Pervasive head-to-tail insertions of DNA templates mask desired CRISPR-Cas9–mediated genome editing events

Boris V. Skryabin 1*, Delf-Magnus Kummerfeld 1, Leonid Gubar 1, Birte Seeger 1, Helena Kaiser 1, Anja Stegemann 1, Johannes Roth 2, Sven G. Meuth 3, Hermann Pavenstädt 4, Joanna Sherwood 5, Thomas Pap 5, Roland Wedlich-Söldner 6, Cord Sunderkötter 7, Yuri B. Schwartz 8, Juergen Brosius 9,10, Timofey S. Rozhdestvensky 1*

CRISPR-Cas9–mediated homology-directed DNA repair is the method of choice for precise gene editing in a wide range of model organisms, including mouse and human. Broad use by the biomedical community refined the method, making it more efficient and sequence specific. Nevertheless, the rapidly evolving technique still contains pitfalls. During the generation of six different conditional knockout mouse models, we discovered that frequently (sometimes solely) homology-directed repair and/or nonhomologous end joining mechanisms caused multiple unwanted head-to-tail insertions of donor DNA templates. Disturbingly, conventionally applied PCR analysis, in most cases, failed to identify these multiple integration events, which led to a high rate of falsely claimed precisely edited alleles. We caution that comprehensive analysis of modified alleles is essential and offer practical solutions to correctly identify precisely edited chromosomes.

INTRODUCTION

Genome editing is a powerful research tool for biology and medicine. In recent years, considerable progress has been made in this area as a result of emerging new technologies that directly modify genes at the stage of single-cell embryos (zygote); stem cells, including induced pluripotent stem cells; or germ cells. The discovery and application of the following sequence-specific programmable nucleases exemplify some of the advances: (i) zinc finger nucleases (1), (ii) transcription activator–like effector nucleases (2), and (iii) CRISPR-Cas9 ribonucleoprotein complexes (3, 4). CRISPR are short, prokaryotic, genomic, palindromic repeats located in clusters. These clusters are transcribed and processed into small RNAs (5) that interact with Cas9 proteins, resulting in a sequence-specific endonuclease (6). The CRISPR-Cas9 complex is composed of two RNA molecules: crRNA (CRISPR RNA) and tracrRNA (transactivator for crRNA) (7). The crRNA contains ~20 nt of recognition sequence complementary to the targeting region of DNA, whereas tracrRNA interacts with Cas9 protein and base pairs with crRNA (8). The minimal “artificial” CRISPR-Cas9 complex consists of a crRNA–tracrRNA molecule hybrid [guide RNA (gRNA)] and Cas9 protein–DNA endonuclease (9). Cas9 is a 1368–amino acid multidomain protein isolated from Streptococcus pyogenes (SpCas9). In conjunction with the crRNA–tracrRNA complex, Cas9 cleaves double-stranded DNA (dsDNA) adjusted to the PAM (protospacer adjacent motif; NGG sequence). The DNA strand complementary to crRNA (target strand) is cleaved by the HNH-like nuclease domain, and the opposite, nontarget strand is cleaved by the RuvC-like domain (10). The CRISPR-Cas9 complex has been broadly used to generate defined site-specific cleavage of genomic DNA; it is a fast, inexpensive, and effective DNA editing system that has a wide range of potential applications. In living cells, the sequence-specific dsDNA breaks are repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. NHEJ often results in small insertions or deletions at the dsDNA break site, which may impair the function of a targeted gene. The NHEJ mechanism is commonly used to generate conventional gene knockout models in a wide range of organisms. The HDR mechanism requires a specific donor DNA template, most often coinjectied together with the CRISPR-Cas9 complex, and results in precise genome editing events. HDR enables the insertion of specific point mutations, the addition of in-frame translated epitopes, the performance of sequence-specific knock-in (KI) events of genes, the generation of conditional knockout (cKO) genetic models, etc. Once refined to perfection, CRISPR-Cas9–mediated HDR-based genome editing holds immense promise for gene therapy. Much of the genome editing community is invested in improving the efficiency and sequence specificity of the CRISPR-Cas9 complexes (11–19). However, several limitations of the technique, such as the low efficiency of HDR, off-target effects, and genomic rearrangements remain challenging obstacles (20, 21).

