**BANDYOPADHYAY & DOUGLASS ET AL.
SUPPLEMENTAL TEXT**

**Directionality of transgene integration affects gene expression**

Although the plasmid contains its own PGK promoter driving the expression of the puromycin resistance marker, we were unable to recover puromycin-resistant cells when the selection cassette’s homology arms were in the ‘Reversed’ orientation of the direction in which EMX1 locus is transcribed (Fig. 1E, data not shown). To further test if the directionality of the transgene relative to the 3’ end of the EMX1 locus matters for transgene expression, we replaced the puromycin selection gene with three other reporter genes (ZsGreen, mCherry, luciferase). These constructs also lacked expression when oriented reverse to the transcription direction of EMX1 (Fig. 1F). For two of these reporter genes, the reverse-oriented reporter gene mRNA levels were measurably lower than the sense-oriented reporter mRNAs (**Figure S1A**). This observation prompted us to hypothesize that the reporter transgene being inserted in the reverse orientation might generate reverse RNAs that cause silencing of both the transgene and endogenous EMX1 transcripts. To test this hypothesis, we used a small hairpin RNA (shRNA) (Zeng *et al.* 2013) to knock down endogenous EMX1 mRNAs to see if this relieved repression of the transgene (Fig. S1B, S1C). Only minimal increase in the expression of two different reporter genes was observed after EMX1 mRNA knockdown (Fig. S1D, S1E), suggesting that the poor expression was not due to the sequestration of the transgene mRNA but rather due to other factors such as unstable 3' UTR sequences in the reverse elements (Hughes 2006). Although we do not think there is promoter interference since the EMX1 homology arms are from the 3’ end of the gene and the plasmid has its own PGK promoter, this result highlights the importance of the orientation of homology arms around the transgene for Cas9-stimulated HDR and is consistent with an earlier observation of directionality affecting the transgene activity (Lombardo *et al.* 2011).

**Process to a label a plasmid repair template with a benzylguanine (BG) moiety.**

To enable tethering of a long transgene DNA to Cas9-SNAP we needed to quantitatively incorporate a BG moiety. We first tried the simplest format of using modified oligonucleotides with BG coupled to the 5' end to generate the transgene by PCR using primers to be then transfected with the Cas9 ribonucleoprotein (RNP). Because a 5' primary amine oligonucleotide was used to react to the BG-NHS-ester, we also tested transgene PCR products bearing the different 5' modifications such as phosphates, primary amines, a 5' phosphorthioate bond, both modifications, and BG coupled to the amine. We then transfected HEK293FT cells with these PCR products in the presence of Cas9-SNAP and performed cell selection with puromycin. Unexpectedly, only those PCR products generated with unmodified or phosphorylated primers resulted in puromycin-resistant cells, indicating successful integration of the transgene, whereas other modifications significantly reduced genomic integration (Fig. S1F). When PCR amplicons versus linear plasmids were compared in a side-by-side transfection and selection experiments, the longer plasmids enabled many more cells to survive puromycin selection (Fig. S1G). Therefore, we decided to develop plasmids further as a repair template because circular plasmids would be less likely to stimulate DNA damage signaling (Lupardus *et al.* 2002; MacDougall *et al.* 2007; Shiotani and Zou 2009).

Next, we developed a method for BG-labeling of plasmids containing the HDR template (**Figure S2A**). For this, we appended the BG moiety to the 5' end of primers and amplified a 2.4 kb amplicon with sense-oriented EMX1 homology arms (Fig. 1A), and used fluorescein-labeled (FL-) oligonucleotides as a proxy for the BG-oligonucleotides inserting and ligating into a plasmid backbone (Fig. S2B). Excess BG- and FL- oligonucleotides efficiently replaced the nicked fragments during a gentle denaturation/renaturation process, and unincorporated oligonucleotides were removed by PEG precipitation (Fig. S2C,2D). After ligation with T4 DNA ligase, we confirmed covalent labeling of the modified oligonucleotides of the plasmid backbone and association with Cas9-SNAP (Fig. S2E).

