In vivo blunt-end cloning through CRISPR/Cas9-facilitated non-homologous end-joining

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ABSTRACT

The CRISPR/Cas9 system facilitates precise DNA modifications by generating RNA-guided blunt-ended double-strand breaks. We demonstrate that guide RNA pairs generate deletions that are repaired with a high level of precision by non-homologous end-joining in mammalian cells. We present a method called knock-in blunt ligation for exploiting these breaks to insert exogenous PCR-generated sequences in a homology-independent manner without loss of additional nucleotides. This method is useful for making precise additions to the genome such as insertions of marker gene cassettes or functional elements, without the need for homology arms. We successfully utilized this method in human and mouse cells to insert fluorescent protein cassettes into various loci, with efficiencies up to 36% in HEK293 cells without selection. We also created versions of Cas9 fused to the FKBP12-L106P destabilization domain in an effort to improve Cas9 performance. Our in vivo blunt-end cloning method and destabilization-domain-fused Cas9 variant increase the repertoire of precision genome engineering approaches.

INTRODUCTION

The ability to make precise double-strand breaks (DSBs) in the genome is extremely useful for genome engineering, as this ability can facilitate directed changes in the genome for applications ranging from studying a gene to engineering an entire biosynthetic pathway. The most popular tools for making DBSs currently are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system (1). Of these, the CRISPR/Cas9 system offers the greatest ease of use.

The CRISPR/Cas9 system, a component of the bacterial RNA-mediated adaptive immune system, consists of transcribed guide RNAs derived from integrated fragments of phage and plasmid DNA that direct the Cas9 RNA-guided DNA endonuclease to targets containing complementary sequences to the CRISPR (1–3). The complex requires a protospacer adjacent motif (PAM) downstream of the target sequence to begin binding (4). Upon binding, Cas9 generates a blunt-ended DSB three to four bases upstream of the PAM through the use of RuvC-like and HNH nuclease domains (3).

Recently, the CRISPR/Cas9 system from Streptococcus pyogenes has been adapted for genome editing (1,3,5,6) and has been successfully used in many eukaryotes (7–12), as well as in disease and therapeutic modeling (13–18). To date, editing and engineering with the CRISPR/Cas9 system has mostly relied on a combination of error-prone repair and homologous recombination, similarly to TALENs and ZFNs, despite the observation that Cas9 generates blunt-ended DSBs (1,5,6). FokI-generated DSBs, relevant for TALENs and ZFNs, are preferentially repaired by error-prone NHEJ, because FokI-induced DSBs are staggered with single-stranded overhangs. However, blunt-ended, chemically unmodified DSBs lack these overhangs and thus could be directly repaired by the precise NHEJ pathway, which has been previously demonstrated using the Tn5 transposon system, which also makes blunt DSBs (19).

Indeed, precise NHEJ has been observed to resolve the majority of CRISPR/Cas9-induced translocation events in human immortalized cells (20). In further support of precise NHEJ repair, relatively low levels of error-prone editing have been observed in human induced pluripotent stem cells (hiPSCs) (5). Thus, while the predominant method of repair of CRISPR/Cas9-induced DSBs remains unclear, the evidence appears to favor the precise rather than error-prone NHEJ repair pathway.

We asked whether CRISPR/Cas9-induced DSBs were predominantly repaired by precise or error-prone NHEJ, and if the mode of repair could be exploited to develop a more precise, homology-independent genome engineering method. The NHEJ repair pathway has previously been exploited to facilitate insertions of plasmid sequences in zebrafish at high efficiency (21) and mammalian cells at low efficiency (22), although both applications displayed low lev-
els of perfect ligation at the genome-plasmid junction. We hypothesized that, given the propensity of Cas9 to remain bound to its target DNA sequence after cleavage (4) and assuming precise NHEJ to be the predominant mode of repair of Cas9-induced DSBs, increasing the turnover of Cas9 through a tunable destabilization domain, such as FKBP12-L106F (23), may lead to higher levels of precise ligation between the genome and exogenous sequences.

Here, we demonstrate that CRISPR/Cas9-induced DSBs are predominantly repaired by precise NHEJ. We examined the repair patterns of deletions generated through the simultaneous use of two chimeric single guide RNAs (sgRNAs), utilizing 15 pairs spanning four genomic loci, three cell types, and two species, human and mouse. We then illustrated that this precise NHEJ repair can be exploited to replace endogenous sequences with exogenous sequences with a high degree of precision at relatively high efficiency in an immortalized human cell line and hiPSCs. We further examined whether the use of a destabilized variant of Cas9 in hiPSCs influenced the level of precise, seamless ligation between the genome and the exogenous cassette.

**MATERIALS AND METHODS**

**sgRNAs and vector construction**

All sgRNAs were chosen using the MIT CRISPR Design tool (http://crispr.mit.edu/). Briefly, genomic regions consisting of up to 250 bp were chosen for each locus, and the highest quality guides were chosen for cloning into the pX330 (Addgene #42230) backbone following the protocol developed by Feng Zhang’s lab (https://www.addgene.org/static/cms/filer_public/95/12/951238bb-870a-42da-b2e8-b2e8-5e38b37d4fe1/zheng_lab_grna_cloning_protocol.pdf). These regions appear in Supplementary Table S1. All oligos, primers, and gBlocks were ordered from Integrated DNA Technologies (IDT, Coralville, IA). A description of the sgRNAs chosen, as well as the oligos used for cloning, appear in Supplementary Table S2. sgRNA/Cas9 vectors, as well as all additional plasmids used in this study, were isolated using the Nucleobond Midi Plus EF kit (Machery-Nagel, Bethlehem, PA). A detailed description of the cloning of the destabilized Cas9 vectors along with subsequent cloning of sgRNA inserts appears in the Supplementary Materials and Methods.

**Knock-in cassette construction**

The DICE-EPv2.0 and PTKmChR cassettes were amplified from a common vector containing the PuroR ΔATK fusion element (from Addgene # 22733) followed by a P2A ribosomal skipping element and mCherry followed by the rabbit beta-globin terminator. This cassette was under the control of the mouse phosphoglycerol kinase (PGK) promoter. Cassette from the pKER series were amplified from their respective plasmid vectors once assembled. Cassettes were amplified via PCR with Q5 high-fidelity polymerase and either subjected to gel electrophoresis, excised, and purified with the MinElute Gel Extraction Kit or pooled, column-purified, digested with FastDigest DpnI (Thermo Fisher Scientific), and purified by chromatography. For the DICE-EPv2.0 cassettes, the phiC31 and Bbx1 attP sites were included in the primers used for amplification. All primers used for cassette amplification appear in Supplementary Table S3. Further details of construction, amplification, and purification of the cassettes appear in the Supplementary Materials and Methods. Phosphorothioate bonds were incorporated into primers during synthesis by the manufacturer (IDT).

