Chimeric RNA: DNA TracrRNA Improves Homology-Directed Repair In Vitro and In Vivo

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Abstract
Nearly 90% of human pathogenic mutations are caused by small genetic variations, and methods to correct these errors efficiently are critically important. One way to make small DNA changes is providing a single-stranded oligo deoxynucleotide (ssODN) containing an alteration coupled with a targeted double-strand break (DSB) at the target locus in the genome. Coupling an ssODN donor with a CRISPR-Cas9-mediated DSB is one of the most streamlined approaches to introduce small changes. However, in many systems, this approach is inefficient and introduces imprecise repair at the genetic junctions. We herein report a technology that uses spatiotemporal localization of an ssODN with CRISPR-Cas9 to improve gene alteration. We show that by fusing an ssODN template to the trans-activating RNA (tracrRNA), we recover precise genetic alterations, with increased integration and precision in vitro and in vivo. Finally, we show that this technology can be used to enhance gene conversion with other gene editing tools such as transcription activator like effector nucleases.

Introduction
Targeted genomic alterations have revolutionized the field of molecular biology. Most recently, efforts have focused on CRISPR and cognate CRISPR-associated proteins (Cas), namely Cas9.1–3 Cas effectors are directed to a region in the genome by a ∼20 nt guide RNA and induce a targeted double-strand break (DSB).3 In the absence of a repair template, the cell typically repairs the lesion by an un-templated repair pathway such as non-homologous end joining (NHEJ)4 or alternative end joining (Alt-EJ).5,6 In the presence of a repair template such as a single-stranded DNA (ssDNA) or a double-stranded DNA (dsDNA) template, precise repair is triggered that is directed by regions of homology on the donor.7

Repair with a dsDNA donor is relatively inefficient in most cell types.8 Alternatively, repair via an ssDNA donor is efficient in multiple systems8–10 and has been demonstrated to be efficient in primary cell types such as T cells for the generation of chimeric antigen receptor T cells11 as well as induced pluripotent stem cells (iPSCs).12 Studies have demonstrated that ssODNs are sufficient for inserting small (<50bp) changes into the genome at appreciable rates.13 Whereas dsDNA-mediated gene replacement uses homologies of ~0.5–1 kbp, ssDNA-mediated gene replacement often proceeds using shorter homologies.14 For example, the length of flanking homologies has been shown to be less important with smaller cargos.14,15 Rates of homology-directed repair (HDR) with Cas9-mediated DSBs and an ssODN in zebrafish are typically modest,16,17 with imprecise sequence changes regularly introduced at the genetic junctions.18 Imprecision at the insertion junctions has been explained partly by the integration of fragments of the ssODN template.19 Though ssODN-mediated changes are sufficient for some applications, the low efficiency and imprecision can make this difficult to deploy in highly precise applications.

Recent work has shown that spatiotemporal localization of an ssODN via covalent linkage to Cas9 increases rates of HDR.20,21 However, these approaches require protein–nucleic acid engineering and can be complex to adopt. The observations using a protein–nucleic acid

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fusion suggest that improving spatiotemporal localization of the repair template to the site of the DSB increases rates of HDR.

Even when targeted, DNA DSBs are still a threat to genomic integrity. Unexpected alterations such as small insertions and deletions (indels) and chromosomal rearrangements are common genetic byproducts. Due to the potentially detrimental consequences, methods have been developed that preclude a targeted DSB coupled with a repair template, including base editing (BE)\textsuperscript{32,23} and prime editing (PE).\textsuperscript{24} Though these technologies do not require DSB-triggered HDR, they are not without drawbacks. Even with several iterations of the BE system, the editing window remains relatively large and renders precision applications difficult. Additionally, prime editing requires several large and potentially toxic or immunogenic components and relies on delivery and activity of a viral reverse transcriptase.

We herein report a methodology that is backwards compatible with CRISPR-Cas9 that uses spatiotemporal components of CRISPR-Cas9 and minimal homologies sufficient for small DNA modifications. We demonstrate that covalent fusion of an ssDNA repair template to the tracrRNA, deliverable as CRISPR-Cas9 RNP, results in precise gene conversion with as little as 10 nt of homology. We demonstrate that our approach, which we term Donorguide, is amenable to both \textit{in vitro} and \textit{in vivo} applications. Additionally, we find that Donorguide can enhance the activity of other gene editing tools such as transcription activator like effector nucleases (TALENs) to increase HDR.

**Methods**

**Cell culture**

HEK293T cells were obtained from ATCC (CRL-3216). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco #11995-040) supplemented with 10% fetal bovine serum (Gibco #26140079) and 1% penicillin/streptomycin (Gibco #15140-122).

