 6e). These results confirm that in trans paired nicking can achieve programmable nuclease-assisted genome editing without concomitantly introducing a high mutagenic load into target cell populations.

Discussion

In this study, we have demonstrated that in trans paired nicking based on combining RGN “nickases” with RGN-targetable donors can trigger robust and seamless chromosomal insertion of small and large genetic payloads into specific genomic sequences in human cells without the catalytic induction of DSBs. We speculate that the rate-limiting HR steps of single-stranded DNA invasion, donor-acceptor synaptic formation and heteroduplex expansion are, to a great extent, overcome by coordinated presentation of 3′ termini on both interacting partners after in trans paired nicking. These events are shared by recent working models invoking SSBs as recombination-initiating substrates30. In addition, recent experiments indicate the involvement of distinct factors underlying canonical and SSB-induced HR pathways. For instance, recombination between donor DNA and a nicked target sequence can proceed through RAD51/BRAC2-independent pathways30. In this regard, the versatility of RGNs for inducing nicks at different positions and strands of HR templates, might constitute a valuable experimental system to dissect SSB-dependent HR pathways and, possibly, further improve genome editing based on in trans paired nicking concepts.

Importantly, we also showed that avoiding the use of DSB-inducing nucleases confers a low mutagenic load to this new genome-editing paradigm. Hence, our research complements and joins those of others on devising high-efficiency genome-editing strategies based on RGN “nickases”31, 32. In particular, a recent study has demonstrated that fusing cytidine deaminase and uracil DNA glycosylase activities to Cas9D10A results in a large “base editor” capable of inducing C→T substitutions within ~65 nt target window31. Another recent study revealed that cleaving and nicking RGNs expose a DNA flap accessible to single-stranded oligodeoxyribonucleotide (ssODN) annealing32. On the basis of this information, rationally designed ssODNs and RGN “nickases” were combined and shown to yield homology-directed gene repair in ~10% of treated 293 reporter cells32. An intrinsic limitation of these approaches is, however, their unsuitability for effecting extensive genetic changes. Moreover, the fidelity of “base editors” depends on the absence of extra cytidines within the ~5 nt “activity window”, while that of coupling RGN “nickases” to ssODNs relies on the lack of adventitious mutations created during synthesis and processing of ssODNs in vitro and in cells, respectively33.

The high specificity and accuracy conferred by in trans paired nicking genome editing coupled to its low mutagenic load should be particularly useful in instances in which the precise genetic manipulation of target cell populations is paramount. Examples include the modeling or the repairing of disease traits in stem/progenitor cells and the unbiased genetic screening of cellular phenotypes based on HR-mediated chromosomal insertion of donor DNA libraries34. Of note, however, regardless of the DNA targeting specificity and fidelity attained by a particular genome-editing procedure, there is always the risk for uncontrollable random chromosomal insertion of the exogenous DNA. Clearly, these unwanted events can take place in cells that lack or harbor the intended genetic modification.

We have confirmed that nicking RGNs are significantly less mutagenic than their cleaving counterparts at on-target sequences (Supplementary Fig. 1). Moreover, experiments done by others have demonstrated that, when compared to cleaving RGNs, nicking RGNs are also significantly less mutagenic at off-target sites23. However, regarding the use of “nickases” specifically, one should caution that SSBs can still trigger some mutagenic events if, for instance, after hitting such lesions, an advancing replication fork collapses resulting in DSB formation. These outcomes will be most problematic at off-target sites. In this regard, sensitive and unbiased assays allowing the genome-wide detection of nick-induced mutagenesis will be instrumental in the future for determining the mutagenic load of gene-editing protocols based on programmable “nickases”. Equally related with off-target activities, programmable “nickases” with improved specificities are in demand. Possible candidates include RGN “nickases” built on recently described high-specificity Cas9 scaffolds such as SpCas9-HF135 and eSpCas99.136. We anticipate that the simple and versatile in trans paired nicking procedure will be compatible with these latest generation tools and, possibly, with other fast-emerging DNA targeting systems.

Concluding, the performance of genome editing depends on its overall efficiency, specificity and fidelity1. In this work, we have shown that testing combinatorial interactions between different types of nucleases and foreign DNA structures, can improve these crucial parameters, expanding the options for high-fidelity genetic manipulation of mammalian cells.