**Cell culture**

HEK293 cells were maintained in 6-well tissue culture plates coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) in high-glucose DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 1× non-essential amino acids (Life Technologies), and 1× GlutaMAX (Life Technologies). Cells were passaged with 0.05% Trypsin-EDTA (Life Technologies) as needed at a 1:12 split. C2C12 cells were grown on 6-well tissue culture plates and maintained in the same medium as HEK293 cells and passaged in the same manner. H9 hESCs were maintained in 0.1% gelatin-coated 6-well tissue culture plates on γ-irradiated mouse embryonic fibroblast feeder cells in human embryonic stem cell medium consisting of DMEM/F12 (Thermo Fisher Scientific), 20% Knockout Serum Replacement (Life Technologies), 1× non-essential amino acids (Life Technologies), 1× GlutaMAX (Life Technologies), 0.1mM β-mercaptopoethanol (Life Technologies), and 8ng/mL bFGF (PeproTech, Rocky Hill, NJ). Cells were passaged as clumps using collagenase IV (Stem Cell Technologies, Vancouver, British Columbia, Canada). JF10 hiPSCs were maintained in 6-well tissue culture plates on recombinant human vitronec (Life Technologies) in Essential 8 medium (Life Technologies). Cells were passaged as clumps every three to four days with Versene (Life Technologies) at a 1:6 split.

**Detection of CRISPR/Cas9-facilitated excision**

HEK293 and C2C12 cells were transfected using FuGene HD (Promega, Madison, WI). Briefly, 150 000 cells were plated 1–2 days prior on poly-L-lysine-coated (HEK293) or 3% mouse phosphoglycerol kinase (PGK) promoter. C2C12) 24-well tissue culture plates and were transfected with 5 μg (HEK293) or 3 μg (C2C12) of each sgRNA/Cas9 vector for a total of 10 or 6 μg per transfection, respectively, in Opti-MEM I (Life Technologies) medium with a 3:1 ratio of FuGene HD:plasmid DNA. Cells were harvested 4 days post-transfection and genomic DNA was isolated using the DNeasy Blood and Tissue Mini Kit (Qiagen). Each transfection was performed at least twice. H9 hESCs were transfected using the Amaxa Nucleofector (Lonz, Basel, Switzerland) with the Human Embryonic Stem Cell Nucleofection Kit 2 (Lonza). Briefly, cells were passaged normally into Matrigel-coated 6-well plates three days prior to nucleofection. Before nucleofection, medium was replaced at least 1 h beforehand with fresh medium containing 10 μM ROCK inhibitor Y-27632 (R&D Systems, Minneapolis, MN). Cells were dissociated with collagenase IV and subsequently electroporated with 1.5 μg of each sgRNA/vector for a total of 3 μg using program B-16 according to the manufacturer’s instructions, and plated on Matrigel-coated 12-well plates in human stem cell medium with 10 μM ROCK inhibitor Y-27632. Each
reaction was carried out at least twice. Genomic DNA was isolated from the cells 2 days later with the DNeasy Blood and Tissue Mini Kit.

PCR amplification of the deletion-containing regions was carried out using OneTaq (NEB) and Q5 high fidelity polymerase using 150 ng of gDNA according to the manufacturer’s instructions. Amplicons were subjected to agarose gel electrophoresis, and subsequently the deletion-containing bands, as well as the full-length bands where applicable, were excised and purified using the MinElute Gel Extraction Kit (Qiagen). Purified amplicons were then subcloned using the CloneJet PCR Cloning Kit (Thermo-Fisher Scientific), and subsequently transformed into α-Select electrocompetent cells (Bioline, Taunton, MA), 10-β chemicompetent cells (NEB), or STBL3 chemicompetent cells according to their manufacturers’ instructions. Colonies were inoculated and grown overnight. Plasmids were isolated the following day using the Miniprep Spin Kit (Qiagen), and subsequently subjected to Sanger sequencing of the inserts. Primers used for amplification of excision events appear in Supplementary Table S4.

Flow cytometry

Cell sorting and flow cytometric analyses for this project was done in instruments in the Stanford Shared FACS Facility. Flow cytometric analysis was carried out on LSRII- and FACScan-class analyzers (BD Biosciences, San Jose, CA). Sorting was carried out on FACSARiaII-class sorters (BD Biosciences), including one obtained using the NIH S10 Shared Instrument Grant S10RR025518-01. Live cells were discriminated on the basis of DAPI exclusion using the NucBlue Fixed Cell Stain ReadyProbes reagent (Life Technologies). Information on fluorescent protein detection appears in the Supplementary Materials and Methods. All flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR). Information on filter sets used for the detection of each fluorescent protein appears in the Supplementary Materials and Methods.

Detection of knock-in blunt ligation and homologous recombination

For HEK293 cells, FuGene HD was used to transfect cells as described above, using 100 ng of cassette and 1.5 μg of each sgRNA/Cas9 vector for a total of 3.1 μg of DNA. 500 000 cells had been plated one to two days prior to transfection. Reactions were carried out in triplicate. Shield1 (Clontech) was added to the cells immediately prior to transfection or 6 days in the case of puromycin-selected hiPSCs. 1 μl of 1:200 diluted WGA of Clover+ cells was used for further genome-cassette junction PCR amplifications. MDA reactions were diluted to 200 μl with TE-EF buffer (Machery-Nagel) and used for further genome-cassette junction PCR amplifications. Primers used appear in Supplementary Table S5.