**In vitro Donorguide RNP preparation**

crRNAs (Supplementary Table S1) were ordered from Integrated DNA Technologies and suspended at a concentration of 200 µM in 1×TE. The chimeric tracrRNAs were ordered from IDT as RNA ultramers (Supplementary Table S1) and suspended at a concentration of 200 µM in 1×TE. To assemble the Donorguide for \textit{in vitro} studies, 3 µL of 200 µM crRNA was combined with 3 µL of 200 µM chimeric tracrRNA and heated to 95°C for 5 min and immediately removed to cool on the bench top to room temperature (RT) for 10–20 min.

Five microliters of the 100 µM crRNA:tracrRNA was added to a new nuclease-free polymerase chain reaction (PCR) tube. Four microliters of Alt-R Cas9 Nuclease 3NLs (62 µM stock; IDT cat. #1074181 1074182) was added to bring to the final volume to 9 µL, and this was incubated at RT for 20–30 min to form the RNP complex.

**Transfections**

For Donorguide HEK293T electroporations, 9 µL of Donorguide RNP complex (see above) was diluted with 16 µL in EP buffer (Etta Biotech Co.) to make a final volume of 25 µL. For ssODN HEK293T electroporations, 5 µL of 100 µM ssODN was added to 9 µL of the CRISPR-Cas9 RNP and diluted to 25 µL with EP. Diluted Donorguide or CRISPR-Cas9 RNP was added to 300 µL HEK293T cells at a concentration of 20E6 cells/mL in EP buffer and mixed thoroughly. Then, 105 µL of the HEK293T cell+ RNP was added to triplicate cuvettes.

For TALEN dCas9 Donorguide electroporations, 9 µL of dCas9 Donorguide RNP or 5 µL of 100 µM ssODN with 4 µL phosphate-buffered saline (PBS) was brought to 25 µL with EP buffer. Donorguide RNP complex or ssODN mix was added to 300 µL HEK293T cells in EP buffer at a concentration of 20E6 cells/mL and mixed thoroughly. Then, 107 µL of the HEK293T cell+ Donorguide cocktail was equally distributed to three Eppendorf tubes. Two-and-a-half micrograms of each of the TALEN arm plasmids were added to each tube and then transferred to an electroporation cuvette.

HEK293T cells were electroporated with Etta H1 electroporator (Etta Biotech Co.) with the following parameters: 200 V, 784 ms interval, six pulses, and 1,000 µs duration. Cells were then transferred to a six-well tissue cell culture plate at a density of about 1.5E6 cells/mL in complete DMEM.

**HEK293T cell DNA isolation and PCR amplification**

DNA from whole-cell populations was purified using the Qiagen DNeasy Blood and Tissue Kit (Qiagen; 69504). PCR amplification was conducted with Platinum SuperFi DNA Polymerase (Thermo Fisher Scientific; 12351010) or Q5® High-Fidelity 2× Master Mix (New England Biolabs; M0492S) and purified with the Qiagen QIAquick PCR Purification Kit (Qiagen; 28104).

**GENEWIZ AmpliconEZ: DNA library preparation and Illumina sequencing**

PCR products were obtained and purified as mentioned above. Illumina partial adapters (forward sequencing read: 5’-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT-3’; reverse sequencing read: 5’-GAC TGG AGT TCA GAC GTG TGC TCT TCG TCC GAT CT-3’) were added to the 5’ end of sequencing primers. DNA
library preparations, sequencing reactions, and initial bioinformatics analyses were conducted at GENEWIZ, Inc. DNA library preparation, clustering, and sequencing reagents were used throughout the process using the NEBNext Ultra DNA Library Prep Kit, following the manufacturer’s recommendations (Illumina).

End-repaired adapters were ligated after adenylation of the 3’ ends followed by enrichment by limited cycle PCR. DNA libraries were validated on the Agilent TapeStation, quantified using the Qubit 2.0 Fluorometer (Invitrogen), and multiplexed in equal molar mass. The pooled DNA libraries were loaded on the Illumina instrument according to the manufacturer’s instructions. The samples were sequenced using a 2 × 250 paired-end (PE) configuration. Image analysis and base calling were conducted by the Illumina in-house software.

Next-generation sequencing analysis
The .fasta files obtained from GENEWIZ AmpliconEZ were analyzed using CasAnalyzer to determine indel efficiency and overall HDR events. The output from CasAnalyzer was further analyzed utilizing NGS Analyzer, an in-house script developed to quantify precise and imprecise HDR events rapidly. NGS Analyzer is hosted at https://github.com/srcastillo/Sequencing under a GNU General Public License v3.0.