Methods

Cells. Human cervix carcinoma HeLa cells (American Type Culture Collection) and its EGFP expressing single cell-derived clone HE237 were cultured in Dulbecco’s modified Eagle’s medium (DMEM; ThermoFisher Scientific) containing 5% fetal bovine serum (FBS; ThermoFisher Scientific). Human embryonic kidney (HEK) 293 T cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS. These cells were kept at 37 °C in an humidified-air 1% CO2 atmosphere. The human embryonic stem cell (ESC) line H1 (ref. 26; WiCell Research Institute) and the induced pluripotent stem cell (iPSC) lines

![Fig. 6](https://www.nature.com/naturecommunications)

**Fig. 6** Homology-directed CCR5 editing after DSB vs. SSB generation. a Diagram of the different DSB-dependent and SSB-dependent genome-editing strategies. pS.DonorR5 and pS.DonorR5,TS have a restriction-fragment length polymorphism (RFLP) flanked by 400-bp CCR5 sequences (“arms”). pS.DonorR5,TS has the gRNA R5.1 target site (TS) bracketing its targeting module (orange boxes). Combining Cas9D10A: gRNA R5.2 and Cas9D10A: gRNA R5.1 complexes generates a targeted DSB by nicking on opposite DNA strands (in cis paired nicking strategy). PAMs boxed sequences; magenta arrowheads, positions of the DSBs and SSBs generated by Cas9 and Cas9D10A, respectively. b Schematics of the CCR5 genotyping assays. DNA products diagnostic for unedited, edited, and mutagenized CCR5 alleles are indicated. RFLA restriction-fragment-length analysis; T7EI mismatch-sensing T7 endonucleases I assay; half arrows, primers c CCR5 genotyping assays. Genotyping of CCR5 sequences by RFLA and T7EI assays in HeLa cells transfected with the indicated plasmid sets. RFLA products specific for unedited and HR-edited CCR5 alleles are identified by open and closed arrowheads, respectively; T7EI digestion products diagnostic for genetic changes induced at CCR5 by HR and NHEJ are equally indicated. The genomic DNA analyses were performed at 3 days post-transfection. d Comparing genome-editing strategies based on single vs. dual RGNs. HeLa cells were co-transfected with the indicated plasmids and 3 days later RFLA was performed on their genomic DNA. Open and solid arrowheads point to unedited and HR-edited CCR5 sequences, respectively. e Comparing CCR5 mutagenesis in cells exposed to RGNs inducing DSBs vs. SSBs. HeLa cells were co-transfected with the indicated plasmids and T7EI genotyping assays were carried out 3 days later. T7EI products diagnostic for indel footprints left after NHEJ-mediated DSB repair are pinpointed by the flat arrowhead.
LUMC0044Ctr4 and LUMC0044Ctr4-h4.9 were cultured in pluripotent stem cell (PSC) growth medium in the presence of irradiated ICR mouse embryonic fibroblasts (MS), and were determined by transfection. The PSC growth medium consisted of DMEM/F12 medium with GlutaMax, 20% KnockOut Serum Replacement (KOSR), 10 mM non-essential amino acids (NEAAs), 25 μM 1 penicillin, 25 μg/ml 1 streptomycin (all from ThermoFisher Scientific), and 10 ng/ml of basic fibroblast growth factor (bFGF; Peprotech). PSCs were cultured at 37 °C in an humidified 5% CO2 atmosphere. The cells used in all the experiments were mycoplasma-free. All human materials were collected based on individual written (parental) informed consent after approval by the “Medical Ethics Committee” of the LUMC (reference numbers P08-087 and P13-080). The experiments were performed according to human materials handling with all the principles outlined in the “Declaration of Helsinki”. All animal experiments were approved by the “Animal Experiments Committee” of the LUMC (reference number: 12133) and were performed according to the recommendations and guidelines set by the LUMC and the Dutch “Experiments on Animals Act”.