Our study examines the generation of six cKO mouse models that used CRISPR-Cas9–mediated HDR mechanism in 10 KI procedures. A comprehensive analysis revealed that direct genome editing of zygotes had resulted in mosaic genotypes of targeted mice (F0 generation). Unexpectedly, more than half of the F1 offspring with modified loci displayed multiple head-to-tail donor DNA integrations. We demonstrated that both HDR and NHEJ mechanisms were used. Conventionally applied polymerase chain reaction (PCR) analyses using the outside targeting homology flanking primers
erroneously displayed integration of the desired single copy template; thus, the analysis failed to identify insert multiplication. If undetected, then this would undermine the validity of studies involving these animal models. To avoid this shortcoming, we suggest methods that improve analyses and verification of correctly targeted loci.

RESULTS
Generation and analysis of F0 founders for cKO mouse models
The strategy to generate cKO mouse models by simultaneous CRISPR-Cas9–mediated insertions of two LoxP sites using two crRNA and two single-stranded oligodeoxynucleotides (ssODN) (2sgRNA-2ssODN), proposed by Yang et al. (22), has been shown to be inefficient in an extensive study involving more than 50 different genomic loci (23). Our alternative “one-step” strategy for the generation of cKO mouse models using CRISPR-Cas9 complexes and long donor DNA templates, containing two LoxP sites, is similar to those recently reported (24–26) and could be demonstrated by S100a8 (calcium-binding protein A8) gene targeting. On the basis of computational analysis, we predicted that genomic elimination of the second exon would result in a translational frameshift leading to S100a8 gene inactivation. Therefore, we designed a donor DNA fragment with LoxP sites flanking the second exon.
 exon of S100a8 gene (Figs. 1A and 2, A to D). Our general strategy for one-step insertion of both LoxP sites relied on the active cellular HDR mechanism. We constructed a DNA template harboring exon-intronic regions flanked by LoxP sites with relatively short (76/83 nt) PAM-mutated homology arms (Figs. 1A and 2B). To select CRISPR-Cas9 complexes that efficiently cut genomic DNA at a chosen position, we designed at least three sequence-specific crRNAs for each flanking region. To gauge whether selected crRNA pairs efficiently guide genomic deletion in vivo, we injected Cas9 components with different combinations of crRNAs for each flanking region. We confirm the correct CRISPR-Cas9 nuclease C1 and C2 cleavage of the genomic locus (Figs. 1A and 2, A to D). Our general strategy for one-step insertion of both LoxP sites relied on the active cellular HDR mechanism. We constructed a DNA template harboring exon-intronic regions flanked by LoxP sites with relatively short (76/83 nt) PAM-mutated homology arms (Figs. 1A and 2B). To select CRISPR-Cas9 complexes that efficiently cut genomic DNA at a chosen position, we designed at least three sequence-specific crRNAs for each flanking region. To gauge whether selected crRNA pairs efficiently guide genomic deletion in vivo, we injected Cas9 components with different combinations of crRNAs for each flanking region. To gauge whether selected crRNA pairs efficiently guide genomic deletion in vivo, we injected Cas9 components with different combinations of crRNAs for each flanking region. To gauge whether selected crRNA pairs efficiently guide genomic deletion in vivo, we injected Cas9 components with different combinations of crRNAs for each flanking region. To gauge whether selected crRNA pairs efficiently guide genomic deletion in vivo, we injected Cas9 components with different combinations of crRNAs for each flanking region.
5′-ends integrated via an NHEJ mechanism (Fig. 3 and fig. S3). As discussed in detail below, head-to-tail multiplications of donor DNA are not unique for S100a8 and were detected in eight additional KI projects involving six different gene loci (Table 1 and figs. S4 to S7).

**PCR analysis of animals with multiple head-to-tail DNA template integration**

As previously mentioned, PCR analysis of F0 animals using primers flanking homology arms of DNA inserts did not reveal the presence of multiple tandem duplications in the targeted locus at various PCR amplification parameters; this includes different primers, as well as various touchdown and annealing temperatures (Fig. 1E and fig. S2). For all other one-step cKO projects, we only detected amplification products indicating “single copy insertion” (fig. S4C).