TALEN design and assembly
A 15-14-15 TALEN design strategy was employed for this assay. Two 15-mer repeat variable di-residue (RVD) TALENs with a 14 bp spacer were designed to target EGFP (Supplementary Table S1) and generated using the FusX assembly system. The RVDs utilized were HD=C, NN=G, NI=A, and NG=T.

Zebrafish husbandry
Wild-type (WT) Mayo Recessive Free (MRF) zebrafish (Danio rerio) were maintained in the Mayo Clinic Zebrafish Core Facility in accordance with standard practices and guidelines from the Institutional Animal Care and Use Committee (A34513-13-R16, A8815-15). MRF fish are WT fish stock that have been maintained through diverse breeding schemes to ensure genetic diversity and have undergone screening processes each generation to ensure that the lines do not carry recessive phenotypes.

Adult zebrafish are kept in 9 L (25–30 fish) or 3 L (10–15 fish) housing tanks at 28.5°C with a light/dark cycle of 14/10 hours. Adult fish pair breeding was set up a day before microinjections, and divider plates were removed on the morning of the injection day. After microinjections, the embryos were transferred to Petri dishes (100 mm) in E2 media (1 ×; 15 mM NaCl, 0.5 mM KCl, 1 mM MgSO4, 150 μM KH2PO4, 50 μM Na2HPO4, 1 mM CaCl2, 0.7 mM NaHCO3) and maintained in an incubator at 28.5°C.

Sexes are not determined in larval fish (2–5 days post fertilization [dpf]) in this study, and thus potential differences based on sex are not assessed.

DNA oligonucleotide preparation
All ssODNs used in this study were purchased from IDT. Stock was prepared at 100 μM in 1×TE, and working stock was prepared at 20 μM to be used for microinjection or left at 100 μM for in vitro studies.

crRNA, tracrRNA, Donorguide, and SpCas9 preparation for zebrafish microinjection
All crRNA, tracrRNA, Donorguide, and SpCas9 were custom ordered from IDT. Stock was prepared at 20 μM in duplex buffer (IDT; cat. #11-05-01-03; 30 mM HEPES, pH 7.5, 200 mM potassium acetate) for crRNA, tracrRNA, and Donorguide. At the 20 μM concentration, crRNA is at 233.9 pg/nL (protospacer length 20 nt), and tracrRNA is at 432.7 pg/nL. SpCas9 stock was prepared at 23 μM (3.73 ng/nL) in PBS (1×).

Microinjection
One-cell stage fertilized zebrafish embryos were harvested and aligned on an agarose plate in E2 media. Three microliters of injection solution was loaded into an injection needle as described previously. A 2 nL of injection solution was delivered into the cell interface layer of the embryo. This injection (2 nL) delivered crRNA:tracrRNA (or Donorguide):SpCas9 complex at approximately 4.52 fmol (52.9 pg):4.52 fmol (97.8 pg):4.52 fmol (733.1 pg) and ssODN (when needed) at 4 fmol. Injected embryos were transferred to a new Petri dish and kept in an incubator at 28.5°C. Dead and nonviable embryos were counted and removed the next day (1 dpf).

Zebrafish experimental replicates
All experiments involving zebrafish microinjection were carried out in three independent experiments. Each experiment utilized a mixed populations of embryos obtained from multiple clutches. Each experiment was ideally conducted on different days. If two experiments were performed on a day, all reagents were prepared from different stock preparations.

Zebrafish genomic DNA extraction
Typically, 40 embryos were individually collected in 36 μL of NaOH (50 mM) 1–2 dpf (if morphological phenotyping was not necessary) and 4–5 dpf (if morphology was assessed). Each embryo was placed in 36 μL 50 mM
NaOH and heated at 95°C for 15 min with regular vortexing. To stabilize NaOH, 4 μL of Tris-HCl (1 mM, pH 8) was added. Eight embryos were pooled into a sample (a biological replicate) by taking 2 μL from each embryo (eight fish per sample), and a 1:3 dilution was made by adding 48 μL of distilled water.

Statistical analyses
We compared between conditions using an unpaired t-test and used the Holm–Sidak method to correct for multiple comparisons. Differences between groups were considered significant at a p-value of <0.05. Statistical analyses were performed with GraphPad Prism v8.4 (GraphPad Software, Inc., San Diego, CA).