Recombinant DNA. The constructs A2U6_pPCaG.Cas9 and A2U8_pPCaG.Cas9.GFPs are hybrid constructs. The “in-one” plasmids A2U5_pPCaG.Cas9GFPs and A2U4_pPCaG.Cas9GFPs encode Cas9 and Cas9GFPs, respectively, together with the AAAS1-targeting gRNA51. To serve as a negative control, construct A1V3_pCas9.gRNAs expresses Cas9 and the non-targeting gRNA NT. This gRNA is irrelevant in human cells as it addresses Cas9 proteins to the recognition sequence of the S. cerevisiae Sre1 homing endonuclease. The annotated maps and full-length nucleotide sequences of A2U6_pPCaG.Cas9, A2U8_pPCaG.Cas9.GFPs, A1V5_pPCaG.Cas9.gRNAs, A1V4_pPCaG.Case9GFPs.aRNA51, A1V3_pCas9.gRNA NT, and A1T6_pCas9.aRNA NT51 could be found in the Supplementary Figs. 17–22, respectively. Likewise for the DMD-targeting donor plasmids AL05_pDonorDMD, AL02_pDonorDMD, AL42_pDonorDMD, and AL25_pDonorDMD (Addgene #100286), AL37_pDonorDMD (Addgene #100287), AM51_pUC.U6.gRNAs (Addgene #100284), AL05_pDonorDMD.TS, and LUMC1044iCtrl4.9 were cultured in pluripotent stem cell (PSC) growth medium supplemented with 10 μM Fasudil and lacking antibiotics. At 24 h post-transfection, the medium was replaced by complete PSC growth medium. After 3–4 days post-transfection, the PSCs were harvested and the transfection efficiencies were determined by EGFP-directed and TRA-1-81-directed flow cytometry. A fraction of the transfected PSCs were harvested and let to divide on MEF cultures for a period of 7 to 10 days, at which stable transfection levels were determined by EGFP-directed and TRA-1-81-directed flow cytometry (see below for details).

Adenoviral vectors. The production, purification and titration of adenovector particles AdVΔ2Cas9.GFP and AdVΔ2Cas9GFP.aRNA NT51, respectively, have been described elsewhere51, 41. The additional set of isogenic donor plasmids pDonorS1, pDonorS1.TS, pDonorS1.1xTS, except that they encode PuroR.T2A.EGFP in place of EGFP (Supplementary Fig. 31). The annotated maps and DNA sequences of Av12_pCas9.gRNAs, Av11_pCas9.gRNAs, and AT61_pCas9H840A can be found in the Supplementary Figs. 17–22. The plasmids hCas9 (ref. 39, 41815) and IcS9.D10A (ref. 39, 41815), hereinafter named pgRNAS1 and Z46_pgRNAGFP2 encoding Cas9 and the non-targeting gRNA NT, and AL05_pDonorDMD.TS encode Cas9 and Cas9D10A, respectively, together with the AAVS1-targeting gRNA51. The plasmids AV13_pCas9.gRNAs and AM51_pUC.U6.gRNAs, herein called pgRNANT and Z46_pgRNAGFP2 encoding Cas9 and the non-targeting gRNA NT, are described elsewhere6, 41. The additional set of isogenic donor plasmids pDonorS1, pDonorS1.TS, pDonorS1.1xTS, except that they contain an EGFP-encoding expression unit present in pDonorS1 and pDonorS1.1xTS, respectively. However, they differ from pDonorS1 and pDonorS1.1xTS in the spacing between their regions of homology (Supplementary Fig. 5a, b). The plasmids together with 1 mg ml−1 of streptomycin (all from ThermoFisher Scientific) and 10% FBS were added to the PSCs 3 days post-transfection, the transfection efficiencies were determined by EGFP-directed and TRA-1-81-directed flow cytometry. A fraction of the transfected PSCs were harvested and let to divide on MEF cultures for a period of 7 to 10 days, at which stable transfection levels were determined by EGFP-directed and TRA-1-81-directed flow cytometry (see below for details).