Analysis of off-target KiBL events

For each sgRNA in the H11 PAMs Out pair, forward and reverse primers were designed to each of the top six target sites as predicted by the MIT CRISPR Design Tool. Each forward and reverse primer was used in conjunction with the 5’ and 3’ pKER-Clover detection primers, for a total of 48 reactions per sample. Whole genome amplification (WGA) was carried out using random phosphorothioate-bond-containing hexamers (IDT), phi29 DNA polymerase (NEB) and 10–50 ng of genomic DNA from sorted Clover+ hiPSCs. 1 μl of 1:200 diluted WGA of Clover+ cells was used in each PCR amplification. PCR was carried out with Q5 high-fidelity polymerase for each possible combination of off-target primer and Clover internal primer with the product being resolved on a 1% agarose gel. Amplification of on-target KiBL was used as a positive control. KiBL events were scored by the presence or absence of the appropriate band and further stratified by relative intensity. Off-target primers appear in Supplementary Table S6.
Statistics

All statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). For MYOD1, PAX3, PAX7, and mUtrn, repair distributions were analyzed using the Kruskal-Wallis test followed by a post hoc Dunn’s multiple comparisons test. For PAX7, each sgRNA pair was only compared between cell types. Otherwise all pairs were compared to all other pairs. For analysis of pKER amplicon retention efficiency in HEK293 cells, a two-way ANOVA with repeated measures followed by post hoc Tukey’s multiple comparisons test was utilized. To analyze amplicon retention efficiency in JF10 hiPSCs, an ordinary one-way ANOVA was employed followed by post-hoc Tukey’s multiple comparisons test.

RESULTS

Patterns of excision and repair resulting from paired PAM orientations

Because Cas9 generates blunt DSBs, we hypothesized that these blunt ends might be preferentially repaired by precise NHEJ. To test this hypothesis, we chose to generate two DSBs within the same locus rather than one, because precise NHEJ mediated by one sgRNA would be indistinguishable from uncleaved DNA at the sequence level. By contrast, if a cell received two sgRNA vectors expressing two different sgRNAs, there would be a high probability of excision of the intervening sequence between the paired sgRNA targets. Ligation-based repair of the genomic junctions would allow subsequent detection and analysis via PCR and Sanger sequencing (Figure 1A). Others have also observed similar excision and rejoining (4,6,26). This excision may occur because Cas9 appears to bind the PAM side of the DSB less strongly after cleavage in vitro (4). The repair consequences of the different PAM orientations resulting from using two sgRNAs simultaneously have not been systematically explored, as the largest study to date focused on only one particular orientation (26). Determining these consequences is important because a functionally significant difference between PAM orientations would impose restrictions upon which pairs of guide RNAs are useful for generating precise deletions. Such an analysis would also be able to identify the preferences of individual guides for cleavage at the third or fourth base upstream of the PAM. For sgRNA pairs, there are four possible orientations: (i) PAMs Out, (ii) PAMs In, (iii) PAMs 5' and (iv) PAMs 3' (Figure 1B). It is important to note that PAMs 5' and PAMs 3' are functionally equivalent at first glance. Because Cas9 can cleave at either the third or fourth base upstream of the PAM, precise rejoining can occur with 0, +1, or −1 bases on each of the repaired genomic ends, depending on which PAM orientation is used, while still being considered precise repair of the resulting ends. As such, each orientation has four possible outcomes for precise repair (detailed in Figure 1B and shown as 5' sgRNA bases/3' sgRNA bases). To this end, we generated multiple sgRNAs against the 3' UTRs of human MYOD1, PAX3, PAX7, and mouse Utrphin (Utrn) to examine the repair patterns and degree of precision utilized by paired PAM orientations (Figure 1C–F). We then used these sgRNAs to address four basic questions about Cas9-mediated deletions: (i) How does PAM orientation affect repair precision and pattern in human cells? (ii) What is the contribution of different pairs of guides in identical orientations to repair pattern and precision at a given locus? (iii) How does repair pattern and precision differ, if at all, for a given pair of guides in different cell types? and (iv) How does the use of paired guides affect repair precision and pattern in mouse cells? These questions and our observations are summarized in Table 1.

We sought to address the first question of differences in repair pattern and precision between PAM orientations by using separately transfected pairs of sgRNAs targeting the MYOD1 3' UTR in HEK293 cells (Figure 1Ci). In comparing repair patterns, we observed a remarkably high level of precision of NHEJ repair as indicated by the high frequency of precise repair (65–96%), regardless of orientation (Figure 1Ci; Supplementary Figure S1). PAMs 5' showed the highest frequency of precise repair, whereas PAMs 3' showed the lowest frequency of precise repair (Figure 1Ci). Imprecise repair was mainly limited to small deletions and occasional inclusion of bases that should have been excised. To determine if PAM orientation significantly affected repair pattern and precision, we compared the deviations from 0/0 precise repair between PAM orientations, which revealed a significant difference between distributions (P < 0.0001). We found no significant difference between PAMs Out and PAMs 3' or between PAMs In and PAMs 5' (P > 0.9999), but all other comparisons between orientations were significantly different (P < 0.0001; Figure 1Ci). PAMs Out and PAMs 3' both displayed a mostly 0/+1 precise repair pattern, possibly because they share the same 3' sgRNA (MYOD1-r2–1; Figure 1Ci), which appears to demonstrate a strong preference for cutting at the fourth base from the PAM rather than the third (Supplementary Figure S1). Interestingly, the predominance of 0/+1 precise repair observed with PAMs 3' appears to be due to 0/0 repair generating a perfect target site for the 5' sgRNA. Additionally, we observed only one stereotypical error-prone event (Supplementary Figure S1). These findings strongly suggested that NHEJ repair of CRISPR/Cas9-induced DSBs was not inherently error-prone. The results also suggested that deviations from 0/0 precise repair were due to the preferences of individual sgRNAs for cleaving at the third or fourth base upstream of the PAM.