Results
Donorguide SpCas9 introduces precise insertions more efficiently than ssODN donors
To assess the feasibility of covalently tethering a DNA template to the 3’ of a tracrRNA to induce precise genetic edits, we employed HEK 293T cells for their ease of transfection (Fig. 1). We previously identified an active Streptococcus pyogenes Cas9 (SpCas9) CRISPR RNA (crRNA):tracrRNA reagent targeting a locus in the safe harbor region AAVS1 (Supplementary Fig. S1).\textsuperscript{29,30} Due to synthetic RNA synthesis length limits, we used separate CRISPR RNA (crRNA) and tracrRNA instead of single-guide RNA.\textsuperscript{31} The tracrRNA was synthesized with a DNA repair template fused to the 3’ end containing 12 nt of homology flanking a 2 nt insertion (Fig. 2A) generating a chimeric RNA:DNA hybrid.

The chimeric tracrRNA was annealed with an SpCas9 crRNA to form what we term the Donorguide and combined with SpCas9 recombinant protein to form the RNP complex. HEK293T cells were electroporated with Donorguide RNP and screened for genetic insertions by next-generation sequencing (NGS; Fig. 1). We found that the Donorguide SpCas9 RNP introduced the precise 2 nt insertion at an efficiency of 1.1 ± 0.50% (mean ± standard deviation; Fig. 2B). In addition, we found that

![FIG. 1. Donorguide composition and experimental workflow. Left: Donorguide consists of a canonical CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) that is fused to a DNA repair template. Top: \textit{In vitro} experiments were carried out by electroporating Donorguide RNP complexes into cells and culturing before DNA isolation for next-generation sequencing (NGS) analyses. Bottom: \textit{In vivo} experiments were carried out by microinjecting single-cell zebrafish embryos with Donorguide RNP complexes and collecting larvae on 1 or 3 days post fertilization (dpf) for NGS. Color images are available online.](image-url)
Donorguide (52 ± 11%) introduced indel mutations at markedly lower rates than a tracrRNA (91 ± 2%). These data suggested that the Donorguide methodology is a viable approach to introduce precise small molecular changes in vitro, albeit at modest rates.

Encouraged by our in vitro results, we sought to see whether Donorguide likewise functions in vivo. We chose zebrafish (Danio rerio) due to their high fecundity, amenability to microinjection, and ease of genotyping (Fig. 1). Single-cell embryos were injected with SpCas9 RNP with a crRNA targeting the N2B exon in the titin gene locus that we have previously shown to be highly active6 (Fig. 2C), along with an ssODN donor providing a 2 nt insertion or CRISPR Donorguide containing the same 2 nt insertion. Injected animals were incubated for 72 h before having their genomic DNA isolated, PCR amplified, and submitted for NGS (Fig. 1).

We found that using Donorguide, compared to SpCas9 and an ssODN donor, results in a similar rate of overall HDR events (9.3 ± 4.3% for ssODN and 13 ± 3% for Donorguide) composed of both precise and imprecise insertion events (Fig. 2D). However, the genetic alterations induced by Donorguide (5.9 ± 1.2%) resulted in a higher rate of precise insertions when compared to an ssODN donor (3.4 ± 2.7%; Fig. 2D) and Donorguide insertions transmit to the offspring (Supplementary Fig. S4).32

Seeking to replicate our appreciable in vivo knock-in results, we sought to determine whether the size of the homology arms accounted for the modest rates of HDR with CRISPR Donorguide in vitro (Fig. 2B). To address this, we increased the homology arm length to 23 nt for both CRISPR Donorguide and its requisite ssODN designed to introduce a 2 nt insertion at AAVS1 (Fig. 2E).
The 23-2-23 Donorguide or SpCas9 and ssODN were electroporated into HEK293T cells and analyzed for HDR by NGS. We found that using larger homologies flanking the insertion resulted in precise HDR rates of 27 ± 2.2% with CRISPR Donorguide compared to Cas9 and an ssODN donor that resulted in 8.1 ± 2.7% precise HDR (Fig. 2F). Taken together, these data suggest that CRISPR Donorguide can introduce HDR at appreciable rates and outperform a comparable ssODN DNA donor.

**Donorguide SpCas9 introduces precise substitutions more efficiently than ssODN donors**

We next set out to assess whether Donorguide could introduce precise genetic substitutions. To this end, we employed an established EGFP-BFP reporter system to measure genetic substitution fluorometrically. EGFP contains 2 aa in its chromophore domain, Thr65 and Tyr66, which when mutated to Ser65 and His66 converts to BFP, which can be recorded by flow cytometry (Fig. 3A). HEK293T cells were transected with an EGFP-expressing plasmid that is flanked by Tol2 inverted terminal repeats as well as a plasmid encoding Tol2 transposase (Supplementary Table S1). Cells were passaged several times and were FACS sorted for the brightest EGFP-expressing cells to establish the HEK-EGFP reporter cell line (Supplementary Fig. S2).

