Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in *Drosophila*

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**ABSTRACT** We and others recently demonstrated that the readily programmable CRISPR/Cas9 system can be used to edit the *Drosophila* genome. However, most applications to date have relied on aberrant DNA repair to stochastically generate frameshifting indels and adoption has been limited by a lack of tools for efficient identification of targeted events. Here we report optimized tools and techniques for expanded application of the CRISPR/Cas9 system in *Drosophila* through homology-directed repair (HDR) with double-stranded DNA (dsDNA) donor templates that facilitate complex genome engineering through the precise incorporation of large DNA sequences, including screenable markers. Using these donors, we demonstrate the replacement of a gene with exogenous sequences and the generation of a conditional allele. To optimize efficiency and specificity, we generated transgenic flies that express Cas9 in the germline and directly compared HDR and off-target cleavage rates of different approaches for delivering CRISPR components. We also investigated HDR efficiency in a mutant background previously demonstrated to bias DNA repair toward HDR. Finally, we developed a web-based tool that identifies CRISPR target sites and evaluates their potential for off-target cleavage using empirically rooted rules. Overall, we have found that injection of a dsDNA donor and guide RNA-encoding plasmids into *vasa-Cas9* flies yields the highest efficiency HDR and that target sites can be selected to avoid off-target mutations. Efficient and specific CRISPR/Cas9-mediated HDR opens the door to a broad array of complex genome modifications and greatly expands the utility of CRISPR technology for *Drosophila* research.

**GENOME** engineering is a powerful tool for dissecting biological mechanisms. The ability to precisely edit genomes has expanded significantly in recent years with the development of sequence-directed nucleases that can generate targeted double-strand breaks (DSBs) in DNA (Bibikova et al. 2002; Liu et al. 2012; Bassett et al. 2013; Gratz et al. 2013a; Yu et al. 2013). Chromosomal DSBs trigger DNA repair via two cellular pathways that can be harnessed for genome editing. Nonhomologous end joining (NHEJ) is an error-prone ligation process that can result in small insertions and deletions (indels) at cleavage sites. By targeting open reading frames, this pathway can be used to disrupt genes through frameshifting mutations. Homologous recombination or homology-directed repair (HDR) employs homologous DNA sequences as templates for precise repair. By supplying a donor repair template, this pathway can be exploited to precisely edit genomic sequence or insert exogenous DNA. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are sequence-directed nucleases that, in pairs, can generate targeted DSBs and have shown promise as genome engineering tools in *Drosophila* (Bibikova et al. 2002; Liu et al. 2012). However, ZFN and TALEN proteins must be custom designed for each genome modification—a costly and uncertain process. In contrast, in the CRISPR/Cas9 system, a common nuclease, Cas9, is directed to targeted loci through simple base pairing by a small RNA molecule. With the ease of producing a targeting RNA, the CRISPR/Cas9 system promises to transform genome engineering.

Endogenous prokaryotic type II CRISPR immune systems are composed of a single polypeptide nuclease, Cas9, that is guided to target sites by a complex of two small RNAs (Gasiunas et al. 2012; Jinek et al. 2012). For use in genome
engineering, the *Streptococcus pyogenes* system was simplified to two components: the Cas9 nuclease and a single chimeric guide RNA (gRNA). The gRNA recognizes a 20-nucleotide target sequence next to a tri-nucleotide NGG protospacer adjacent motif (PAM) in the genome to direct Cas9-mediated cleavage of both DNA strands within the target sequence (Jinek et al. 2012). We and others have recently demonstrated that the CRISPR/Cas9 system can be employed to make targeted modifications to the *Drosophila* genome that are efficiently transmitted through the germline (Bassett et al. 2013; Gratz et al. 2013a; Kondo and Ueda 2013; Ren et al. 2013; Sebo et al. 2013; Yu et al. 2013). Nearly all the work conducted to date in *Drosophila* has taken advantage of aberrant NHEJ to generate frameshifting mutations in open reading frames. While this is an effective approach for disrupting gene function, it is stochastic and limited to the generation of indels. NHEJ does not permit the precise incorporation of exogenous DNA, including visual markers to aid in screening, limiting its application. Further, more complex genome engineering applications such as the incorporation of recombination sites for making conditional alleles, endogenous protein tagging, or precise editing of genomic sequences require HDR (Golic 2013; Gratz et al. 2013b; Bassett and Liu 2014; Beumer and Carroll 2014). Here we present tools and techniques that overcome these limitations. We demonstrate efficient methods for CRISPR/Cas9-mediated HDR using double-stranded DNA (dsDNA) donors that facilitate the incorporation of large exogenous sequences, including easily screened and removable visible markers. We also present a web-based tool for identifying and evaluating CRISPR target sites and demonstrate that gRNAs designed with this tool are highly specific. These advances enable broad application of the CRISPR/Cas9 system for complex genome engineering in *Drosophila*.

**Materials and Methods**

**Generation of vasa-Cas9 lines**

To generate transgenic flies that express Cas9 in the germ-line, Cas9 was placed under the regulatory control of *vasa*. We began with the p3xP3-EGFP/vasa-ΦC31NLSattB plasmid (gift from Konrad Basler, University of Zurich, Zurich, Switzerland). The coding sequence for ΦC31 was replaced with an XbaI-containing multiple cloning site through amplification of the *vasa* regulatory elements and plasmid backbone. Sequence encoding 3xFLAG-NLS-Cas9-NLS was amplified as an XbaI-flanked fragment from pX330 (Cong et al. 2013) and ligated into the XbaI site of the modified p3xP3-EGFP/vasa-ΦC31NLS-attB plasmid. The resulting p3xP3-EGFP/vasa-3xFLAG-NLS-Cas9-NLS plasmid was used for ΦC31-mediated integration (Supporting Information, Figure S1 and Table S1).