The transfections were initiated by mixing each of the appropriate vector particles AdVΔ2Cas9.GFP and AdVΔ2Cas9GFP.aRNA NT51, respectively, with the appropriate donor plasmids AV11_pDonor.EPS1 (Addgene #100296) and AV12_pDonor.EPS1.TS (Addgene #100292; Supplementary Fig. 30). The plasmids hCas9 (ref. 39, 41815) and IcS9.D10A (ref. 39, 41815), hereinafter named pgRNAS1 and Z46_pgRNAGFP2 encoding Cas9 and the non-targeting gRNA NT, and AT61_pCas9H840A can be found in the Supplementary Figs. 17–22. The plasmids hCas9 (ref. 39, 41815) and IcS9.D10A (ref. 39, 41815), hereinafter named pgRNAS1 and Z46_pgRNAGFP2 encoding Cas9 and the non-targeting gRNA NT, are described elsewhere6, 41. The additional set of isogenic donor plasmids pDonorS1, pDonorS1.TS, pDonorS1.1xTS, except that they contain an EGFP-encoding expression unit present in pDonorS1 and pDonorS1.1xTS, respectively. However, they differ from pDonorS1 and pDonorS1.1xTS in the spacing between their regions of homology (Supplementary Fig. 5a, b). The plasmids together with 1 mg ml−1 of streptomycin (all from ThermoFisher Scientific) and 10% FBS were added to the PSCs 3 days post-transfection, the transfection efficiencies were determined by EGFP-directed and TRA-1-81-directed flow cytometry. A fraction of the transfected PSCs were harvested and let to divide on MEF cultures for a period of 7 to 10 days, at which stable transfection levels were determined by EGFP-directed and TRA-1-81-directed flow cytometry (see below for details).

Detection of DSBs after AdV-mediated delivery of RNAs. Hela cells were seeded in wells of 24-well plates at a concentration of 6 × 104 cells per well. The next day, they were transduced with different amounts and combinations of adenoviral vector particles AdVΔ2Cas9.GFP or AdVΔ2Cas9GFP.aRNA NT51, respectively, and let to divide for 3 days. Hela transduced cells and cells transduced exclusively with each of these vectors alone served as negative controls. Transduction experiments were carried out in duplicate to generate parallel samples for genotyping and western blot analysis. At 3 days post-transduction, genomic DNA was extracted and T7EI-based genotyping assays were performed as detailed under the previous section. Likewise, at 3 days post-transduction, protein lysates were prepared under ice-cold conditions for western blot analysis. At 3 days post-transduction, protein lysates were prepared under ice-cold conditions for western blot analysis. At 3 days post-transduction, protein lysates were prepared under ice-cold conditions for western blot analysis. At 3 days post-transfection, the transfection efficiencies were determined by EGFP-directed and TRA-1-81-directed flow cytometry. A fraction of the transfected PSCs were harvested and let to divide on MEF cultures for a period of 7 to 10 days, at which stable transfection levels were determined by EGFP-directed and TRA-1-81-directed flow cytometry (see below for details).

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combined with the aforementioned secondary antibody. These primary and secondary antibodies were diluted 1:5000 and 1:10,000 in blocking buffer, respectively.

**Sanger sequencing.** The amplicons specific for translocation events between AAVS1 and DMD sequences (Supplementary Fig. 1e) and for AAVS1-exogenous DNA junctions (Supplementary Fig. 8) were amplified, isolated and purified from agarose gel by using the JETquick Gel Extraction Spin Kit (Genomd) according to the manufacturer’s recommendations. The PCR mixtures and cycling conditions used are described in Supplementary Tables 23 and 24, respectively. All the recovered fragments were inserted into pETI2/blunt cloning vector provided in the Clone/JET PCR Cloning Kit (ThermoFisher Scientific) following the manufacturer’s instructions. After transformation, randomly selected clones were grown and subjected to Sanger sequencing (Baseclear, Leiden, the Netherlands). The AAVS1-specific PCR products derived from randomly selected iPS cells (Fig. 3f) were purified and sequenced to Sanger sequencing for identifying insertional polymorphism generated after RGN activity. All nucleotide sequence reads were aligned and analyzed with the aid of AlignX, Vector NTI Advance R 11.5.0 software. The Sanger sequencing chromatograms were generated by using the Chromas Lite 2.1.1 software (Technelysium Pty).

**Flow cytometry.** The frequencies of cells expressing EGFP, mTurquoise2 and/or the TRA-1-81 antigen, characteristic of uncommitted PSCs, were determined by using a BD LSR II flow cytometer (BD Biosciences). The TRA-1-81 labeling was carried out by incubating single-cell suspensions of PSCs with a phycocytochrome-conjugated TRA-1-81 antibody (eBioscience) diluted 1:100 in a buffer consisting of PBS supplemented with 0.5% BSA and 2 mM ethylenediaminetetraacetic acid (EDTA). After an incubation period of 30 min at 4 °C in the dark, excess antibody was removed by thorough successive washes with large volumes of the aforementioned buffer. Data was analyzed with the aid FlowJo 7.2.2 software (Tree Star). Non-transfected cells were used to set background fluorescence levels. At least 10,000 events, each representing a single viable cell, were measured per sample.