HEK-EGFP cells were transected with Donorguide or SpCas9 with an ssODN containing the EGFP-BFP substitutions flanked by 10 nt of homology (Fig. 3A and B). Cells were incubated at 37°C for 72 h and measured for BFP expression by flow cytometry. Compared to SpCas9 and an ssODN (0.49 ± 0.11% BFP+), Donorguide converted EGFP to BFP at a significantly greater rate (1.7 ± 0.48% BFP+) as measured by flow cytometry (Fig. 3C and D) and NGS (Supplementary Fig. S3). These results suggest that when compared to a free ssODN of similar size, CRISPR Donorguide results in a quantitative improvement of HDR induction in vitro.

To assess whether conversion of EGFP-BFP observed in our HEK-GFP reporter can also be deployed in vivo, we employed a previously established transgenic zebrafish line that has an EGFP reporter stably integrated into its genome (Fig. 3E) that transmits at a Mendelian ratio. Single-cell embryos were injected with either SpCas9 and an ssODN or Donorguide encoding the EGFP-BFP transition. Injected animals were pooled, had their DNA isolated, were barcoded, and were submitted for NGS. We found that CRISPR Donorguide (1.7 ± 1.3%) results in more precise insertion events compared to an ssODN of a similar size (0.13 ± 0.17%; Fig. 3F). These results suggest that CRISPR Donorguide functions cross-model to enhance gene substitution relative to an ssODN.

**Donorguide SpCas9 introduces precise deletions more efficiently than ssODN donors**

Small insertions comprise about 3% of known human pathogenic genetic variants, and being able to correct these aberrant sequences to restore the WT gene is highly sought after. Targeting insertion regions with CRISPR-Cas9 to resolve the lesion can result in additional loss of function alleles as a result of inadvertent activation of NHEJ or Alt-EJ. Therefore, precise deletions are required to address such conditions. To this end, we set out to determine if Donorguide could introduce precise deletions at higher rates than a comparable ssODN. To assess precise deletion, we developed a reporter cell line such that unperturbed cells will be EGFP+, but if a precise deletion of the stop codon is introduced the cells will be EGFP+/mRFP+ (Fig. 4A). HEK EGFP cells were transected with either SpCas9 and an ssODN encoding the 3 nt stop codon deletion or with CRISPR Donorguide, incubated for 72 h, and assessed for precise deletion by NGS. We found that CRISPR Donorguide introduced the precise deletion at nearly the same rate as an ssODN (11 ± 3.5% ssODN and 10 ± 4.0% Donorguide). However, in agreement with previous observations (Fig. 2B), we note that Donorguide resulted in a significantly decreased amount of overall indel mutations compared to SpCas9 (89 ± 10% ssODN and 41 ± 15% Donorguide).

Accordingly, Donorguide resulted in a significantly greater percentage of precise deletion alleles, defined as the number of desired alterations/overall indel mutations (25 ± 1.7% Donorguide and 13 ± 2.9% ssODN; Fig. 4B). These results suggest that CRISPR Donorguide can introduce a precise deletion at a comparable rate to an ssODN, but the overall fraction of desired alleles is significantly greater in the mutant allele population.

**Modeling clinically relevant mutations in animal models is a top priority in the field of precision genomics.** Therefore, we sought to determine if we could employ CRISPR Donorguide to model small molecular changes in a clinically relevant gene. To this end, we chose the gene nr3c1, which is a cognate cortisol receptor and encodes a glucocorticoid receptor that mediates hypothalamic–pituitary–inter-renal axis activation in zebrafish and is used to model the human acute stress response. We targeted nr3c1 with SpCas9 and provided an ssODN or Donorguide consisting of 12 nt of homology designed to delete 8 bp (Fig. 4C).

As noted in the in vitro deletion study, we found comparable rates of overall precise deletion between ssODN
and Donorguide (0.33 ± 0.21%) when considering all alleles. In agreement with our previous studies with SpCas9 and an ssODN, SpCas9 resulted in significantly more indels (88 ± 8.4%) compared to Donorguide (9.8 ± 5.3%). Accordingly, when considering the proportion of desired alleles in the mutant allele population, Donorguide resulted in a significantly greater (3.4 ± 1.4%) percentage of mutant alleles when compared to an ssODN (0.96 ± 0.31%; Fig. 4D). These results suggest that although Donorguide may introduce a similar overall number of precise deletions compared to an ssODN, Donorguide-mediated deletions comprise a greater proportion of the mutant allele population.