**Long single-stranded DNAs and Mini-circles also underperform in Cas9-stimulated HDR**

 If strand invasion via a single homology arm from a Cas9 cut site explains how ssODNs are such efficient templates for Cas9-stimulated HDR, we considered this notion that long single-stranded DNA (lssDNA) might also promote better HDR at the EMX1 locus, as has been suggested by other studies (Quadros *et al.* 2017; Minev *et al.* 2019). Therefore, we re-engineered our plasmid templates such that after enzymatic nicking, denaturation, and agarose gel purification, we could generate three different forms of lssDNAs: a 2kb lssDNA with two homology arms (2A) and two ~1.4 kb lssDNAs with a single homology arm, located either upstream (UA) or downstream (DA) of the transgene (**Figure S4**). Unexpectedly, all of the lssDNAs exhibited poor Cas9-stimulated HDR compared to the double-stranded plasmid (Fig. S4B, S4C). Whereas the plasmid template routinely yielded Amplicons-A and -B, only the 2A lssDNA yielded Amplicon-B and not -A, with no junction amplicons with the UA or DA single homology arm lssDNAs. Finally, the transgene-spanning Amplicon C' remained elusive with lssDNA repair templates. Thus, our data could not replicate the efficiency of lssDNAs as suggested by others (Quadros *et al.* 2017; Minev *et al.* 2019), but rather are consistent with the prevailing issues recently described in the Cas-9 genome editing literature (Gurumurthy *et al.* 2019).

 Based on these results, we rationalized that long dsDNA may still be a better repair template than lssDNAs. To avoid bacterial vector sequence integration, we generated ‘Mini-circles’ (MCs) of the 2A, UA, and DA repair template plasmids targeting EMX1 (Fig. S4D). MCs lacking bacterial vector sequence have been shown to express transgenes more efficiently and persist longer in transfected mammalian cells (Chen *et al.* 2003; Gracey Maniar *et al.* 2013) . Similar to lssDNAs, the two-homology arm MC (2-A), but no single homology arm MCs (UA, DA), showed modest Cas9-stimulated HDR when tested by detection of transgene junction sites (2-A) (Fig. S4F). However, no clear integration of the full-length transgene was observed for any of the three MCs.

**SUPPLEMENTARY FIGURE AND TABLE LEGENDS

Figure S1. Directionality of homology arms can impact selection marker expression, while blunt end dsDNA PCR amplicons are less suitable templates for Cas9-stimulated HDR.**
A) Quantitative RT-PCR analysis of reporter gene expression from “Direct” (Dir) and “Reversed” (Rev) pCNE-based plasmids. B) Design of shRNAs; C) and effective knockdown of endogenous EMX1 transcripts in HEK293FT cells. D) Top panels with GFP expression confirm shRNA transfection, but this does not improve the poor expression of mCherry cloned into pCNE-plasmid. E) Similar lack of luciferase expression that is not improved by knocking down EMX1 with shRNAs. F) Comparisons of unmodified dsDNA PCR amplicons versus amplicons with primers that have a 5' end modifications. Error bars represent standard deviation of three biological replicates measuring amount of puromycin-selected cells. Agarose gel showing similar levels of each dsDNA PCR amplicon with modified 5' ends are comparable to the unmodified dsDNA amplicon. G) Comparison of a PCR amplicon versus a plasmid template, where transfections contained the same molar amount of puromycin marker as well as balanced same mass of DNA with carrier plasmid added to the PCR amplicon.

**Figure S2. Procedure to label plasmids with a BenzylGuanine (BG) coupled oligonucleotide.**(A) Chemical coupling of BG-NHS ester to the primary amine of an internal dT with a C6-amino group. (B) The pCNEPm3 plasmid has homology arms to EMX1 flanking a PGK promoter and puromycin acetyltransferase. A tandem array of Nb.BbvCI sites in the backbone is nicked and heated to liberate the cut fragments on one strand, which can then be quantitatively replaced with an oligonucleotide that is coupled with BG or a tracer amount of Fluorescein (FL) for tracking the labeling reactions. (C) Retention of RNT-FL and RNT-BG oligos onto nicked and refolded plasmids. The fluorescent RNT-FL oligo serves as a proxy for plasmid labeling with RNT-BG. (D) Removal of unincorporated oligo by precipitating plasmids with PEG followed by additional magnetic bead based reverse oligo depletion. Quantitation of fluorescent signal of gel scan on the right. (E) Demonstration of covalent linkage of RNT-BG + RNT-FL oligos to plasmids after T4 DNA ligase reaction, because ligated oligos do not get denatured away from plasmid during heating with urea. Gel on the left is a fluorescence scan for the RNT-FL tracer oligo, while the right gel is later stained with SYBR gold to image total plasmids. Graph is quantitation of gel on the left.