Having observed that all four orientations were capable of facilitating precise repair at one locus, we next asked if different paired PAMs in the same orientation would display the same patterns and degrees of repair precision at a given locus. To address this question, we utilized four pairs of sgRNAs in the PAMs In orientation at the PAX3 3' UTR in HEK293 cells (Figure 1Di). We observed that all four pairs displayed a remarkably high level of precise repair, ranging from 88 to 100%, and an absence of insertions (Figure 1Di; Supplementary Figure S2). We found a significant difference between the distributions of deviations from precise repair (P < 0.0001), driven solely by PAMs In #1 tending toward 0/−1 precise repair (P < 0.004 for PAMs In #1 versus each other pair; Figure 1Dii). Upon closer examination, this difference appeared to be driven by the PAX3-1 sgRNA. These findings provided additional evidence both for the high degree of precision involved in CRISPR/Cas9-
Figure 1. Analysis of deletion repair patterns generated by paired sgRNAs. (A) Schematic showing excision of sequence between two sgRNAs followed by how deletion repair was analyzed. (B) Each paired PAM orientation has four possible outcomes for precise repair (depicted below each orientation) based on the ability of SpCas9 to cleave at the third or the fourth base upstream of the PAM. (C–F) (i) Schematic diagrams of the targeted locus with sgRNA targets depicted as sgRNA/Cas9 complexes with largest deletion size for each locus. Red indicates exonic sequence and purple indicates 3′ UTR. (ii) Cumulative percentage of precise (green), excision+insertion (orange), and imprecise (red) repair observed in sequenced amplicons for each tested sgRNA pair. (iii) Dot plot of deletion-containing amplicons described in sub-panel (ii). Each dot represents the sum of bases beyond 0/0 precise repair. The numbers of amplicons analyzed for each sgRNA pair appear on in subpanel (iii). Data from subpanel iii were analyzed with the Kruskal–Wallis test followed by post-hoc Dunn’s multiple comparisons test. For MYOD1, PAX3, and mUtrn, all sgRNA pairs were compared against all pairs. For PAX7, each pair was only compared between cell types. ****P < 0.0001; **P < 0.004.

Table 1. Summary of findings from paired sgRNA-mediated deletion experiments

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<th>Question</th>
<th>Answer</th>
<th>Observations</th>
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<tr>
<td>Does PAM orientation affect level/pattern of repair precision?</td>
<td>No</td>
<td>Vast majority of deletions repaired precisely. NHEJ not inherently error-prone.</td>
</tr>
<tr>
<td>Are there significant differences in level/pattern of repair precision between different pairs in the same orientation at the same locus?</td>
<td>No</td>
<td>Variation in repair pattern between pairs still results in precise repair. Individual sgRNAs have strong preferences for cleaving at third or fourth base upstream of PAM.</td>
</tr>
<tr>
<td>Will a given pair of sgRNAs behave similarly in different cell types?</td>
<td>Yes</td>
<td>Deletions repaired with similarly high level of precision and similar patterns between HEK293 and H9 hESCs.</td>
</tr>
<tr>
<td>Are murine cells capable of repairing Cas9-mediated deletions with a high degree of precision?</td>
<td>Yes</td>
<td>Precise repair observed; imprecise deletions also observed.</td>
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induced NHEJ repair and that individual sgRNAs have different preferences for which base they cleave at.

Because CRISPR/Cas9-induced NHEJ repair did not appear to be inherently error-prone, we next sought to address whether a given pair of sgRNAs would generate similar repair patterns in different human cell types. Thus, we designed four pairs of sgRNAs against the PAX7 3′ UTR, which were subsequently transfected into HEK293 cells and H9 human ESCs (hESCs) (Figure 1Ei). For H9 hESCs, only one pair resulted in <75% precise repair. We observed more variation in HEK293, with levels ranging from 44–90% (Figure 1Eii; Supplementary Figure S3 for HEK293; Supplementary Figure S4 for H9 hESCs). Additionally, we observed only two typical error-prone events for HEK293, a single and a double error-prone event (Supplementary Figure S3). For H9, we observed two typical error-prone repair events, both of which were single sgRNA events (Supplementary Figure S4). In spite of this variation, we found that none of the four pairs of sgRNAs generated significantly different repair patterns between the two cell types (Figure 1Eiii). This result demonstrated not only that deletions generated by a given pair of guides were repaired with a high degree of precision regardless of cell type, but also were repaired in similar ways between cell types.

Given the high degree of precise repair in human cells across three loci and 12 pairs of sgRNAs, we sought to examine whether murine cells would display similar repair behavior. Paired sgRNAs have previously been utilized to generate deletions in murine embryos, although there was significant variability in deletion size between embryos (27). To examine repair patterns in murine cells, we transfected C2C12 murine immortalized myoblast cells with three pairs of sgRNAs in different orientations against the mUtrn 3′ UTR (Figure 1Fi). We observed a varied repair pattern with levels of precision ranging from 42 to 100% (Figure 1Fii). We also observed a high degree of repetition in the imprecise excision repair amPLICons in their deviation from 0/0 precise repair (Figure 1Fiii; Supplementary Figure S5). However, there was no significant difference between the distributions of deviation from 0/0 precise repair for the three orientations ($P = 0.1354$). These results indicated that murine cells were capable of repairing paired-guide-induced deletions with a high level of precision.

Knock-in blunt ligation: CRISPR/Cas9-mediated in vivo blunt-end cloning

Because of the high level of precise NHEJ repair we observed in generating deletions, we asked if we could exploit this apparent in vivo blunt-end ligation to use CRISPR/Cas9 to replace endogenous sequences precisely with linear exogenous sequences in a homology-independent manner (Figure 2A). A similar method was developed in zebrafish in which the same sgRNA cleaved both the genomic locus and a donor plasmid, ultimately resulting in efficient homology-independent knock-in of the plasmid, albeit with reduced levels of perfect ligation (21). We reasoned that introducing linear, double-stranded DNA might result in a higher level of precise ligation. Thus, we constructed an expression cassette consisting of the mouse PGK promoter driving expression of the Puro$^\Delta$TK fusion gene linked via a P2A skipping peptide to mCherry, followed by the rabbit beta globin terminator sequence (PTKmChR; Figure 2B). We also generated cassettes through PCR amplification containing a phiC31 attP site in the sense primer and a Bxb1 attP site in the antisense primer for the purpose of constructing a selectable landing pad for the DICE system (28) that did not rely on homology arms for integration (DICE-EPv2; Figure 2B). Each cassette was generated with 5′ phosphorylated primers, purified, and subsequently co-transfected into HEK293 cells with various sgRNA/Cas9 constructs targeting the PAX7 3′ UTR (Figure 2C), singly or in pairs. Cells were analyzed by flow cytometry and by bulk junction PCR, followed by Sanger sequencing (Figure 2D).