**PCR analyses of gene-editing experiments.** The composition of the PCR mixtures and thermocycling parameters used for the analyses of genome-modifying events are discriminated in the Supplementary Tables 23, 24, 26 and 27. These analyses were performed on whole target cell population as well as on individually sorted cells. The sorting of EGFP+ HeLa and 293 T cells was conducted by using a FACSAria II flow cytometer (BD Biosciences). The EGFP+ cells were recovered into pETI2/blunt cloning vector provided in the Clone/JET PCR Cloning Kit (ThermoFisher Scientific) following the manufacturer’s instructions. After transformation, randomly selected clones were grown and subjected to Sanger sequencing (Baseclear, Leiden, the Netherlands). The AAVS1-specific PCR products derived from randomly selected iPS clones (Fig. 3f) were purified and sequenced to Sanger sequencing for identifying insertional polymorphism generated after RGN activity. All nucleotide sequence reads were aligned and analyzed with the aid of AlignX, Vector NTI Advance R 11.5.0 software. The Sanger sequencing chromatograms were generated by using the Chromas Lite 2.1.1 software (Technelysium Pty).

**Generation of iPSs.** Human fetal fibroblasts were isolated and reprogrammed to iPSs as detailed elsewhere45. In brief, the cell reprogramming was induced by transducing 2 × 104 human fetal fibroblasts seeded in a 12-well plate with the multi-cistronic lentiviral vector LV.Rl.PPT.SFK.OsK.MdTomato.preFRT46. The vector particles, encoding OCT3/4, KLF4, SOX2, and cMYC, were removed 24 h later. At 6 days post-transduction, the cells were harvested and 103 of them were seeded on a 10-cm dish with 2 × 106 MEFs cultured in KOSR PSC growth medium (ThermoFisher Scientific) and incubated at 37 °C for 2 days. The unbound probes were removed by a series of post-hybridization washes and the samples were subsequently dehydrated by exposing them to increasing concentrations of ethanol as aforementioned. Finally, the chromosome specimens were sealed and counterstained with Citifluor AF1 mounting solution (Citifluor Ltd.) containing 500 ng ml−1 of the DNA dye DAPI. Digital images were processed with the aid of Cell® 3.4 imaging software (Olympus) or LAS AF software (Leica).

**COBRA-FISH karyotyping.** Combined binary ratio labeling (COBRA)-FISH analysis was carried out for determining the karyotype of iPSs essentially following the instructions indicated in a previously published protocol47. In short, slides with metaphase chromosome spreads from iPSs were mounted in VectaMount Air™ (Vector Laboratories) and then air-dried. Following denaturation at 80 °C for 5 min, the slides were rehydrated in 70%, 90% and 100% ethanol and counterstained with DAPI and Hoechst 33342 dye. The slides were then analyzed using a fluorescence microscope equipped with an IX71 Peltier-cooled digital color camera (Olympus) or a Confocal laser scanning microscope TCS SP8 (Leica). The images were processed with the aid of Cell® 3.4 imaging software (Olympus) or LAS AF software (Leica).

**Immunofluorescence microscopy.** The acquisition of pluripotency markers (i.e., TRA-1-81, SSEA4, OCT3/4, and NANOG) and differentiation markers (i.e., AFP, CD31, and TUBB3) by iPSs and iPS-derived cells, respectively, was assessed via immunofluorescence staining. In brief, cells were first fixed for 15 min in 4% PFA. After several washes with PBS, they were exposed for 1 h to a PBS solution containing 0.1% Triton X-100 and 4% normal swine serum (NSS) Jackson Immunologic Products) and subsequently incubated overnight at 4 °C. Next, the cells were incubated according to the supplier’s instructions. After transformation, randomly selected clones were grown and subjected to Sanger sequencing (Baseclear, Leiden, the Netherlands). The AAVS1-specific PCR products derived from randomly selected iPS clones (Fig. 3f) were purified and sequenced to Sanger sequencing for identifying insertional polymorphism generated after RGN activity. All nucleotide sequence reads were aligned and analyzed with the aid of AlignX, Vector NTI Advance R 11.5.0 software. The Sanger sequencing chromatograms were generated by using the Chromas Lite 2.1.1 software (Technelysium Pty).
assessed by using the aforementioned AP detection kit following the manu-
facturer’s instructions.