Considering difficulties in identifying head-to-tail insertions when relatively long donor templates were used (from 550 bp to 1.65 kb), we tested the HDR-mediated integration of a single-stranded DNA (ssDNA) harboring one LoxP site (~210 nt) during the construction of an Il4 gene conditional mouse model (Fig. 4A). Multiple head-to-tail integrations of a single LoxP site were verified in the F1 mouse offspring. A total of 49 mice were PCR-analyzed using primers (SD1 and SR1) flanking the LoxP site in the homology arms (Fig. 4A). Tandem multiplication of the LoxP-harboring DNA template was detected in five mice: numbers 34, 40, 42, 44, and 48; all other mice revealed a PCR product corresponding to a single copy LoxP integration into the Il4 gene locus using the HDR-HDR mechanism (Fig. 4B). This relatively low frequency of head-to-tail amplification was suspicious. Hence, we developed and performed additional control PCR amplification by using nonoverlapping bi-directional primers (SD1r and SR1d) that would specifically detect head-to-tail LoxP repeats (Fig. 4C). Unexpectedly, a total of 30 mice containing multiple copies of donor DNA were detected, indicating that ~83% of mice harboring LoxP head-to-tail multiplications were not verified by standard, commonly used PCR detection methods (Fig. 4, C and D).
Southern blot analysis of targeted genomic loci

Alerted by the high false-positive rate of conventional PCR analysis, we turned to Southern blot hybridization to test the frequency of multiple head-to-tail integrations. Southern blot hybridization analyses characterized locus-specific targeting of the following mouse gene loci: *S100a8*, *Trek1*, *Inf2*, *Trpc6*, and *Ccnd2* (Fig. 2E and figs. S4 to S7). In all cases, 32P-labeled donor DNA templates were used as a specific probe for hybridization (table S2). To facilitate the correct detection of single copy integrations, we incorporated additional restriction endonuclease recognition sites adjacent to the introduced *LoxP* sequences (Fig. 2B and figs. S4 to S7A). Restriction endonuclease recognition sites were chosen depending on the presence of the same sites in the targeted locus, assuming that following genomic DNA digestion, the resulting fragments would be unambiguously identifiable by size during electrophoresis in 0.8% agarose gels. For example, in the chosen region of the *S100a8* conditionally targeted locus, the flanking Bam HI endonuclease sites were located 4 kb apart in the wild-type allele (Fig. 2A). Complete digestion of genomic DNA of the correctly targeted locus should reveal 3.2-, 0.7-, and 0.3-kb DNA fragments (Fig. 2C), while the observed 1.1- and 0.2-kb fragments indicated multiple head-to-tail integrations of donor DNA via the NHEJ-HDR mechanisms (Fig. 2D). Using this strategy, we could clearly identify multiple copy integrations of donor DNA template during the generation of cKO mouse models, both in F0 and F1 offspring (Table 1, Fig. 2E, and figs. S4 to S7). Our analyses also revealed that multiple head-to-tail donor DNA template integrations arose via HDR-NHEJ, HDR-HDR, or NHEJ-NHEJ mechanisms (Table 1, Fig. 3, and figs. S4 to S7). Overall, we conclude that the repetitive head-to-tail integration of the donor DNA template is a common by-product of the CRISPR-Cas9–mediated HDR-based genome editing process, regardless of the donor DNA template size, sequence composition, or strandedness of the template (dsDNA or ssDNA) (Table 1). Southern blot hybridization analysis enabled the identification of single copy, positively targeted mice already in the F0 generation (fig. S7 and Table 1). However, because of the mosaic nature of donor DNA integration for some of the F0 mice, which indicated multiple copy integrations, we were, after crossing, able to identify offspring that harbored the desired single copy targeted allele.
DISCUSSION

CRISPR-Cas9 endonuclease has rapidly emerged as a state-of-the-art tool for genome editing in model organisms from all kingdoms of life (27). From the assembly of the CRISPR-Cas9 complex and the discovery of direct targeting of specific genomic sequences in vitro (9, 28), it took only 6 months to experimentally verify in vitro findings in bacterial and mammalian cells (3, 4, 29). The establishment of genetically modified mouse models to study the potential functional roles of genes and their products in human diseases is an important aspect of biomedical studies (30–34). cKO mouse models constitute a powerful approach that enables the investigation of gene functions in specific cell types and/or in a development-specific manner (35, 36).