For the DICE-EPv2 cassette, flow cytometric analysis revealed that the percentage of mCherry$^+$ cells was increased above cassette alone (0.20 ± 0.15%) for sgRNAs PAX7r2-1 and PAX7r3-2, both alone and paired (referred to as PAMs Out Large) with a range of 1.6–2.7% two days post-transfection (Figure 2E and F). We then confirmed that the selection cassette was functional by treating the cells for 4 days with puromycin, which increased the percentage of mCherry$^+$ cells to 18–25%, albeit while also increasing selection for random integration (Supplementary Figure S6A). When using the PTKmChR cassette, we also observed that the percentage of mCherry$^+$ cells was increased above cassette alone (0.56 ± 0.32%) in the presence of all sgRNAs tested, which consisted of two pairs of PAMs 5′, two pairs of PAMs Out (the four pairs previously characterized in Figure 1F), and the PAMs Out Large pair, together and singly for a range of 2.6–5.7% (Figure 2G). Four days of puromycin selection resulted in a lower percentage of randomly integrated cells (6.57 ± 1.64%) than we observed with the DICE-EPv2 cassette and enriched the percentage of mCherry$^+$ cells for the PAMs 5′ and PAMs Out pairs, ranging from 17–33% (Supplementary Figure S6B). These data are also summarized in Table 2.

Subsequent PCR analysis of bulk unsorted cells for all possible junctions of genomic and cassette sequences revealed that knock-in blunt ligation (KiBL) of the PTKmChR cassette resulted in a high level of precise genomic junction repair (94%) and an appreciable degree of precise cassette junction repair (38%; Figure 2Hi and ii). This set of transfections utilized the PAX7-r1 pairs of sgRNAs as shown in Supplementary Figure S7. By contrast, KiBL of the DICE-EPv2 cassette using the PAX7-r2-1 and r3-2 sgRNAs led to more imprecise repair of the genomic junction (60% of sequenced amplicons) and an absence of precise cassette junction repair. We speculated that the lack of precise cassette junction repair with DICE-EPV2 was due to the formation of hairpin structures by the palindromic attP sites. Such hairpins could be unfavorable for direct end-joining and may be more susceptible to nuclease degradation. We also note that the lack of precise cassette junction repair could be due to larger distance between the PAMs-Out-Large pair when compared to the PAX7-r1 pairs. The overall results indicated that the blunt DSBs generated by the CRISPR/Cas9 system could be exploited in mammalian cells to knock-in PCR cassettes in a homology-independent manner. We observed both sense and antisense...
Figure 2. Knock-in blunt ligation (KiBL) in human immortalized cells. (A) The use of two sgRNAs at the same locus should facilitate ligation of exogenous sequence in place of the excised sequence. (B) Schematic of the PGK-PTKmChR and DICE-EPv2 cassettes. (C) Workflow for KiBL experiments: HEK293 cells are transfected with sgRNA/Cas9 vectors and cassettes before flow cytometric analysis and sequence analysis. (D) Schematic diagram of the PAX7 3' UTR region with sgRNAs used in these experiments. (E) Representative flow cytometric data displaying the difference in the percentage of mCherry+ cells between the DICE-EPv2.0 cassette transfected alone and with sgRNA/Cas9 vectors after two days. (F) Quantification of the DICE-EPv2 transfection experiments in terms of percentage of mCherry+ cells using sgRNAs PAX7r2-1 and PAX7r3-2. n = at least two independent experiments consisting of three replicates each for each transfection. Data is shown as the mean ± SEM. (G) Cumulative percentage of sequenced amplicons categorized by repair status of the genome-cassette junction. n for each is displayed below. (H) (i) Cumulative percentage of sequenced genomic/precise cassette junction for the PTKmChR cassette. Boxed sequence denotes PGK promoter sequence, PAM is underlined, and the first four bases of sgRNA target are overlined.
orientations of cassette knock-in, indicating that ligation by precise NHEJ lacked inherent directionality, as expected due to the absence of homologous sequences in the cassettes (Supplementary Figure S7).

To avoid the relatively large size of the PTKmChR and DICE-EPv2 cassettes and the somewhat weak fluorescence of mCherry, we developed a second series of smaller, brighter cassettes. These cassettes consisted of the Ef1α promoter driving the expression of a fluorescent protein, followed by the rabbit β-globin terminator sequence. Our fluorescent proteins of choice were Clover, mRuby2 (both from 29), mCardinal (31), and mCerulean (32) due to minimal spectral overlap and general brightness. These cassettes were less than 2 kb in size, could readily be amplified from template plasmid, and were easily purified. We called these cassettes the pKER (polymerase-chain-reaction-based Knock-in EF1α-RBG terminator) series, and they are depicted in order of brightness in Figure 3A.

To assess quickly the suitability of the pKER series for KiBL, we used pKER-mKOx to investigate the effects of the orientation of the paired PAMs the phosphorylation status of the cassette on the efficiency of KiBL, using the percentage of positive cells as a proxy. We speculated that unphosphorylated cassettes might have an advantage for KiBL, as they may not be detected as free, broken ends of DNA as readily as phosphorylated cassettes, and they may decrease the possibility of amplicon concatemerization. For these experiments, we generated four sgRNAs against the human H11 locus on chromosome 22 (Supplementary Figure S8A). H11 was previously identified as a safe harbor site in the human and mouse genomes (28,33) whose gene structure appeared to be highly conserved in mammals and vertebrates in general (Supplementary Figure S9). We transfected HEK293 cells with phosphorylated or unphosphorylated pKER-mKOx cassettes and pairs of sgRNA/Cas9 vectors in all four paired PAM orientations and analyzed the cells by flow cytometry two days later (Supplementary Figure S8B). In this experiment, we observed a higher percentage of mKOx+ cells in cultures co-transfected with sgRNA/Cas9 vectors (ranging from 8.2 to 12%) than those transfected with cassette alone (1.3–1.5%); Supplementary Figure S8C; also summarized in Table 2). Flow cytometry did not reveal any significant differences between PAM orientations or cassette phosphorylation status in terms of percentage of mKOx+ cells or relative normalized median fluorescence intensity (MFI), other than that the presence of Cas9 increased both measures (Supplementary Figure S8D; P < 0.003 for all comparisons when comparing PAMs treated to cassette alone). These results suggested that the pKER cassettes were well-suited for use in KiBL.