**Fly stocks**

*hs-Cre, hs-flp DSH3PX1°Fy05068*, and *lig4°Fy04506* lines are available at the Bloomington Drosophila Stock Center (Ahmad and Golic 1996; Siegal and Hartl 1996; Bellen et al. 2004; McVey et al. 2004).

**Molecular reagents**

*pBS-hsp70-Cas9*: To generate a high-copy number version of the Cas9 vector for injection, a NotI fragment containing Cas9 and the hsp70 regulatory regions was excised from phsP70-Cas9 (Gratz et al. 2013a). This fragment was ligated into the NotI site of pBluescript SK+ (Agilent Technologies).

*pU6-gRNAs*: Target-specific sequences for *rosy* and *DSH3PX1* gRNAs were synthesized as 5'-phosphorylated oligonucleotides, annealed, and ligated into the *BbsI* sites of pU6-BbsI-chiRNA (Gratz et al. 2013a).

**In vitro transcribed gRNAs**: Following the protocol of Bassett et al. (2013), *DSH3PX1* gRNAs S1 and S2 were generated by PCR (Phusion polymerase; New England Biolabs, Beverly, MA) amplification of two oligonucleotides that anneal to create a template for *in vitro* transcription by T7 polymerase. PCR products were purified using the QiaexII agarose gel extraction kit (QIAGEN, Valencia, CA) and *in vitro* transcription was performed with the MegaScript T7 Kit (Ambion). Transcripts were purified by phenol-chloroform extraction and isopropanol precipitation.

**Donor templates**: The pH-DsRed-attP vector was designed for rapid generation of gene-specific donor templates and custom synthesized (DNA2.0; Figure S1). The vector contains an attP ΦC31 docking site for subsequent modification of the targeted locus and a 3xP3-DsRed marker that drives expression of DsRed in the eye, flanked by loxp recombination sites for its removal. Multiple cloning sites on either side of the replacement/marker cassette are flanked by type IIS restriction sites, *AarI* and *SapI*, respectively, to facilitate seamless incorporation of homology arms. The multiple cloning sites of pH-DsRed-attP were designed to conserve as many restriction sites as possible from pGX-attP to make the vector compatible with homology arms previously generated for ends-out homologous recombination (Huang et al. 2009).

To generate the *DSH3PX1* replacement donor, 1.1-kb homology arms flanking the S1 and S2 cleavage sites were amplified and incorporated via *AarI* and *SapI*, respectively (Figure S1 and Table S2). The *DSH3PX1* conditional allele donor was constructed by assembling three DNA fragments into a pUC19 vector. A 1.5-kb 5'-homology arm leading up to the S1 cleavage site was amplified (Phusion polymerase, New England Biolabs) with a forward primer containing a *SpeI* site and a reverse primer incorporating a partial (up to the XbaI site) FRT site. This fragment was cloned into the XbaI site of pUC19. A 2.5-kb region beginning downstream of the S1 cleavage site and extending to the second intron of *DSH3PX1* was amplified with a forward primer incorporating a partial (beginning at the XbaI) FRT sequence and a reverse primer containing a *HindIII* site. This fragment was cloned into the XbaI and *HindIII* sites of the pUC19-fragment 1 plasmid. A third fragment consisting of the 3xP3-DsRed-FRT sequence was amplified from a transgenic fly line with
a pBac(SAstopHD-DsRed) insertion, using primers containing NgoMIV site overhangs. The PCR product was ligated into the pU19-fragments 1 and 2 plasmid at the NgoMIV site present in the previously cloned intronic sequence of DSH3PX1 (Figure S1 and Table S2). We have since generated an additional donor cloning vector containing 3xP3-DsRed but no attP site (pHD-DsRed) for rapid cloning of similar dsDNA donors that will be available through Addgene.

**Embryo injections**

Preblastoderm embryos were injected through the chorion membrane, using standard protocols. Injections were carried out at 18° and embryos were shifted to 25° immediately following injection. pBS-hsp70-Cas9 was injected at a concentration of 250 ng/μl. The pU6-gRNA targeting constructs were injected at 250 ng/μl for single gRNAs and 100 ng/μl each when two gRNAs were injected. In vitro transcribed gRNAs were injected at 25 or 50 ng/μl. dsDNA donor templates were injected at a concentration of 500 ng/μl. All injection mixtures were prepared in water. Lig4 injections were performed by Genetic Services Inc.

**Screening**

To assess germline transmission of targeted modifications, adults that developed from injected embryos were outcrossed to y¹; ry¹ or w¹¹¹⁸. Offspring were screened for 10 days after the first flies emerged for progeny with rosy or RFP+ eyes, indicating transmission of the dsDNA donor. Transmission rates were calculated both as a percentage of fertile crosses that produced one or more transgenic progeny and as a percentage of total progeny. Note that in nearly every case, crosses that yielded transformed progeny did so within the first few days of screening, suggesting that screening effort can be minimized by limiting screening to 3–5 days with little risk of missing independent transformants. Biallelic events were recognized by either transmission of modified loci to >50% of progeny or modification of two discernable chromosomes (e.g., the vasa-Cas9 and balancer chromosomes).