Increased rates of precision editing with Donorguide enable phenotype screening in animals
As we observed that Donorguide generated a greater proportion of desired deletions in mutant alleles, we tested whether we could leverage this phenomenon to streamline the generation of animal models. We developed an assay to remove WT alleles effectively from the population while simultaneously interrogating whether the remaining mutant alleles were generated by random indels or precise HDR.

To this end, we targeted the tyrosinase (tyr) gene in zebrafish, which is required for converting tyrosine into the pigment melanin that confers skin color.36 We inserted a 6 nt AvrII restriction site with either an ssODN encoding the EGFP-BFP substitution or with CRISPR Donorguide into the tyr locus (Fig. 5A). If precisely inserted, the cargo will generate an in-frame stop codon and should lead to the development of zebrafish with pigmentation phenotypes (Fig. 5B). Zebrafish were injected, and the resulting pigmentation mutants segregated and pooled. Their genomic DNA was isolated, PCR amplified, and subsequently submitted for NGS.

Interestingly, while Cas9 and the ssODN generated a strong albino-like phenotype (Fig. 5D), we observed only apparent WT zebrafish in the Donorguide condition.
This observation was supported by sequencing analysis that showed the Cas9- and ssODN-injected albino zebrafish consisted of almost entirely (97.0% ) indel alleles (Supplementary Fig. S5). The Donorguide-injected animals did not have a visible pigmentation phenotype (and were therefore randomly pooled without segregation based on phenotype) and had only 24.2% indel alleles, which was likely not sufficient to produce an apparent pigmentation phenotype (Supplementary Fig. S5). We found that the DNA portion of the tyr chimeric tracrRNA was designed complementary to the crRNA target strand. We reasoned that the DNA region of the chimeric tracrRNA and the crRNA were aberrantly annealing during Donorguide assembly and preventing the formation of the crRNA:Donorguide duplex. We then designed the tyr chimeric tracrRNA DNA region to be of the same strand as the crRNA target strand, and therefore prevent aberrant base pairing. Upon switching the strandedness of the DNA donor portion, we observed strong albino phenotypes and requisite nuclease activity using the same strand Donorguide (Fig. 5F and Supplementary Fig. S5) at approximately the same rates as the Cas9- and ssODN-injected conditions (Supplementary Fig. S6).

We note that when comparing the ssODN and Donorguide of the same strand, Donorguide resulted in significantly more overall HDR events (23.6%) than Cas9 and an ssODN did (5.5% ; Fig. 5G). Additionally, we found Donorguide introduced significantly more precise HDR events (11.6%) compared to a canonical
ssODN (1.5 ± 0.74%; Fig. 5G). These results suggest that when eliminating WT alleles by phenotypic screening, CRISPR Donorguide-mediated insertions at the tyr locus comprise approximately a quarter of the mutant allele population. Importantly, the HDR events that are introduced with Donorguide are significantly more precise than those from an ssODN.

Donorguide works in concert with other gene editing technologies

Even with the advent of CRISPR orthologs such as Cas12a and enhanced CRISPR-Cas9 variants with decreased protospacer adjacent motif (PAM) stringency, there is still a PAM and gRNA requirement that could preclude the utilization of CRISPR. This is particularly problematic when performing HDR, where it has been demonstrated that gene conversion is greatly enhanced when the genetic change is introduced proximally to the DSB. TALENs have only a 5′ thymidine requirement, which can be overcome with mutant variants. TALENs have been shown to function efficiently as gene editing tools in vivo and in vitro for both knock-out and knock-in applications.

To this end, we assessed whether Donorguide could enhance TALEN-mediated HDR. We designed TALENs targeting the EGFP chromophore domain using our previously established FusX assembly (Supplementary Table S1). We designed a catalytically inactive Cas9 (dCas9) Donorguide containing the EGFP-BFP substitution with a crRNA targeting 80 bp downstream of the TALEN target site to prevent steric interference between dCas9 and the TALENs. HEK EGFP cells were transfected with the EGFP TALENs and an ssODN or with dCas9 Donorguide containing the EGFP-BFP transition (Fig. 6A) and measured for BFP expression 72 h later by flow cytometry.

We found that TALENs with dCas9 Donorguide resulted in an approximate threefold increase in BFP+ cells compared to TALENs with an ssODN (7.6 ± 3.6% Donorguide and 1.3 ± 0.51% ssODN). Taken together, these data suggest that CRISPR Donorguide results in greater gene conversion than an ssODN and can be used in concert with non-CRISPR gene editing tools such as TALENs.