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<th>Table 2. Summary of KiBL efficiencies</th>
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Testing Cas9 fusion proteins and nuclease protection to facilitate precise ligation between genome and cassette

In vitro, the sgRNA–Cas9 complex has been observed to remain bound strongly to its target DNA site following cleavage (4). We hypothesized that facilitating the dissociation of this complex might increase the efficiency or precision of KiBL by allowing the ligation reaction to proceed before significant degradation of the PCR cassette occurred. Accordingly, we sought to generate a less stable version of Cas9. We utilized fusion of the FKBP12-L106P destabilization domain to Cas9 for this purpose. We chose FKBP12-L106P for several reasons. It is relatively small and has been used effectively to destabilize a wide range of proteins. In particular, this domain was beneficial for cell viability when fused to ZFNs (34). The stabilizing ligand for the FKBP12-L106P destabilization domain, Shield-1, is potent, relatively inexpensive, possesses good intracellular availability, and has little effect on cell viability (23,35). We fused the FKBP12-L106P destabilization domain to either the N- or C-terminus of Cas9, generating ‘DD–Cas9′ and ‘Cas9–DD’ respectively (Figure 3B; Supplementary Figure S10A). We then examined the relative stability of Cas9-DD and DD–Cas9 via Western blotting. HEK293 cells were transfected with constructs encoding the H11-r1–2 sgRNA and WT–Cas9, Cas9–DD, or DD–Cas9. After 29 h, we observed a higher level of protein present for both Cas9–DD and DD–Cas9 in cells treated with 0.5 μM Shield-1 compared to cells treated with the DD-containing Cas9 vectors alone (Supplementary Figure S10B). These results suggested that the destabilization domain-containing Cas9s were more sta-
Figure 3. Destabilized Cas9 and nuclease protection effects on precise, seamless genome-cassette ligation. (A) Schematic diagrams of the pKER series of cassettes organized by brightness. Each cassette consists of a fluorescent protein under the control of the EF1α promoter and followed by the rabbit beta-globin terminator sequence. (B) Diagrams of Cas9-DD and DD-Cas9 showing the location of the destabilization domain on SpCas9. The domain is near the PAM-binding domain at the C-terminus. (C) Schematic diagram displaying the workflow for comparing WT-Cas9 to Cas9-DD. HEK293 cells are transfected with pKER-Clover cassette and the PAX7 PAMs Out-Large pair of vectors encoding either WT-Cas9 or Cas9-DD. Two days post-transfection, a portion of the cells are subjected to flow cytometric analysis and the remainder are reserved for genomic DNA isolation for sequence analysis. (D) Representative flow cytometric data for the pKER-Clover cassette and the PAX7 PAMs Out-Large sgRNAs with destabilized Cas9-DD. (E) Quantification of the percentage of Clover+ cells for phosphorylated and unphosphorylated cassettes for WT-Cas9, destabilized Cas9-DD, and stabilized Cas9-DD (1 μM Shield-1 for 24 h). n = 5 independent experiments each consisting of three technical replicates. Data is displayed as the mean ± SEM of the averages of each experiment. **P < 0.001. (F) Diagram of the modified PAX7 3′ UTR locus. This diagram displays pKER-Clover placed at the locus in the 5′ to 3′ orientation. Arrows indicate the primers used to generate amplicons for the sequence analysis in (G). (G) Analysis of genome-cassette junctions for the comparison of WT-Cas9 with destabilized Cas9-DD and the determination of the effect of protecting the cassette from nuclease degradation. Numbers of amplicons analyzed appear beneath their respective treatments. Also depicted are the sequences of the amplicons from the destabilized Cas9-DD junction analysis. Black denotes genomic sequence, turquoise denotes cassette sequence, PAM is underlined, and dashes denote unobserved sequence.
able in the presence of Shield-1 than in its absence. On the other hand, in the context of HEK293 cells, we noticed that destabilized Cas9-DD appeared to be similar in protein level compared to WT-Cas9 and that DD-Cas9 displayed a higher level of protein than WT-Cas9, regardless of whether it was stabilized or destabilized.

Because of the level of excision we observed with our paired sgRNAs in HEK293 cells, we sought to characterize how Cas9–DD might affect KiBL efficiency. Thus, we transfected HEK293 cells with our PAX7 PAMs Out Large sgRNA/Cas9 vector pair encoding either WT–Cas9 or Cas9–DD, and pKER-Clover cassettes that were either phosphorylated or unphosphorylated. Two days after transfection, these cells were subjected to flow cytometric analysis and harvested for genomic DNA (Figure 3C). We chose PAX7 because we had already successfully carried out KiBL and analyzed the pattern of repair at this locus (Figure 2). Flow cytometric analysis confirmed that co-transfection with the sgRNA/Cas9 vectors increased the percentage of Clover+ cells (18–36%) over that of cassette alone [2.4–2.6%; Figure 3D–E; \( P < 0.002 \) for all comparisons]. In terms of the percentage of Clover+ cells, we did not detect any significant difference between WT–Cas9, stabilized Cas9–DD (1 \( \mu \)M Shield-1), and destabilized Cas9–DD, or between phosphorylated or unphosphorylated cassettes (Figure 3E). These data are also summarized in Table 2.

We next sought to determine whether there was an effect of Cas9–DD on the precision of ligation on the molecular level. To this end, we examined the 5′ PAX7 genomic-5′/polymerase-Clover junction resulting from KiBL using unphosphorylated cassettes with WT–Cas9 or destabilized Cas9–DD and the PAMs Out Large sgRNAs (Figure 3F), a pair which had previously failed to yield precise cassette junctions (Figure 2Hi). We also examined whether the addition of three phosphorothioate bonds at the 5′ ends of the cassettes affected the precision of the cassette side of the junction by potentially protecting the cassette from nuclease degradation. We observed that unprotected cassettes co-transfected with WT–Cas9 resulted in precise genomic junctions, but imprecise cassette junctions in 100% of observed amplicons (n = 6/6; Figure 3G). Protecting the 5′ ends of the cassette resulted in an increase in precise genomic/predictive cassette junctions (40% of amplicons; n = 2/5) when using WT–Cas9 (Figure 3G). When destabilized Cas9–DD was used in conjunction with the cassette with phosphorothioate bonds, we observed a similar increase in precise genomic/predictive cassette junctions (60% of amplicons; n = 6/10). We also observed a small increase in imprecise genomic/imprecise cassette junctions (20% of amplicons; n = 2/10; Figure 3G). These doubly imprecise junctions were identical to each other. Because the highest proportion of events with precise ligation occurred when we used protected 5′ ends, these results supported the use of nuclease protection of pKER cassettes to facilitate precise, seamless ligation in KiBL in HEK293 cells. However, we did not detect a significant difference in precise ligation between WT–Cas9 and destabilized Cas9–DD.