**Molecular characterization of engineered and off-target loci**

Genomic DNA was isolated from individual F1 flies. PCR was performed using primers flanking the targeted modifications or potential off-target cleavage site. Amplified products were purified and sequenced.

**Fli and Cre approach**

To remove the DsRed visible marker, DSH3PX1 DsRed-attP males were crossed to hs-Cre virgin females. Progeny were reared either at 25° without heat shock or at 18° with a single heat shock of 1 hr at 37°. To remove the first exon of DSH3PX1, DSH3PX1 conditional flies were crossed to hs-FLP. Progeny were heat-shocked every 24 hr until pupation at 37° for 1 hr.

**Development of CRISPR Optimal Target Finder**

Euchromatic regions of the dm3/BDGP release 5 Drosophila melanogaster genome were indexed as in Iseli et al. (2007). PHP code was developed to (1) parse user-inputted DNA sequence to detect CRISPR targets on both strands, (2) execute fetchGWI (Iseli et al. 2007) to identify similar sequences elsewhere in the genome, (3) employ algorithms based on empirical rules and user-selected parameters to identify potential off-target cleavage sites, and (4) return CRISPR target sites ranked by specificity along with location information and a Gbrowse link for each potential off-target site. The following invertebrate genomes were processed identically: D. simulans (annotation DroSim1), D. yakuba (DroYak2), D. sechellia (DroSec1), D. virilis (DroVir3), two strains of Anopheles gambiae (AgamM1 and AgamS1), Aedes aegypti (AaegL1), Apis mellifera (apiMel3), Tribolium castaneum (TriCas2), and Caenorhabditis elegans (ce10). A detailed user manual is available at http://tools.flycrispr.molbio.wisc.edu/targetFinder/CRISPRTargetFinderManual.pdf.

**Results**

**Germline expression of Cas9 increases targeting efficiency**

Previous work has shown that germline expression of FpsC31 phage recombinase yields increased transformation efficiency over injection protocols (Bischof et al. 2007). To determine whether this is the case for Cas9, we generated transgenic fly lines that express Cas9 in the germline under the control of vasa regulatory regions (Figure 1A). These lines are homozygous viable, fertile, and healthy, suggesting low toxicity from germline expression of Cas9.

CRISPR/Cas9 targeting efficiency varies significantly between loci and even between target sites within the same locus (Bassett et al. 2013; Gratz et al. 2013a; Ren et al. 2013; Yu et al. 2013). Therefore, to directly compare the efficiency of targeting in vasa-Cas9 flies to injection of Oregon-R flies with hsp70-Cas9, a method we previously employed in targeting yellow, we assessed two separate gRNAs targeting the rosy locus (Figure 1B) (Gratz et al. 2013a). We found that germline expression of Cas9 yielded the highest efficiencies (Table 1). This effect was most pronounced for the R3 guide, which was moderately efficient in the injection paradigm with 8% of fertile injectees (founders) transmitting rosy mutations to 1.4% of all progeny and highly efficient in vasa-Cas9 flies with 53% of fertile injectees transmitting rosy mutations to 15% of progeny. Interestingly, we did not observe a difference in the rate of biallelic indel formation, with both injected and integrated Cas9 approaches yielding founders in which both alleles were mutated 29% of the time. Overall, our results demonstrate high levels of NHEJ with germline-expressed Cas9 and are in agreement with recent investigations of vasa-Cas9 in the targeting of integrated GFP and RFP plasmids; of nanos-Cas9 in the targeting of white; and, as reported by Hui-Min Chen

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and Tzumin Lee at CRISPRflydesign.org, of UAS-Cas9 under the control of a germline Gal4 driver (Ren et al. 2013; Sebo et al. 2013).

To assess the nature of the rosy mutations, we amplified and sequenced the target sites in F1 flies. We observed similar indels in both cases, indicating that repair of targeted Cas9-induced cleavage by NHEJ occurred in both the injection and the germline expression approaches (Figure 1C).

CRISPR/Cas9-mediated HDR with dsDNA donors

Most of the initial experiments employing the CRISPR/Cas9 system in Drosophila have depended on easily scored phenotypes for identification of targeted events (Bassett et al. 2013; Gratz et al. 2013a; Kondo and Ueda 2013; Ren et al. 2013; Sebo et al. 2013; Yu et al. 2013). To develop a more universal method to screen for targeted events, we optimized CRISPR/Cas9-mediated HDR using dsDNA donors that enable the incorporation of larger DNA sequences, including visible markers. We generated a donor vector that contains multiple cloning sites for rapid incorporation of flanking homology arms, an attP F3C1 phage recombination site for subsequent access to the locus, and the visible marker 3xP3-DsRed flanked by loxP sites for CRE-mediated removal (Figure 2A).

To assess dsDNA-mediated HDR, we targeted the DSH3PX1 locus for replacement with an attP docking site (Figure 2B). Our general strategy was to employ two gRNAs targeting sites 5’ and 3’ of the gene and our dsDNA donor with homology arms of ~1 kb immediately flanking the targeted cleavage sites (Figure 2B). Homology arm length was selected to balance efficiency and ease of cloning based on a detailed study of ZFN-mediated HDR (Beumer et al. 2013). To optimize efficiency, we directly compared three approaches for delivery of CRISPR components: (1) injection of dsDNA donor and DNA plasmids encoding gRNAs and Cas9 into w1118 embryos, (2) injection of dsDNA donor and DNA plasmids encoding gRNAs into vasa-Cas9 embryos, and (3) injection of dsDNA donor and in vitro transcribed gRNAs into vasa-Cas9. Based on the incorporation of the DsRed marker, the overall efficiency of HDR was very high (Table 2). Injection of dsDNA donor and DNA plasmids encoding gRNAs into vasa-Cas9 flies yielded the highest efficiency of germline transmission (18% founder rate), while gRNA injection as RNA into vasa-Cas9 yielded the lowest efficiency (3% founder rate). Furthermore, in vasa-Cas9 targeting
experiments 70% of founders exhibited biallelic HDR vs. 0% for injections into w1118 embryos, consistent with high targeting efficiency with germline expression of Cas9.