**Figure S3. Promiscuous HDR and transgene integration with nicked plasmids in HEK293FT cells.**

(A) Crystal violet staining of HEK293FT cells transfected with pCNEPm3 plasmid that is intact versus linearized or nicked with NtBbvCI. Top plate compares having only one component of Cas9 or sgRNA, where the transgene is able to integrate without Cas9-stimulation. Bottom plate further compares complete Cas9-SNAP versus no sgRNA. (B) Genomic PCR assays in the same format as Fig. 3 and 4, detecting the left junction (Amp.-A), right junction (Amp.-B) and transgene-spanning products (Amp.-C). Blue arrows point to specific lanes lacking sgRNA, where the Intact plasmid does not integrate at the EMX1 locus, but the Nicked plasmid is able to integrate in an HDR-like manner without Cas9 stimulation. The black arrowheads point to the 1.2 kb size of Amplicon-A and –B and the 2.2 kb size of Amplicon-C from the endogenous EMX1 locus. A desired HDR event generating a 3.3 kb Amplicon-C' is not observed (green arrowhead). (C) The BG- and FL- labeled plasmids after T4 DNA ligase repair as detailed in Fig. S2, then subjected to digestion with λ-Exonuclease that removes nicked plasmids but leaves behind fully-repaired intact plasmids. (D) Crystal violet staining of puromycin-selected cells after transfected with BG- and FL-labeled pCNEPm3 plasmid with or without λ-Exonuclease treatment. (E) Genomic PCR assays in the same format as (B).

**Figure S4. Long single-stranded DNAs (lssDNAs) and Mini-circles (MC) also underperform in Cas9-stimulated HDR.**A) Schematic of generating lssDNAs from nicking of plasmids and denaturing and resolving smallest linear fragment on an agarose gel. Left panel shows the constructs with both homology arms (2-A) or a single homology arm. (B) Genomic PCR of puromycin-selected cells after transfecting just the 2-Arm lssDNA, Cas9-SNAP and sgRNA\_21. The respective Amplicons- A, -B, and –C are the same as in Fig. 3 and 4, where the black arrowheads point to the 1.2 kb size of Amplicon-A and –B and the 2.2 kb size of Amplicon-C from the endogenous EMX1 locus. A desired HDR event generating a 3.3 kb Amplicon-C' is not observed (green arrowhead). (C) Genomic PCR analysis of lssDNA templates using two sgRNAs, with format of assay similar to (B). (D) Diagram of initial concept to generate MC repair templates where vector backbone bacterial sequences are recombined out to yield a MC DNA, and in this case a single homology arm. The formats of the Two-arm (2A), Upstream-arm (UA) and Downstream-arm (DA) constructs are as detailed in A). (E) Gel showing efficiency of generating the MCs after the 4.4 kb plasmid backbone is removed. Although the MC is the dominant product, larger contaminating recombinants still persist in the purification. (F) Genomic PCR of puromycin-selected cells after transfecting just the 2A, UA, and DA MCs along with Cas9-SNAP and sgRNAs. The respective Amplicons- A, -B, and -C are the same as in Fig. 2 and 3, where the black arrowheads point to the 1.2 kb size of Amplicon-A and -B and the 2.2 kb size of Amplicon-C from the endogenous EMX1 locus. A desired HDR event generating a 3.3 kb Amplicon-C' is not readily observed (green arrowhead).