We also sought to apply KiBL to human iPSCs (hiPSCs), because successful application of this method might provide a viable alternative to traditional nuclease-mediated homologous recombination (HR). For these experiments, we used the JF10 line of hiPSCs generated from a healthy donor and chose H11 as our target locus (Supplementary Figure S11A). To minimize the effect of untransfected cells on downstream analyses, we isolated fluorescent-protein-positive cells via FACS four to seven days after electroporation with unphosphorylated, nuclease-protected pKER cassettes, the PAMs Out sgRNA/Cas9 vector pair, and stabilized Cas9–DD or DD–Cas9 (Supplementary Figure S11B–D). To analyze the degree of precise ligation at the junctions, we carried out targeted genomic amplification across the H11 locus on pooled Clover+ cells in order to capture all of the KiBL events (Supplementary Figure S11E–F) and identified precise, seamless ligation at both the 5′ and 3′ junctions of the cassette (Supplementary Figure S11F–G). These data are summarized in Table 2 and further discussed in the legend of Supplementary Figure S11. Although the efficiency of the reaction was low compared to HEK293 cells, these results demonstrated that KiBL was feasible in hiPSCs and that stabilized Cas9–DD facilitated precise, seamless ligation for KiBL in hiPSCs.

**Paired sgRNAs combined with short homology arms facilitate homologous recombination in hiPSCs**

We then sought to characterize the potential application of our pKER cassettes to HR in such a way that we could track the number of individual events. We employed a two-step approach to generate pKER-Clover cassettes containing 6-base barcodes and 50-base homology arms (Supplementary Figure S12A). Homology arms of this size have previously been used with the CRISPR/Cas9 system to facilitate homologous recombination in human immortalized cell lines (36) and mouse ESCs (37). We note that this method of generating HR cassettes could lead to an excess of cassettes possessing overhangs rather than blunt ends, making direct comparison with KiBL rather difficult. After co-transfection with the H11 PAMs Out sgRNA pairs and Cas9-DD, followed by isolation with FACS, sequence analysis of the 5′ genomic-5′ Clover junction revealed that HR occurred several times (Supplementary Figure S12B). After isolation with FACS, PCR amplification of the 5′ genomic-5′ Clover junction, and subsequent subcloning, we observed 11 different barcodes in 12 sequenced amplicons (Supplementary Figure S12B). Of these 11, we observed that HR occurred 10 times due to the possession of a thymine (the human reference allele, which we had placed in the 5′ homology arm) instead of cytosine (the allele possessed by JF10) in the position prior to the PAM that differed from reference, indicating that homologous recombination did indeed occur. We also observed that both alleles could undergo HR, as indicated by detection of both alleles of our allele-specific T/C SNP on the 5′ side of the locus. Additionally, we observed one non-barcoded KiBL event and a barcoded KiBL event where the cassette appeared to lack at least the 5′ homology arm. We did not observe any events where the homology arm was incorporated via NHEJ rather than HR. These results indicated that several independent events occurred in the Clover+ populations. To compare the frequency of KiBL to HR, we calculated the normalized fold change of Clover+ cells observed in KiBL or HR to the cassette-alone control for each set of experi-
ments, focusing on Cas9–DD as we did not test the ability of WT–Cas9 or DD–Cas9 to facilitate HR. This comparison suggested that the frequency of KiBL may be higher than the frequency of HR, which is consistent with the preference for NHEJ over HR (Supplementary Figure S12C). These data demonstrated that PCR amplicons possessing short homology arms combined with the CRISPR/Cas9 system facilitated homologous recombination in hiPSCs, which had not, to our knowledge, previously been demonstrated.

Analysis of off-target KiBL events

Because KiBL utilizes the CRISPR/Cas9 system, there was a concern that off-target cleavage could result in the uptake of cassettes at sites other than the targeted locus. Indeed, such events have been observed in HEK293T cells using the GUIDE-seq method for unbiased identification of CRISPR/Cas9 off-target cleavage events (38). We reasoned that KiBL utilizing the pKER cassettes would allow us easily to identify off-target cleavage and repair in hiPSCs. Thus, we chose to examine the top six off-target sites for each sgRNA in the H11 PAMs Out pair as determined by the MIT CRISPR Design tool. For each off-target site, we attempted to amplify the 5′ genomic-5′ cassette, 3′ genomic-5′ cassette, 5′ genomic-3′ cassette, and 3′ genomic-3′ cassette junctions, which led to 48 off-target reactions per sample. In order to carry out this large number of assays on the number of Clover+ cells isolated by FACS sorting, we performed whole genome amplification as described in the Materials and Methods to ensure that there would be enough DNA. We analyzed seven pools of Clovers+ cells: one pool treated with WT-Cas9, two pools treated with destabilized Cas9–DD, two pools treated with Cas9–DD and 0.5 μM Shield-1, and two pools treated with Cas9–DD and 1 μM Shield-1. We were unable to detect off-target KiBL events at the vast majority of predicted junctions, with the exception of faint detection of the 3′H11 sgRNA OT4 genomic reverse primer-3′ pKER cassette junction (Figure 4). We also note that obtaining Clover+ cells from the WT–Cas9 was rather difficult in hiPSCs in contrast to HEK293 cells. These data suggested that, at least in hiPSCs, KiBL occurred predominantly at the on-target locus, with the caveat that the frequency of off-target KiBL events might be below the sensitivity of our assay.

DISCUSSION

We demonstrated in this work that the blunt-end DSBs generated by the CRISPR/Cas9 system mediate repair with a high degree of precision and can be exploited to insert exogenous sequences precisely into the genome through knock-in blunt ligation. When using two sgRNAs simultaneously, we observed that all four paired PAM orientations were capable of facilitating precise repair, which was observed in both human and mouse cells. We then demonstrated that PCR amplicons lacking homology arms can be used to replace the sequence between sgRNA targets precisely and that this in vivo blunt-end cloning functioned in an immortalized human cell line and in hiPSCs. Moreover, we developed a destabilized variant of Cas9 and increased precise ligation efficiency through the incorporation of nuclease protection in the form of phosphorothioate bonds in the cassette.