To confirm that the precisely targeted events had occurred in the DsRed-positive flies, we amplified and sequenced the engineered region in at least one F1 fly per founder. In 11/12 cases, HDR occurred as anticipated. Thus, with DNA injection into vas-Cas9, 16% of crosses yielded precise HDR. In 1 of 9 independent cases where DsRed-positive flies were recovered from vas-Cas9 flies injected with CRISPR components as DNA, the donor was not incorporated at the targeted locus but rather elsewhere in the genome. A previous study in zebrafish demonstrated that homology-independent integration of DNA occurs preferentially at CRISPR-induced DSBs, so we assayed predicted off-target cleavage sites for donor integration by PCR (Auer et al. 2014). We found no evidence of integration at any of the eight predicted off-target sites (see Figure 5), suggesting either a random integration event or a complex rearrangement of the target site, both of which have been observed with traditional ends-in and ends-out homologous recombination methods.

In previous studies employing ZFNs, mutation of Lig4, a DNA ligase in the NHEJ pathway, biased endogenous DNA repair toward HDR (Beumer et al. 2008). To determine the effect on CRISPR/Cas9-mediated genome engineering, we injected the same dsDNA donor and gRNA plasmids as above along with Cas9 into Lig4 mutants. Of 28 fertile crosses, none yielded DsRed-positive progeny. However, we did find that injection into a Lig4 background increased HDR efficiency over injection into wild-type in analogous experiments targeting

### Table 1 Germline transmission rates of NHEJ

| gRNA(s) | Injection line | Male crosses | | Female crosses |
|---|---|---|---|---|
| | % (no.) founders | % (no.) mutant progeny | % (no.) founders | % (no.) mutant progeny | % germline transmission | % mutant progeny per founder |
| R2 | w1118 | 27 (3/11) | 22 (88/404) | NA | NA | 27 (3/11) | 28 (2–67) |
| R3 | w1118 | 9.7 (3/31) | 1.7 (27/1621) | 5.3 (1/19) | 0.49 (2/406) | 8 (4/50) | 23 (5–56) |
| R2 | vas-Cas9 | 21 (3/14) | 5 (68/1264) | 36 (4/11) | 10 (40/399) | 28 (7/25) | 41 (5–100) |
| R3 | vas-Cas9 | 39 (9/23) | 6 (93/1548) | 71 (12/17) | 37 (236/630) | 53 (21/40) | 20 (1–100) |

The indicated gRNAs were either co-injected with pBS-hsp70-Cas9 into w1118 flies or injected into vas-Cas9 flies. Injected flies were crossed to y1; ry1 and progeny screened for rosy eye color. The percentage of crosses producing one or more rosy progeny (founders) is indicated along with the percentage of total mutant progeny. At least one progeny per founder was sequenced to confirm the presence of a targeted lesion.
a 25-kb gene (4% vs. 0% founder rate, data not shown). Thus, mutations in Lig4 can facilitate CRISPR/Cas9-mediated HDR. Note, however, that in targeting this gene, injection of CRISPR components as DNA into vasa-Cas9 flies again yielded the highest efficiency HDR (7% founder rate, data not shown).

Finally, we confirmed the CRE-mediated removal of the 3xP3-DsRed marker (data not shown). As previously reported, we found that hs-Cre is leaky and succeeded in removing the marker in germ cells by either rearing crosses at 25°C without heat shock or rearing them at 18°C. Two of 16 crosses yielded DsRed-positive embryos (Figure 3A). Two of 16 crosses yielded DsRed-positive embryos along with DNA plasmids encoding fl. Upon molecular characterization, we found that one of the flies transmitted the intended repair event, yielding an HDR founder rate of 6%. The imperfect repair event reflected accurate incorporation of the donor at the 5‘ end through the DsRed marker and an unknown rearrangement at the 3‘ end.

Our donor template was designed such that the Flp-mediated removal of exon 1 also results in the removal of the DsRed marker (Figure 3A). We followed a standard heat-shock protocol and observed the loss of DsRed in the majority of the eye, indicating successful removal of the FRT-flanked sequences (Figure 3B).

### Targeted deletions can be rapidly identified through negative screening

An advantage of the CRISPR/Cas9 system is the fact that CRISPR components can be introduced into any genetic background through injection. We reasoned that we could capitalize on this to facilitate the identification of targeted deletions by injecting into a fly line that contains a marked transposable element in the targeted locus and screening for its loss. To test this, we targeted the DSH3PX1 locus for deletion in DSH3PX1fET08084 flies, which contain a 10.9-kb element in the first exon (Figure 4A). We employed flanking gRNAs, which were injected as plasmid DNA along with hsp70-Cas9 into DSH3PX1fET08084 embryos. The DSH3PX1 locus is 3.3 kb, bringing the total size of the targeted deletion to 14.2 kb. We observed loss of the w+ marker in progeny of 1/17 crosses (6%). Molecular analysis confirmed that the targeted deletion had occurred in these flies (Figure 4B).