Discussion

We report a facile approach to introduce small genetic changes in vitro and in vivo. Our in vitro results suggest that Donorguide can be adapted to other cell types to study protein function, validate variants of uncertain significance, or generate isogenic cell lines. Additionally, Donorguide lends itself to the possibility of a gene...
therapy tool due to its decreased background indel mutations compared to SpCas9. We further show that this approach is amenable to editing in vivo using the zebrafish system. The success of Donorguide in zebrafish further suggests that this technology can be readily used to improve the generation of other in vivo models such as mice, rats, and larger animal models.

We found both a qualitative and quantitative improvement over standard ssODN methods using Donorguide in vivo. In zebrafish, our data suggest that for small genetic modifications it would be preferable to use the Donorguide to increase both the overall amount and precision of gene integration events. This feature enables researchers to make better animal models and should require less screening for allelic alterations. Imprecision of insertion events in vivo is well documented in the field and has been partially explained by several phenomena, but a unifying explanation remains elusive. We observe genetic imprecision in zebrafish in agreement with previous reports using ssODN donors.

We find that using Donorguide partially ameliorates some of the imprecision observed with ssODN donors. This could be explained, at least in part, by one of two factors. First, the nature of the DNA:RNA hybrid of the Donorguide could be less accessible to cellular nucleases and reduce the presence of donor fragments aberrantly integrating into the genomic lesion. Second, the Donorguide could be physically protected by the RNP complex likewise sparing it from cellular exonucleases that ssODNs are otherwise susceptible to similarly reducing the presence of donor DNA fragments.

There are limited reports of similar imprecision in mammalian systems. Accordingly, we observe little imprecision in our in vitro assays. We find that in our in vitro studies, the improvement of HDR with Donorguide compared to an ssODN is primarily an overall increase in desired mutant alleles without a significant change in precision of the integration events. We note one exception to this net increase of HDR events with Donorguide in our small deletion study. While we find

**FIG. 6.** CRISPR Donorguide functions synergistically with other gene editing tools in vitro. (A) Schematic diagram of transcription activator-like effector nucleases (TALENs) targeting the EGFP chromophore domain coupled with dCas9 Donorguide. (B) dCas9 Donorguide targets the EGFP locus ~ 80 nt downstream of the TALEN target site and encodes the EGFP-BFP transition flanked by 21 nt of homology. (C) Quantification of EGFP-BFP conversion by flow cytometry. HEK GFP cells were transfected with TALENs and an ssODN encoding the EGFP-BFP transition or with TALENs and dCas9 Donorguide encoding the EGFP-BFP mutation. Transfected cells were measured for BFP expression 72 h post transfection by flow cytometry. Individual values of three independent experiments. Color images are available online.
that while the net amount of precise deletion events is comparable between the ssODN and Donorguide, our approach results in a greater proportion of desired alterations in the pool of mutant alleles.

We recapitulated this increased mutant deletion allele proportion phenomena at the nr3c1 locus in zebrafish. Our nr3c1 observations, however, stood out for several reasons. First, the rate of precise deletions was surprisingly low for both ssODN and Donorguide. This low rate of HDR from both approaches suggests a locus-specific phenomenon contributing to poor HDR. Despite this, the trend of increased proportion of deletion alleles held consistent. Second, there was a sizable discrepancy of nearly a tenfold difference in indel induction between the ssODN and Donorguide at the nr3c1 locus.

The relative decrease in Donorguide-mediated indels is consistent throughout this study. However, the difference was particularly profound at this locus. This dramatic decrease in indel induction suggests that there is likely some component of the DNA portion of the Donorguide that is preventing either the formation of the RNP or the nuclease activity of the RNP.

The presence of DNA fused to the 3′ end of the tracrRNA could disrupt the formation of the crRNA:tracrRNA duplex or Cas9 RNP complex. We calculated the change in entropy (ΔS) and Gibbs free energy (ΔG) by using the mfold web server (http://www.bioinfo.rpi.edu/applications/mfold) with default parameters except for prohibiting a string of consecutive bases from pairing in the first 20 bases (20 nt spacer). The ΔS and ΔG for crRNA:tracrRNA of each loci were predicted to be −650.65 cal/K/mol and −23 kcal/mol, respectively, upon duplexing. The magnitudes of ΔG for Donorguide (crRNA:chimeric tracrRNA) duplexes were considerably higher than the standard crRNA:tracrRNA complex (Supplementary Table S2).