Nevertheless, our study uncovered serious pitfalls exemplified in 10 separate KI procedures during the construction of six cKO mouse models that need to be taken into account. All gene-targeting protocols were performed by direct injection of CRISPR-Cas9 components together with donor DNA template into fertilized oocytes. Eight KIs were performed with relatively long donor DNA fragments (~700 to 1650 nt). Seven procedures used ssDNA and three dsDNA templates (Table 1). Three KI attempts with ssDNA and one with dsDNA templates did not yield the intended single copy integration of donor template (Table 1).

Efficiencies of donor DNA integration were variable and correlated with template size; in general, longer templates integrated less efficiently (Table 1). We noticed that most edited mice obtained from CRISPR-Cas9–modified zygotes (F0 generation) exhibited mosaic genotypes, harboring subpopulations of cells derived from different DNA integration events, and contained diverse copy numbers in the targeted loci. Our data suggest that PCR amplification of short genomic flanking regions in conjunction with inserted donor DNA is the most efficient and reliable approach for the identification of F0 mice with correctly targeted loci. Positive PCR results on both flanks indicated that a certain subpopulation of cells contains HDR-integrated DNA template (Fig. 1, C and D). However, longer PCR products representing subpopulations of cells with target DNA integrated via HDR-NHEJ or NHEJ-NHEJ are difficult to amplify. Nevertheless, in some cases, most probably depending on the degree of mosaicism and PCR primer locations, these arrangements could be detected as well (Fig. 1D, numbers 10 and 18).

When the selected F0 founders were crossed with wild-type mice for F1 offspring production, we often detected animals harboring multiple head-to-tail integrations of the donor template at the targeted loci (Fig. 3). We observed template multiplication irrespective of size, nucleotide composition, or the utilization of dsDNA or ssDNA (Table 1). A commonly applied PCR verification method in heterozygotic animals using template-specific primers in most cases erroneously identified those as single copy integration events. Moreover, in cases of multiple-copy HDR-HDR–based integrations of donor DNA, it proved impossible to correctly identify the desired single copy mice by amplification with primers set in the genomic flanking regions followed by PCR product sequencing.

To correct this error, we propose methods that can be used for the successful identification of HDR-HDR–based single copy targeted mouse loci. The first approach is based on a combination of PCR analyses: F0 and F1 founders harboring an HDR-HDR–based insertion of donor DNA could be identified using PCR amplification of flanking regions including elements of the insert (Fig. 1, C and D). A repeated head-to-tail template could be detected by a second PCR step using bidirectional, nonoverlapping primers (Fig. 4C). Furthermore, candidates for singly targeted loci should be sequenced to confirm the absence of possible mutations in the inserted donor DNA template. This relatively simple strategy could be useful for verification of any genome KI models, including point mutations in genes, specific deletions, or insertions in all species. Notably, identification of F0 founders with positive PCR results on both flanks does not guarantee that offspring will contain the correctly targeted single copy locus. On the other hand, identification of single copy positively targeted mice in the F0 generation is relatively rare. Since the mosaic nature of donor DNA integration often results in subpopulations of germ cells with correctly targeted loci, we therefore recommend crossing F0 candidates displaying HDR-HDR–integrated donor DNA template with wild-type animals and to perform a second PCR step using bidirectional, nonoverlapping primers on F1 offspring.
As shown in this study, Southern blot analysis is an additional method to reliably identify intended F1 founders. Below, we outline a strategy to design donor templates that permits the unambiguous identification of single copy targeted loci. We recommend the incorporation of two specific restriction endonuclease sites flanking the LoxP regions. This will allow the detection of small DNA fragments on Southern blots in the event that multiple donor template copies are integrated (Fig. 2E and figs. S4 to S7). Notably, the fragments should not be too small, as Southern blots poorly detect small size DNA fragments; this is illustrated by the failure to expose the 0.2-kb signal in the Trpc6 gene cKO project (fig. S6C).

Despite the advantages of CRISPR-Cas9–based genome editing, a number of potential problems such as target specificity and off-target effects still impede the CRISPR-Cas9 technology for use in biomedical research; further efforts are necessary to overcome these hurdles. Our study examines problems that are not unique for the CRISPR-Cas9 system but instead generally affect direct KI genome targeting. In multiple cases, we documented that the insertion of donor DNA via the HDR mechanism results in mosaicism yielding subpopulations of cells with head-to-tail template amplification in the modified loci. Our findings and strategies are important elements that will aid in unlocking the full potential of the CRISPR-Cas9–mediated genome editing protocols for the generation of custom-designed gene variants for biomedical research and gene therapy.