### Optimizing CRISPR/Cas9 specificity

To facilitate the identification of highly specific CRISPR targets in Drosophila, we developed a freely available web-based tool to identify and evaluate CRISPR targets within a sequence of genomic DNA (tools.flycrispr/molbio.wisc.edu/targetFinder). First, CRISPR Optimal Target Finder identifies all possible CRISPR target sites in user-provided genomic sequence. Users can specify whether they want the program to identify all CRISPR targets in the input sequence, only those that start with a G for efficient expression from the U6 promoter when supplying gRNA as plasmid DNA, or only those that start with GG for in vitro transcription of gRNAs from the T7 promoter. Once the pool of target sites is identified, the program rapidly assesses the potential for off-target cleavage for each identified CRISPR target using user-defined criteria and rules based on empirical findings. To find all genomic sequences with sequence similarity to each CRISPR target query, CRISPR Optimal Target Finder employs the fetchGWI algorithm, which can exhaustively search complete genome-sized databases for similar sequences with subsecond speed, while allowing a specified number of mismatches (Iseli et al. 2007). Similar sequences are then filtered via algorithms based on large-scale analyses of CRISPR/Cas9 specificity in cell lines (Craddock et al. 2013; Fu

### Table 2 Germline transmission rates of HDR

| gRNAs | dsDNA donor | Injection line | Male crosses | Female crosses |
|-------|-------------|----------------|--------------|---------------|
|       |             |                | % (no.) founders | % (no.) HDR progeny | % (no.) founders | % (no.) HDR progeny | % germline transmission | % HDR progeny per founder |
| S1, S2 (DNA) | attP | vasa-Cas9 | 22 (6/27) | 11 (493/4424) | 13 (3/23) | 3 (106/3233) | 18 (9/50) | 75 (36–100) |
| S1, S2 (DNA) | attP | w1118 | 22 (2/9) | 3 (48/1616) | 0 (0/9) | 0 (0/1400) | 11 (2/18) | 19 (11–28) |
| S1, S2 (DNA) | attP | lig4 | 0 (0/11) | 0 (0/2599) | 0 (0/17) | 0 (0/3066) | 0 (0/28) | 0 |
| S1, S2 (DNA) | attP | vasa-Cas9 | 5 (1/21) | 0.1 (2/3711) | 0 (0/10) | 0 (0/3862) | 3 (1/31) | 2 |
| S1, S3 (DNA) | FRTs | vasa-Cas9 | 11 (1/9) | 1 (17/1561) | 14 (1/7) | 4 (28/716) | 13 (2/16) | 22 (12–32) |

The indicated gRNAs were co-injected into vasa-Cas9 flies. For injections into w1118 and lig4 injections, the indicated gRNAs were co-injected with pBS-hsp70-Cas9. Injected flies were crossed to w1118 and progeny screened for DsRed-positive eyes. The percentage of crosses producing one or more transgenic progeny (founders) is indicated along with the percentage of HDR progeny. At least one DsRed-positive progeny per founder was sequenced to confirm the targeted HDR.
et al. 2013; Hsu et al. 2013; Mali et al. 2013b; Pattanayak et al. 2013; Yang et al. 2013) or animals (Chiu et al. 2013; Yang et al. 2013) to identify potential off-target cleavage sites for each CRISPR target. These and previous studies demonstrate that the PAM-proximal region of the CRISPR target sequence (frequently referred to as the “seed”) is more critical for specificity than the distal eight nucleotides, so our search and filtering strategies consider both the number and the location of mismatches to evaluate all potential off-target cleavage sites (Semenova et al. 2011; Wiedenheft et al. 2011; Cradick et al. 2013; Fu et al. 2013; Hsu et al. 2013; Mali et al. 2013b; Pattanayak et al. 2013; Yang et al. 2013; Cho et al. 2014). These studies also suggest that off-target cleavage, or its aberrant repair, may be greater in cell lines than in animals, so the program allows the user to select the stringency of the algorithm employed. Maximum stringency criteria are based on the composition of off-target cleavage sites observed in large-scale analyses conducted in animals (Chiu et al. 2013; Yang et al. 2013) and define potential off-target sites as those with (i) perfect matches (zero mismatches) to the seed sequence or (ii) one mismatch in the seed sequence when there are only one or zero mismatches in the distal sequence. Finally, the program allows the user to choose whether or not NAG-adjacent sites are considered in the evaluation of CRISPR target sequence specificity. CRISPR sequences adjacent to an NAG PAM sequence can be cleaved at one-fifth the efficiency of those adjacent to a canonical NGG PAM sequence in transformed cell lines, although this has not been observed in animals to date (Hsu et al. 2013). Once potential off-target cleavage sites are found, the program returns all identified CRISPR targets sorted by the likelihood of off-target nuclease activity along with the sequence and genomic location, including a Gbrowse link, of each predicted off-target site.

CRISPR Optimal Target Finder also provides the ability to search additional invertebrate genomes. To facilitate the adoption of CRISPR in nonmodel systems, we included organisms with relevance to agriculture and global health: D. simulans, D. yakuba, D. sechellia, D. virilis, A. mellifera, T. castaneum, two strains of A. gambiae, A. aegypti, as well as C. elegans.