The more negative ΔG values for the Donorguide duplexes suggest that the Donorguide complexes are generating more energetically favorable structures than the crRNA:tracrRNA duplex. Therefore, we hypothesize that the DNA portion of the Donorguide complex establishes stable local secondary structures at the 3′ end of the duplex. The stable Donorguide structure may alter the local topology around the classical terminal hairpins in the crRNA:tracrRNA duplex and result in inefficient RNP complex formation by disrupting protein–nucleic acid interactions.

In our in vitro studies, we note that increasing the lengths of homology flanking the insertion resulted in a marked increase in HDR events at AAIVSJ. We are currently generating these chimeric molecules from commercial manufacturers. While it is of interest to increase the length of the DNA portion of Donorguide systematically, chemical synthesis constraints are a limiting factor to broad access.

Current synthesis limits only allow for ~50 nt of DNA to be added to the 3′ end of the tracrRNA, restricting the possibility of cargos and lengths of flanking homology. In-house synthesis of these chimeric molecules could afford the addition of larger homology arms flanking diverse cargos such as epitope tags or fluorescent reporter genes. Further study is needed to identify the ideal length that minimizes the formation of undesired structures and maximizes the use as a homologous repair template.

We leveraged the increased proportion of mutant alleles with Donorguide to streamline the generation of animal models by using the tyr locus in zebrafish. Screening for albino mutants showed significantly more insertion events in the Donorguide condition compared to ssODN. Donorguide can therefore be used to simplify the generation of animal models by decreasing the number of animals screened to find a desired mutant.

Being able to narrow down the editing outcomes to either a desired alteration or no editing at all may be desirable in the context of gene therapies such as for sickle cell disease (SCD) in which a point mutation causes the formation of sickled hemoglobin. Though sickled hemoglobin is causal for the pathology of SCD, total loss of function via an inadvertent indel mutation would likely be more harmful than the original point mutant. In SCD and similar pathologies, Donorguide would therefore be a preferred method of correction in which the genetic outcome is more likely to be no editing at all or a precise alteration with less collateral genetic damage.

In this study, all the DNA portions of Donorguide are of the same strand as the crRNA. We find when using a crRNA complementary to the DNA portion of Donorguide, we observe poor editing in vivo (Fig. 5E and Supplementary Fig. S5). This phenomenon can likely be explained by the crRNA abnormally base pairing with the DNA portion of the Donorguide and preventing either the formation of the Donorguide (crRNA:chimeric tracrRNA) duplex or with the formation of the Donorguide RNP complex.

We therefore developed several guidelines for Donorguide experiments: (1) identify a crRNA that is at least 50% active with SpCas9, as Donorguide tends to have decreased nuclease activity; (2) design the DNA repair template of the Donorguide to be of the same strand as the crRNA; and (3) for zebrafish, 12 nt of homology flanking a cargo of 2–6 nt should be a starting point for Donorguide design.

To our knowledge, the DNA portion of Donorguide follows the same rules as ssODN design, and therefore
an HDR design tool (IDT, horizon discovery, etc.) can be used to simplify the Donorguide design. We show that Donorguide results in either an overall increase of HDR events or an increased proportion of desired mutant alleles both in vitro and in vivo relative to a comparable ssODN. Divergent research applications could therefore leverage Donorguide for its desired editing outcomes. We likewise include design guidelines to deploy CRISPR Donorguide in diverse model systems for enhanced precision genome editing applications.

Conclusions
The ability to target the genome effectively for precise genetic alterations for gene therapy or to generate transgenic animal models is highly sought after. Here, we demonstrate precise genetic alterations in zebrafish and human cells with our newly developed CRISPR Donorguide. We show that Donorguide is a streamlined technology to introduce small genetic alterations both in vitro and in vivo with few custom reagents that are required for activity and is therefore a simple-to-adopt technology for most research labs.

In human cells, we show that Donorguide results in a greater amount of desired mutagenic events than a comparable ssODN. In zebrafish, we show CRISPR Donorguide introduces both qualitative and quantitative improvement over an ssODN donor by increasing both the overall amount and precision of HDR events. To assess the precision of HDR events, we developed NGS Analyzer to discern between precise and imprecise HDR events. Additionally, we show that Donorguide introduces less background indel mutations than standard CRISPR-Cas9.

Finally, we show that Donorguide can be used to enhance other gene editing technologies such as TALENs. The features of Donorguide described in this study suggest that with further optimization, Donorguide has the potential to be applied to clinical gene correction applications and enable the streamlined generation of transgenic animal models.

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Author Disclosure Statement
B.W.S. and S.C.E. have a financial interest in LEAH Laboratories and Mettaforge Therapeutics. S.C.E. and K.J.C. have a financial interest in LifEngine Biotechnologies and Recombinetics, Inc.

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Supplementary Material
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