MATERIALS AND METHODS
Cytoplasmic microinjections of the CRISPR-Cas9 components into fertilized oocytes
For the preparation of CRISPR-Cas9 microinjection solution, commercially synthesized crRNA (table S1), tracrRNA and, Cas9 protein [Integrated DNA Technologies (IDT), USA] were mixed as follows: 100 pmol of crRNA were mixed with 100 pmol of tracrRNA (when two crRNAs were used, the concentration of tracrRNA was increased to 200 pmol) in 10 mM potassium acetate and 3 mM Hepes (pH 7.5) buffer and incubated at 95°C for 2 min, followed by cooling to room temperature. The annealed crRNA/tracrRNA complex was mixed with Cas9 mRNA, Cas9 protein, and DNA target fragment. The final concentrations of CRISPR-Cas9 components in 0.6 mM Hepes (pH 7.5) and 2 mM potassium acetate microinjection buffer were as follows: crRNA (2 pmol/µl), tracrRNA (2 pmol/µl) or 4 pmol/µl of tracrRNA if two crRNAs were used, Cas9 mRNA (10 ng/µl), Cas9 protein (25 ng/µl), and DNA target fragment (from 0.05 to 0.01 pmol/µl). The final injection solution was filtered through Millipore centrifugal columns and spun at 20,000 g for 10 min at room temperature.

Microinjections were performed in B6D2F1 (hybrid between C57BL/6J and DBA strains) fertilized one-cell oocytes. Oocytes were removed from oviducts of superovulated B6D2F1 female mice in M2 media supplemented with hyaluronidase (400 µg/ml), washed twice for removal of cumulus cells in M2 media, transferred to KSOM media, and kept at 5% CO₂ and 37°C before injection. Cytoplasmic microinjections were performed in M2 media using the Transjector 5246 (Eppendorf), and Narishige NT-88NE micromanipulators attached to a Nikon Diaphot 300 inverted microscope. Oocytes that survived microinjections were transferred to oviducts of pseudopregnant CD1 foster mice and carried to term. Positively targeted F0 animals were identified by PCR and Southern blot analysis of genomic DNA isolated from tail biopsies.

Donor DNA template preparation
Donor DNA templates for microinjection (table S3) were synthesized and cloned into pUC57 or pBlueScript vector (Biomatie). dsDNA templates were sequenced and directly digested from the CsCl2 gradient purified plasmid vector using Xho I restriction endonuclease. The resulting donor dsDNA fragments were separated using 1% agarose gel electrophoresis, extracted with 6 M NaI, and stored in double-distilled H₂O (ddH₂O). ssDNA templates were either purchased from IDT or MWG or amplified from the aforementioned plasmid vectors using asymmetric PCR with 500 M excess of one of the primers. PCR amplification was performed in 50-µl reaction volume containing 200 ng of plasmid DNA template, primers (1 and 0.002 pmol/µl) (table S3), 50 U of Taq polymerase, 2 U of Phusion DNA polymerase (NEB), and 0.2 mM deoxy nucleoside triphosphates (dNTPs). The resulting ssDNA fragments were separated using 1% agarose gel electrophoresis, extracted with 6 M NaI, and stored in ddH₂O.

PCR analysis of the targeting events for HDR, NHEJ, and multiple copy integration
PCR analysis was performed in 50-µl reaction volume containing 1 µM each gene specific primer (table S3), 5 U of Taq polymerase, 100 ng of genomic DNA, 5% dimethyl sulfoxide, 1 M betaine, and 0.2 mM dNTPs. The resulting DNA amplicons were separated using 1% agarose (1× tris-acetate-EDTA buffer) or 6% (w/v) polyacrylamide gel (1× tris-borate–EDTA buffer) electrophoresis, followed by ethidium bromide staining.