Southern blot analysis. Genomic DNA was extracted from individual
EGFP/mTurquoise2+ HeLa cell clones and from a control EGFP+ HeLa cell clone
according to a standard organic solvent-based protocol as follows. The cells were
collected and incubated overnight at 50 °C in 250 μl of lysis buffer containing
10 mM Tris- HCl (pH 8), 25 mM EDTA (pH 8), 0.5% (w/v) SDS, and 100 mM
NaCl. Prior to use, the lysis buffer was supplemented with freshly added protease
K (ThermoScientific) at a final concentration of 0.1 μg μl⁻¹. The resulting
cell lysates were extracted twice by gentle pipetting in a 1:1 mixture with buffer-
swatched saturated phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase
was recovered and was subsequently subjected to one additional extraction cycle by
gentle pipetting in a 1:1 mixture with chloroform. Next, the chromosomal DNA
present in the aqueous phase was precipitated in 2.0 and 0.5 volumes of ethanol
and 7.5 ammonium acetate (pH 5.5), respectively. The recovered genomic DNA
pellets were washed with 70% (v/v) ethanol, gently air-dried, and were finally
dissolved in DNase-free sterile water at a concentration of 1–2 μg μl⁻¹. Subse-
sequently, DNA samples (10 μg each) were digested overnight with BglII (New
England BioLabs) and were resolved through a 1.0% agarose gel in 1× Tris-acetate-
EDTA buffer. The DNA was transferred by capillary action onto an Amersham
Hybond-XL membrane (GE Healthcare Life Sciences) using an alkaline trans-
fer buffer consisting of 4.0 NaOH and 1 M NaCl. After overnight transfer, the
membrane was neutralized with a pH 7.2 solution containing 0.5 M Tris-HCl and
1 M NaCl. The EGFP-specific and mTurquoise2-specific probes (994 bp each) were
isolated from agarose gel after AgeI/HindIII double digestion of AAX6_pDonor
and AX28_pDonor_Turq 31, respectively. The purified DNA probes were radi-
olabeled with [α-32P]dATP (GE Healthcare Life Sciences) by using the DecaLabel
DNA labeling Kit following the manufacturer’s instructions (ThermoScientific).
The Pre-hybridization and hybridization steps, 2 h and overnight, respectively, were performed at 65 °C in Rapid-Hyb Buffer (GE Healthcare). Next,
the membrane was washed at 65 °C once with a 2 × SSC solution supplemented
with 0.1% (w/v) SDS (20 min) and twice with a 0.5 × SSC solution supplemented
with 0.1% (w/v) SDS (20 min each). Finally, the membrane was gently air-dried,
wrapped in Saran film and exposed to an Amersham Hyperfilm MP (GE
Healthcare). The autoradiogram film was obtained by using standard developing
solutions.

Statistical analyses. The researchers were not blinded to sample allocation during experiments and data analyses. One-way ANOVA combined with Bonferroni tests were used for the statistical analyses of data sets obtained from three independent experiments comparing the performance of donors with no, one, or two gRNA

Restriction-fragment length analyses. Amplicons spanning the AAVS1 and
CCR5 target sites, and the Bpl polymorphism in the mTurquoise2-coding sequence
were generated with the PCR reagents and subsequently exposed to the restriction enzymes specified in Supplementary Table 28. The corresponding primers and
PCR cycling conditions are indicated in Supplementary Tables 23, 24, 26, and 27.

Data availability. All relevant results generated in this study are available within the paper and respective Supplementary Information or are available from the

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Author contributions

X.C. designed experiments, generated reagents, performed experiments, analyzed the data, and wrote the paper together with M.A.F.V.G. J.M.J. generated reagents, performed experiments, and analyzed the data. J.L. generated reagents and performed experiments. I.M. generated reagents and analyzed the data. H.M.M.M. and A.E.J.J. generated and characterized the iPSCs. H.M.M.M. and A.E.J.J. designed, performed and analyzed the experiments in iPSCs with the help of X.C. and M.A.F.V.G. H.M.M.M. wrote the portions about the generation and differentiation of iPSCs in the Methods section. M.A.F.V.G. designed and supervised the research, analyzed the data, and wrote the paper together with X.C.

Additional information

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