With the exception of gRNA S3, all of the guides used in this work were designed to avoid off-target sequences by the
animal-based, or “high-stringency”, criteria located next to a canonical NGG PAM (Figure 5A). To assess the specificity of our gRNAs, we amplified and sequenced the 14 potential off-target sites defined by the more stringent cell culture-based, or “maximum”, criteria and adjacent to a canonical PAM for gRNAs R2, R3, S1, and S2 in 23 independent F1 flies and observed intact sequence in every case (Figure 5). We also identified and assessed four potential off-target sites with perfect seed matches to gRNAs R2, S1, and S2 adjacent to noncanonical PAMs and again observed no indels (Figure 5). These results demonstrate that careful selection of target sites using the high-stringency criteria enables the generation of lines carrying highly specific targeted mutations.

To further probe the potential for off-target cleavage with CRISPR/Cas9, gRNA S3 was designed to challenge the system. S3 has six PAM-adjacent perfect matches to its seed sequence elsewhere in the genome (Figure 5A). The overall identity of these off-target sequences ranges from 16/20 to 12/20 (Figure 5B). We amplified and sequenced each potential off-target site in DSH3PX1 conditional F1 flies and found no evidence of cleavage. Similarly, no evidence of off-target cleavage was observed at the one NAG-adjacent perfect seed match in the genome (Figure 5). This demonstrates that the CRISPR/Cas9 system can be employed for highly specific genome editing in the Drosophila germline even when perfect matches to the CRISPR target seed sequence are found elsewhere in the genome and reinforces the role of the more distal nucleotides in targeting specificity.

Discussion

Our results demonstrate that the CRISPR/Cas9 system can catalyze complex genome engineering with high efficiency and specificity. We present a universal approach for identifying targeted events through HDR with dsDNA donors containing positive markers and demonstrate that this approach can be used in conjunction with germline expression of Cas9 to efficiently replace a gene or generate a conditional allele. Through our analysis of off-target cleavage, we show that target site selection with our web-based tool facilitates highly specific modification of the genome free from unintended mutations.

The broad application of CRISPR/Cas9 genome engineering requires tools for rapid identification of targeted events. In most cases, phenotypic screening will not be an option, necessitating alternative approaches. PCR-based molecular screening methods are a universal option and have been demonstrated with CRISPR/Cas9-mediated NHEJ, but the associated generation and maintenance of candidate fly stocks is time and labor intensive (Yu et al. 2013). We demonstrate two alternative approaches: negative screening through the removal of marked elements in targeted lines and positive screening through HDR with dsDNA donors containing visible markers. The simplicity of the negative screening approach combined with the presence of marked elements in most Drosophila genes makes it an appealing option for some applications. On the other hand, HDR with dsDNA donor templates offers both the opportunity for incorporating screenable markers and the capacity to introduce more complex and larger-scale modifications.

To optimize CRISPR/Cas9-mediated HDR approaches in Drosophila, we performed a direct comparison of the efficiency of multiple approaches. We found that injection of gRNAs as DNA into vasa-Cas9 flies maximized efficiency over either injection of in vitro transcribed gRNAs into vasa-Cas9 flies or coinjection of gRNA and Cas9 DNA into w1118 flies. Ren et al. (2013) also observed increased efficiency with germline-expressed Cas9 when targeting white for mutation via NHEJ. Our survival rate for both NHEJ and HDR in vasa-Cas9 flies was ∼10%. This is lower than observed for nanos-Cas9 and may indicate that nanos-driven Cas9 is less toxic than vasa-driven Cas9, possibly due to more restricted expression in preblastoderm embryos. Alternatively, the difference may reflect variability in the gRNAs, the targeted locus, or the injection technique. Note also that our vasa-Cas9 lines were not homozygous; thus ∼25% of the embryos were homozygous for the balancer chromosome and genetically invisible. Consistent with high targeting efficiency, we observed a high rate of biallelic HDR in vasa-Cas9 founders (70%), while this was not observed in Cas9-injected wild-type or transposon-expressing founders. It is important to note that for some applications, such as the targeting of genes required in the germline, it will be desirable to avoid higher cleavage rates that frequently yield biallelic breaks and would, thus, decrease the likelihood of recovering a targeted event.
It is somewhat surprising that we observed lower rates of HDR when injecting gRNAs as DNA since previous studies have reported very high rates of CRISPR/Cas9-mediated NHEJ with injection of in vitro transcribed gRNAs (Bassett et al. 2013; Yu et al. 2013). This could be attributable to differences in injection protocols and handling or may reflect the different gRNA concentrations employed. We were unable to co-inject dsDNA donor at our standard concentration (500 ng/μl) with in vitro transcribed gRNAs at concentrations >25 ng/μl each without rapidly clogging the injection needle. As a result, nearly all of the flies we analyzed were injected with half as much gRNA as in previous studies. In either case, our experiences highlight a key advantage of DNA and transgenic approaches: RNA is more difficult to handle, particularly when sending CRISPR components to injection companies as most laboratories do, as well as significantly more expensive to generate.

We also used CRISPR/Cas9-mediated HDR to generate a conditional allele through the incorporation of FRT sites flanking the first exon on DSH3PX1. This experiment demonstrates that donor templates can be designed for the incorporation of discontinuous exogenous DNA sequences—an approach that enables careful placement of the visible marker for minimal disruption. Analogous donors can be designed for the integration of in-frame protein tags or editing of specific base pairs with visible markers placed in adjacent introns.