Southern blot DNA analysis
Genomic DNA was obtained from tail biopsies. Tail tissue was lysed in buffer containing 100 mM tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and proteinase K (100 µg/ml) (Roche) overnight at 55°C. Genomic DNA was extracted by phenol-chloroform and chloroform, followed by precipitation with 2.5 volumes of isopropanol and washing with 70% ethanol. The DNA pellet was dissolved in TE buffer [10 mM tris (pH 7.9) and 0.2 mM EDTA]. Positively targeted F1 animals were analyzed using Southern blot hybridization. Approximately 10 to 20 µg of genomic DNA was digested with the corresponding restriction endonuclease, fractionated on 0.8% agarose gels, and transferred to GeneScreen nylon membranes (NEN DuPont). The membranes were hybridized with 32P-labeled specific DNA probes (table S2). DNA labeling was performed using a random prime DNA labeling kit (Roche) and [α-32P] deoxyctydine-5’ triphosphate (PerkinElmer). Membranes were washed with 0.5× saline sodium phosphate EDTA buffer [1× saline sodium phosphate EDTA buffer is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA (pH 7.7)] and 0.5% SDS at 65°C and exposed to MS film (Kodak) at −80°C.

Mice
All animal procedures were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany. F1 heterozygous mice were produced by breeding F0 DBAXC57BL/6J founders to C57BL/6J mice. Pups were weaned at 19 to 23 days after birth, and females were kept separately from males. The mice were housed in standard individually ventilated cages. General health checks were performed regularly to ensure that any findings were not the result of deteriorating physical conditions of the animals.
Supplementary materials

Supplementary material for this article is available at advances.sciencemag.org/cgi/content/full/6/7/eaax2941/DC1

Supplementary Material and Methods

Fig. S1. Evaluation of in vivo $50008$ crRNA cleaving efficiency in mouse embryos.

Fig. S2. PCR analysis of genomic DNA from F0 founder number 6 after HTT integration in the $50008$ locus at different touch down/annealing temperature conditions using primer pair (d4/d4) (Fig. 1D).

Fig. S3. Sequence analysis of heterozygous animal (F1) number 45 with MC head to tail (d4/r4) (Fig. 1D).

Fig. S4. Analysis of the In2 targeted locus.

Fig. S5. Analysis of the Inf2 targeted locus.

Table S1. List of crRNAs used.

Table S2. Designed donor DNA templates.

Table S3. List of oligonucleotides used for ssDNA donor template generation by asymmetric PCR and PCR analyses of targeted loci.

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Acknowledgments: We thank S. Klco-Brosius for editing assistance. Funding: This work was supported by the Deutsche Forschungsgemeinschaft (RO5622/1-1 to T.S.R., SK259/2-1 to B.V.S., SU 195/4-1 to C.S., SFB/CRC 1009-809 to J.R., and SFB/CRC 1009-810 to H.P. and R.W.-S.), BMBF (16GW0055 to S.G.M.), DZHK (81X2800174 to B.V.S.), the IZKF Muenster (Wed2/022/18 to R.W.-S.), Cancerfonden (CAN 2016/524 to Y.B.S.), Strategic Research grant from Medical Faculty, Umeå University (to Y.B.S.), and an IMF grant of the Medical Faculty of WWU (SH121608 to J.S.). Core Facility TRAM is an institution of the Medical Faculty of WWU. The support of the Medical Faculty is thankfully recognized. Competing interests: The authors declare that they have no competing interests. Author contributions: B.V.S. and T.S.R. conceived and designed the study. L.G., B.S., H.K., A.S., B.V.S., and T.S.R. were involved in experimental work for all cKO projects. D.-M.K. cloned and analyzed junction sites of repeats. J.R., S.G.M., Y.B.S., and C.S. were involved in the generation of S100a8, Trek1, Ccnd2, and Il4 cKO mice, respectively. H.P. and R.W.-S. participated in the Inf2 cKO project. J.S. and T.P. were involved in Trpc6 cKO mice generation. T.S.R., B.V.S., and J.B. analyzed the data and wrote the paper. All authors provided input and approved the final manuscript. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 11 March 2019
Accepted 25 November 2019
Published 12 February 2020

Citation: B. V. Skryabin, D.-M. Kummerfeld, L. Gubar, B. Seeger, H. Kaiser, A. Stegemann, J. Roth, S. G. Meuth, H. Pavenstädt, J. Sherwood, T. Pap, R. Wedlich-Soldner, C. Sunderkötter, Y. B. Schwartz, J. Brosius, T. S. Rozhdestvensky, Pervasive head-to-tail insertions of DNA templates mask desired CRISPR-Cas9-mediated genome editing events. Sci. Adv. 6, eaax2941 (2020).