Other strategies utilizing the CRISPR/Cas9 system to facilitate insertion of exogenous sequences into the genome have relied on knocking in plasmid DNA through NHEJ (21,22) or microhomology-mediated end-joining (39). In general, the plasmid-based methods rely on in vivo cleavage of a plasmid donor to mediate integration, which was previously demonstrated with ZFNs (40). These methods introduce additional undesired sequence into the genome due to inclusion of the plasmid backbone, whereas KiBL only introduces the knock-in cassette. Additionally, the Cas9 nicks have been used to knock-in a double-stranded oligonucleotide with compatible cohesive overhangs in a strategy similar to the ObLiGaRe method used with ZFNs, albeit with relatively low efficiency (41,42). The ObLiGaRe method relies on the creation and accessibility of such overhangs, limiting its ease of use in modifying the genome, which is not a concern with KiBL. However, we note KiBL should theoretically result in the desired knock-in orientation in 50% of modified alleles, although we have not directly tested this.

Another set of methods utilizing PCR amplicons has been developed for HR and homology-directed repair (HDR). In cultured Drosophila cells, the use of PCR amplicons containing homology arms has been used to great effect (43), which is similar to what has been observed with human immortalized cell lines and mouse ESCs (36,37). A subset of these methods has focused on increasing HDR frequency through cell cycle synchronization (44) and by inhibition of the NHEJ machinery (45–47), which itself was first successfully used with zinc finger nucleases in Drosophila (48,49). Our method offers the advantage of potentially functioning during the G1, S, and G2 phases of the cell cycle, whereas the HR and HDR strategies are restricted to late S and G2 phases. Thus, our method might be useful in non-dividing cells such as neurons, where the application of Cas9 has been limited to generating mutations through error-prone repair (50). Moreover, some cells such as aged hematopoietic stem cells and aged skeletal muscle stem cells retain the capacity to precisely repair DSBs through NHEJ, which might make KiBL a useful method for genome engineering in them, particularly in their quiescent, i.e., non-dividing, state (50–53). Additionally, KiBL may prove amenable to high-throughput applications such as saturation editing, whereas HR would be labor-intensive due to the necessity of constructing homology arms for each targeted locus (54).

Strategies for controlling Cas9 at the protein level include using less sgRNA and Cas9, controlling Cas9 expression through the inducible Tet-ON system, and splitting Cas9 in half (55–58). Inducible expression does not provide posttranslational control, whereas the split-Cas9 variants, while potentially controllable post-translationally, rely on either the sgRNA or rapamycin to bring the two halves together (57,58). Both approaches also increase the number of individual components in the system. Our destabilized Cas9 retains the simplicity of requiring only the Cas9 and sgRNA expression cassettes. Fortunately, Shield-1 does not induce any undesired responses when applied to cells in culture...
Figure 4. Targeted analysis of off-target KiBL events in hiPSCs. Genomic DNA from various CRISPR/Cas9-treated UPT-Clover+ cells was subjected to whole genome amplification and used for analysis of the top six off-target sites (denoted OT1–6) for both the sgRNAs of the H11 PAMs Out pair. Each off-target site was analyzed using a sense (denoted F) and an antisense (denoted R) primer designed to amplify the off-target locus. Each F and R primer were used in two amplifications, one with the 5′ pKER cassette detection primer and the other with the 3′ pKER cassette detection primer. H11 F and H11 R correspond to the primers H11-X1-Fwd and H11-X1-Rev respectively and act as on-target controls. White indicates lack of detection, grey indicates faint detection, and black indicates strong detection. Each Cas9 treatment originated from an independent pool of cells.

or in animals, unlike rapamycin, (23,35,59). Recently, an additional method was described using an evolved ligand-dependent intein to restrict the activity of Cas9. Binding of the 4-hydroxytamoxifen ligand resulted in the cleavage of the intein and activation of Cas9 (60). Such a system is elegant, but possesses the drawback of being unable to restrict the activity of Cas9 once the intein is cleaved. It would be interesting to combine the destabilization domain with this intein, because such a combination could allow for an unprecedented level of control of Cas9 activity.

In this work, we chose to identify off-target KiBL events through a targeted, candidate-based method utilizing off-target sites possessing the fewest number of mismatches. While this method provides a good starting point, it does not fully account for the finding that SpCas9 has been reported to tolerate bulges, mismatches, insertions, and deletions in the target DNA and the sgRNA, cleave at non-canonical PAMs, and tolerate more than four mismatches (38,51,61–68). Whole genome sequencing would identify the off-target KiBL events, in addition to error-prone off-target cleavage repair, but would be more appropriate for characterizing individual hiPSC clones. Fortunately, error-prone off-target activity appears not to occur at high frequency in primary stem cells and hiPSCs (69–72) and rarely in CRISPR/Cas9-generated mutant mice (73). Our results are in agreement with these reports. We also note KiBL could itself be used to identify off-target cleavage events in an unbiased manner similar to that of the unbiased detection methods of GUIDE-seq (38) and utilization of integrase-deficient lentiviral vectors (74). Ideally, this would be combined with a linear amplification-based method to maximize sensitivity, similar to the strategies used in identifying nuclease-generated translocations (75) and in measuring the frequency of adeno-associated virus-mediated promotorless gene targeting (76).

We have presented a method for utilizing the CRISPR/Cas9 system to facilitate in vivo blunt-end cloning in precise, homology-independent manner through NHEJ. This method, KiBL, is enabled by the ability of Cas9 to generate blunt-end DSBs, which we and others have exploited using two sgRNAs simultaneously to facilitate precise excision of the intervening sequence (5,6,26,36). The main advantages of our method are its high level of precise ligation, lack of incorporation of additional exogenous sequences, freedom from the need to clone homology arms, and potential ability to function throughout the cell cycle. The main drawbacks of the method include the challenge of generating large amounts of cassette DNA, the lower electroporation efficiency of linear dsDNA, and the theoretical caveat that only 50% of events should occur in the desired orientation. The limitation of the small amount of cassette DNA generated by PCR greatly affects directly comparing KiBL to CRISPR/Cas9-mediated plasmid-based HR, because HR uses at least 10-fold more donor vector than our method (≥2μg versus 200ng) (77). Thus, there is more donor vector available for incorporation in HR relative to KiBL. Together, our analysis of paired sgRNA-mediated deletions and observations by others...
underscore that NHEJ resulting from blunt-end, chemically unmodified DSBs, such as those made by Cas9, are repaired with a high degree of precision. These observations suggest not only that NHEJ is not as error-prone as commonly thought, but also that precise repair may be the predominant NHEJ repair mechanism (78). In summary, KiBL is a versatile method capable of facilitating advanced genome engineering strategies with a high degree of precision.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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