In all our HDR experiments, we employ circular dsDNA donor plasmids. We did not compare the efficiency of circular to linear plasmids in our paradigm as injected linear donors were previously shown to be ineffective, possibly due to degradation (Beumer et al. 2008). A recent report demonstrated that CRISPR/Cas9-mediated cleavage of a target site with a single gRNA could improve ends-out homologous recombination catalyzed by a linearized dsDNA donor template (Baena-Lopez et al. 2013). However, this approach requires the co-introduction of Flp and I-SceI enzymes and the efficiency appears to be low. A second notable feature of our approach is the use of two gRNAs flanking the targeted locus. While we have not conducted a rigorous comparison of one vs. two gRNAs in HDR, the idea that two gRNAs improve efficiency is consistent with studies demonstrating significantly higher rates of NHEJ-mediated mutation with the introduction of a second gRNA (Gratz et al. 2013a; Kondo and Ueda 2013; Ren et al. 2013).

Off-target cleavage has been raised as a serious concern for CRISPR/Cas9-mediated genome engineering (Gradick et al. 2013; Fu et al. 2013). Unlike TALENS and ZFNs, which function as obligate dimers, Cas9 generates DSBs with its two nuclease domains, so only 22 nucleotides of sequence determine cleavage specificity. Further, it has been shown that the CRISPR/Cas9 complex can tolerate mismatches particularly at the 5’ end of the targeting sequence (Semenova et al. 2011; Wiedenheft et al. 2011; Gradick et al. 2013; Fu et al. 2013; Hsu et al. 2013; Mali et al. 2013b; Pattanayak et al. 2013; Yang et al. 2013; Cho et al. 2014). It is possible to mutate one nucleotide domain, rendering Cas9 capable of cleaving only one DNA strand. This nickase version of Cas9 can be targeted in pairs to increase specificity or singly to generate DNA nicks capable of promoting HDR but not NHEJ, although at a cost of reduced flexibility and efficiency (Cong et al. 2013; Hsu et al. 2013; Mali et al. 2013a). To balance efficiency and specificity, we developed an online tool for identifying optimal CRISPR targets. While several
online resources have recently been developed for identifying CRISPR targets, they are not available for Drosophila, do not make the functionally important distinction between mismatches in seed and distal sequences in their algorithms, and/or do not provide the transparent off-target analysis and reporting of CRISPR Optimal Target Finder (Hsu et al. 2013; Ren et al. 2013). Our tool is designed to provide the user with maximum understanding of and control over the evaluation criteria and to supply the information necessary to select optimal CRISPR targets for their specific genome engineering project. When deleting a gene, there are likely to be many suitable CRISPR targets, making target selection straightforward. However, for applications such as inserting an in-frame tag at a precise location, employing a target site as close to the desired change as possible is highly advantageous and might warrant, for example, selecting an optimally located target with a potential for off-target cleavage over a less ideally located target with no predicted off-target sites. Through a comprehensive analysis of off-target cleavage, we demonstrate that CRISPR Optimal Target Finder facilitates complex genome engineering with high specificity.

The development of approaches for highly specific and efficient CRISPR/Cas9-mediated HDR makes a broad range of precise genome modifications possible anywhere in the genome. Coupled with the ease of generating unique targeting gRNAs, these advances significantly expand the prospects for complex genome engineering in Drosophila.

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Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in Drosophila

Scott J. Gratz, Fiona P. Ukken, C. Dustin Rubinstein, Gene Thiede, Laura K. Donohue, Alexander M. Cummings, and Kate M. O'Connor-Giles
A. vasa-Cas9 (p3xP3-EGFP/vasa-3xFLAG-NLS-Cas9-NLS) insert

Spacer
3XP3 enhancer/hsp70 promoter
GFP
SV40 3´ UTR
vasa promoter and 3´ UTR
Flag
NLS
Cas9

GGATCTAATTCAATTGAGACTAATTTCAATTGAGACTAATTTCAATTGAGACTAATTCAATTGAGCTACGTGACGACGGCAAGCTTATCGATTTCGAGCCCTCGACCGGCACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCATCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA
B. pHD-DsRed-attP donor vector

Spacer
AarI
SapI
Multiple cloning sites
attP site
LoxP site
3XP3 enhancer/hsp70 promoter
DsRed
SV40 3’ UTR

TGCTGAAAGGAGATGAGAAGTTTAGGAGCTGTTTGAGTTTATGAGGATGTGACGATTTAAATAAATGCTTGCGAACAAAATATGGTTACAGCTCGGTAAATACATATTTACCAAATGTCACACAAATATTAACGTATGGTAATGTGACAATTTACGGACTTTCGTAAATTCCAAGTTTCTTTTGGAAAGCTGTAGATTTGGTTCAGTTTCTACCTTCACTAGGATTTGTAATGATATTTGAAACAGATCTTTTATGCCAACTCGATACTTCGATGCATCCAGATACATCGACAGCTGAGCCGTGCTCTGCAGCCCTGGCACTCGGCGCTTATCAGTAGGGGGAGACCGTAATGTAGTTGAAACGCCTCGCATTTGGTGCAATATAAAATTATGATCATAAATTTGTCCTTATATTTTACTTGATTTATTACTATAAAATTGTATACCCACCTA

C. DSH3PX1 replacement donor vector (in pHD-DsRed-attP)

Homology arm
PAM
Target sequence corresponding to the nt that remain after cleavage
attP site
LoxP site
3XP3 enhancer/hsp70 promoter
DsRed
SV40 3’ UTR