**Figure S5. Improved Cas9-stimulated HDR at EMX1 with Long 3' Single-Stranded Overhang (L3SSO) DNAs.**

A) Schematic of method to generate L3SSO DNAs from multiple nicking of plasmid and denaturing gel electrophoresis. Final panel shows the model for improved HDR. B) Representative experiment of Crystal violet staining and quantitation of puromycin-selected cells nucleofected with Cas9SNAP-RNPs and either the intact pCHEP2 plasmid or the L3SSO cut and purified from pCHEP2. C) Schematic of poison-primer approach to detect proper HDR event at EMX1. D) Genomic PCR analysis of the EMX1 locus for integration of L3SSO Format for detecting Amplicons –A and –B is the same as in Fig. 2C. Gel for Amplicon-C2' are only showing the products of the 2o PCR while the 1o PCR with poison primers is not shown. E) Diagrams of the sequenced Amplicon-C2' products compared to the L3SSO DNA repair template. UA: Upstream arm, DA: Downstream arm, E: EcoRI, C: ClaI, P: PstI, H: HindIII, NbB: Nb.BbvCI, X: XmaI restriction sites. Patterned arrows represent L3SSO segments, while green arrows represent the endogenous EMX1 locus sequence.

**Figure S6. Comparing BL3SSO template efficiency at other genomic loci.**Schematics of GFP BL3SSO to target: A) the Piwi5 gene locus in *Aedes aegypti* mosquito Aag2 cells, and B) the ISG56/IFIT1 locus in human cells. Flow cytometry analysis of GFP positive cells after transfection of BL3SSO constructs and Cas9/sgRNA RNPs targeting C) the Piwi5 and Ago3 loci in Aag2 cells, and D) ISG15, ISG56 and ACE2 loci in HEK293 cells. E) Direct comparison of BL3SSO to L3SSO and plasmids that contain the cloned L3SSO sequence in Cas9 directed HDR at human AGO2, Viperin, and ISG15 loci. Schematic shows targeting to the second exon of AGO2, four flow cytometry plots as examples, and the quantitation of GFP-positive cells across the samples in the bar graph below.

**Table S1. Oligonucleotides, sgRNA templates and primers used in this study.**

**Supplemental references**

Chen, Z. Y., C. Y. He, A. Ehrhardt and M. A. Kay, 2003 Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. Mol Ther 8**:** 495-500.

Gracey Maniar, L. E., J. M. Maniar, Z. Y. Chen, J. Lu, A. Z. Fire *et al.*, 2013 Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Mol Ther 21**:** 131-138.

Gurumurthy, C. B., A. R. O'Brien, R. M. Quadros, J. Adams, Jr., P. Alcaide *et al.*, 2019 Reproducibility of CRISPR-Cas9 methods for generation of conditional mouse alleles: a multi-center evaluation. Genome Biol 20**:** 171.

Hughes, T. A., 2006 Regulation of gene expression by alternative untranslated regions. Trends Genet 22**:** 119-122.

Lombardo, A., D. Cesana, P. Genovese, B. Di Stefano, E. Provasi *et al.*, 2011 Site-specific integration and tailoring of cassette design for sustainable gene transfer. Nat Methods 8**:** 861-869.

Lupardus, P. J., T. Byun, M. C. Yee, M. Hekmat-Nejad and K. A. Cimprich, 2002 A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. Genes Dev 16**:** 2327-2332.

MacDougall, C. A., T. S. Byun, C. Van, M. C. Yee and K. A. Cimprich, 2007 The structural determinants of checkpoint activation. Genes Dev 21**:** 898-903.

Minev, D., R. Guerra, J. Y. Kishi, C. Smith, E. Krieg *et al.*, 2019 Rapid in vitro production of single-stranded DNA. Nucleic Acids Res 47**:** 11956-11962.

Quadros, R. M., H. Miura, D. W. Harms, H. Akatsuka, T. Sato *et al.*, 2017 Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol 18**:** 92.

Shiotani, B., and L. Zou, 2009 Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. Mol Cell 33**:** 547-558.

Zeng, M., M. S. Kuzirian, L. Harper, S. Paradis, T. Nakayama *et al.*, 2013 Organic small hairpin RNAs (OshR): a do-it-yourself platform for transgene-based gene silencing. Methods 63**:** 101-109.