TGCTGAAAGGAGATGAGAAGTTTAGGAGCTGTTTGAGTTTATGAGGATGTGACGATTTAAATAAATGCTTGCGAACAAAATATGGTTACAGCTCGGTAAATACATATTTACCAAATGTCACACAAATATTAACGTATGGTAATGTGACAATTTACGGACTTTCGTAAATTCCAAGTTTCTTTTGGAAAGCTGTAGATTTGGTTCAGTTTCTACCTTCACTAGGATTTGTAATGATATTTGAAACAGATCTTTTATGCCAACTCGATACTTCGATGCATCCAGATACATCGACAGCTGAGCCGTGCTCTGCAGCCCTGGCACTCGGCGCTTATCAGTAGGGGGAGACCGTAATGTAGTTGAAACGCCTCGCATTTGGTGCAATATAAAATTATGATCATAAATTTGTCCTTATATTTTACTTGATTTATTACTATAAAATTGTATACCCACCTA
D. *DSH3PX1* conditional allele donor vector

Homology arm

**PAM**

Target sequence corresponding to the nt that remain after cleavage

**FRT site**

3XP enhancer/hsp70 promoter

**DsRed**

**SV40 3’ UTR**

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Figure S1  DNA sequence of Cas9 and donor constructs used in this study.

Annotated sequences of (A) codon-optimized Cas9 (Cong et al., 2013) under the control of the Drosophila *vasa* promoter and 3′ UTR, (B) pH-DsRed-attP dsDNA donor vector, (C) the *DSH3PX1* donor vector used to replace *DSH3PX1* with attP, and (D) the *DSH3PX1* donor vector used to make a conditional allele of *DSH3PX1*. 
Table S1  CRISPR/Cas9 transgenic fly lines

| Description                          | Full genotype                                                                 | BDSC stock # |
|--------------------------------------|-------------------------------------------------------------------------------|--------------|
| vasa-Cas9 on X                       | \(y[1] \text{M}\{\text{vas-Cas9}\}\text{ZH-2A w[1118]/FM7c}\)               | 51323        |
| vasa-Cas9 on 3                       | \(w[1118]; \text{PBac}\{y[+mDint2]=\text{vas-Cas9}\}\text{VK00027}\)          | 51324        |
| vasa-Cas9, U6-tracrRNA on X          | \(y[1] \text{M}\{\text{vas-Cas9, U6-tracrRNA}\}\text{ZH-2A w[1118]}\)         | 51326        |
| vasa-Cas9, U6-tracrRNA on 3          | \(w[1118]; \text{PBac}\{y[+mDint2]=\text{vas-Cas9, U6-tracrRNA}\}\text{VK00027}\) | 51325        |
| U6-tracrRNA on X                     | \(y[1] \text{M}\{w[\text{GMR.PHb}]=\text{U6-tracrRNA}\}\text{ZH-2A w[1118]/FM7c, P}\{w[+mC]=\text{GAL4-Kr.C}\}\text{DC1, P}\{w[+mC]=\text{UAS-GFP.S65T}\}\text{DC5, sn[+]}\) | 51322        |
| U6-tracrRNA on 2                     | \(w[1118]; \text{PBac}\{y[+mDint2] w[\text{GMR.PHb}]=\text{U6-tracrRNA}\}\text{VK00037/CyO, P}\{\text{Wee-P.pho}\}\text{2}\) | 51321        |

The transgenic fly lines generated for this study, along with related fly strains are detailed. All lines are available at the Bloomington Drosophila Stock Center (BDSC) through the laboratories of Melissa Harrison, Kate O’Connor-Giles and Jill Wildonger. Chimeric gRNAs should be used with vasa-Cas9 flies, while only the targeting crRNA should be used with lines that also carry the common tracrRNA component.
## Table S2  Primers used to generate donor homology arms

| Name                  | Sequence                                                                 | Donor          |
|-----------------------|--------------------------------------------------------------------------|----------------|
| SH3PX1 5’ Arm AarI F  | GGTACACCTGCGAGTCGCTATGGTAATTAGGACGTGACG                                  | DsRed-attP     |
| SH3PX1 5’ Arm AarI R  | GGACCACCTGCGCCCTCCTACAGAGGACACTCTCTAATTGATAAT                            | DsRed-attP     |
| SH3PX1 3’ Arm SapI F  | GAGTGCTCTTCTTATCAACGGGCACACACTCCACAC                                  | DsRed-attP     |
| SH3PX1 3’ Arm SapI R  | GGCTGCTCTCGGACTGTTAAACACAAACAGAGAGC                                   | DsRed-attP     |
| SH3PX1 5’ Arm F SpeI  | GGACTAGTGTTCAACACGACTCTTTAATTGATCG                                     | DsRed-FRT      |
| SH3PX1 5’ Arm R XbaI  | TTTCTAGAGAATAGGAACCTCCAGAGGCACTCTCTAAATTG                               | DsRed-FRT      |
| SH3PX1 3′ F Arm XbaI  | TCTCTAGAAAGTATAGGAACCTTTCAAGTGAGATAGCAGTT                                | DsRed-FRT      |
| SH3PX1 3′ F Arm HindIII| CCAAGCTTCTTATAGAGGCTAACACCTCAGTT                                      | DsRed-FRT      |
| 3xP3 DsRed NgoMIV F   | ATTCGCCGGCAATTCGAGCTCCGGCCGGGA                                          | DsRed-FRT      |
| 3xP3 DsRed NgoMIV R   | GGCGCGCGAATTCCTATATTCTTATAGAG                                           | DsRed-FRT      |

Primers used to generate dsDNA donors used in this study. DSH3PX1-specific sequences are underlined and incorporated enzyme sites are noted in primer names.