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

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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-nt target sequence next to a trinucleotide 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 germline, Cas9 was placed under the regulatory control of *vasa*. We began with the p3xP3-EGFP/*vasa*- Φ C31^{NLS}attB plasmid (gift from Konrad Basler, University of Zurich, Zurich, Switzerland). The coding sequence for Φ C31 was replaced with an *Xba*I-containing multiple cloning site through amplification of the *vasa* regulatory elements and plasmid backbone. Sequence encoding 3xFLAG-NLS-Cas9-NLS was amplified as an *Xba*I-flanked fragment from pX330 (Cong *et al.* 2013) and ligated into the *Xba*I site of the modified p3xP3-EGFP/*vasa*- Φ C31^{NLS}attB plasmid. The resulting p3xP3-EGFP/*vasa*-3xFLAG-NLS-Cas9-NLS plasmid was used for Φ C31-mediated integration (Supporting Information, Figure S1 and Table S1).

Fly stocks

hs-Cre, *hs-flp* *DSH3PX1*^{EY08084}, and *lig4*¹⁶⁹ 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 *Not*I fragment containing Cas9 and the hsp70 regulatory regions was excised from phsp70-Cas9 (Gratz *et al.* 2013a). This fragment was ligated into the *Not*I 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 *Bbs*I 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 pHD-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 Φ C31 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, *Aar*I and *Sap*I, respectively, to facilitate seamless incorporation of homology arms. The multiple cloning sites of pHD-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 *Aar*I and *Sap*I, 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 *Spe*I site and a reverse primer incorporating a partial (up to the *Xba*I site) FRT site. This fragment was cloned into the *Xba*I 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 *Xba*I) FRT sequence and a reverse primer containing a *Hind*III site. This fragment was cloned into the *Xba*I and *Hind*III 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{SAsstopHD-DsRed} insertion, using primers containing NgoMIV site overhangs. The PCR product was ligated into the pUC19-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.

Flp and Cre induction

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 ΦC31 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

Figure 1 Germline expression of Cas9 efficiently generates DSBs repaired by NHEJ. (A) A *Streptococcus pyogenes*-derived Cas9 codon optimized for eukaryotic expression (Cong *et al.* 2013) was placed under the control of the *Drosophila vasa* promoter and 3'-UTR and incorporated via Φ C31 to generate transgenic flies that express Cas9 in the germline. (B) Schematic of the *rosy* locus indicating R2 and R3 gRNA target sites. The predicted cleavage site for each gRNA is indicated (red arrows). (C) Sequences of the independent *rosy* mutants generated by Cas9-induced DSBs. The predicted R2 and R3 cleavage sites are indicated with a red arrowhead. Three independent *rosy* mutants generated using the R3 gRNA have larger indels and for two we did not identify an indel at the cleavage site.

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 Φ C31 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 cleavages 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

Table 1 Germline transmission rates of NHEJ

gRNA(s)	Injection line	Male crosses		Female crosses		% germline transmission	% mutant progeny per founder
		% (no.) founders	% (no.) mutant progeny	% (no.) founders	% (no.) mutant progeny		
R2	<i>w¹¹¹⁸</i>	27 (3/11)	22 (88/404)	NA	NA	27 (3/11)	28 (2–67)
R3	<i>w¹¹¹⁸</i>	9.7 (3/31)	1.7 (27/1621)	5.3 (1/19)	0.49 (2/406)	8 (4/50)	23 (5–56)
R2	<i>vasa-Cas9</i>	21 (3/14)	5 (68/1264)	36 (4/11)	10 (40/399)	28 (7/25)	41 (5–100)
R3	<i>vasa-Cas9</i>	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 *vasa-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.

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 *vasa-Cas9*, 16% of crosses yielded precise HDR. In 1 of 9 independent cases where DsRed-positive flies were recovered from *vasa-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

Figure 2 CRISPR/Cas9-induced HDR with a dsDNA donor vector. (A) Diagram of the pHD-DsRed-attP vector. pHD-DsRed-attP contains an attP ΦC31 docking site for subsequent access to a targeted locus and a 3xP3-DsRed marker, which drives expression of DsRed in the eye, flanked by loxP recombination sites for its removal. Along with traditional restriction sites, the multiple cloning sites are flanked by type IIS restriction sites, AarI and SapI, respectively, to facilitate seamless incorporation of homology arms. For compatibility with donors generated for ends-out homologous recombination, the multiple cloning site conserves as many restriction sites as possible from pGX-attP (Huang *et al.* 2009). (B) Schematic of the *DSH3PX1* locus and the HDR strategy utilized to replace the gene with an attP docking site. The S1 and S2 target sites are indicated (red arrows). Homology arms of ~1 kb immediately flanking the cleavage sites were cloned into pHD-DsRed-attP.

Table 2 Germline transmission rates of HDR

gRNAs	dsDNA donor	Injection line	Male crosses		Female crosses		% germline transmission	% HDR progeny per founder
			% (no.) founders	% (no.) HDR progeny	% (no.) founders	% (no.) HDR progeny		
S1, S2 (DNA)	attP	<i>vasa-Cas9</i>	22 (6/27)	11 (493/4424)	13 (3/23)	3 (106/3233)	18 (9/50)	75 (36–100)
S1, S2 (DNA)	attP	<i>w¹¹¹⁸</i>	22 (2/9)	3 (48/1616)	0 (0/9)	0 (0/1400)	11 (2/18)	19 (11–28)
S1, S2 (DNA)	attP	<i>lig4</i>	0 (0/11)	0 (0/2599)	0 (0/17)	0 (0/3066)	0 (0/28)	0
S1, S2 (RNA)	attP	<i>vasa-Cas9</i>	5 (1/21)	0.1 (2/3711)	0 (0/10)	0 (0/862)	3 (1/31)	2
S1, S3 (DNA)	FRTs	<i>vasa-Cas9</i>	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.

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° without heat shock or rearing them at 18° with a single heat shock of 1 hr at 37° (Hampel *et al.* 2011).

Generation of conditional alleles with CRISPR/Cas9-mediated HDR

The replacement of a gene with an attP recombination docking site provides ongoing genetic access to a targeted locus, allowing for an unlimited array of subsequent modifications (Gao *et al.* 2009; Huang *et al.* 2009). However, in many circumstances, including the targeting of genes required for viability or the precise insertion of exogenous DNA sequences, it will be preferable to directly target a locus for specific modifications. To investigate the possibility of using our marked dsDNA donor strategy to mediate such modifications we sought to create a conditional allele of *DSH3PX1* through the site-specific integration of FRT sites flanking the first exon. We injected a dsDNA donor containing the first exon and a visible marker flanked by FRT sites along with DNA plasmids encoding flanking gRNAs into *vasa-Cas9* embryos (Figure 3A). Two of 16 crosses yielded DsRed-positive flies for a founder rate of 13% (Table 2). Upon molecular characterization, we found that one of the two founder 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 *DSH3PX1^{EY08084}* flies, which contain a 10.9-kb EP element in the first exon (Figure 4A). We employed flanking gRNAs, which were injected as plasmid DNA along with *hsp70-Cas9* into *DSH3PX1^{EY08084}* 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^{+mC}* 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 (Gradick *et al.* 2013; Fu

Figure 3 dsDNA donors can be used to generate positively marked conditional alleles. (A) Schematic of the *DSH3PX1* locus including the S1 and S3 target sites flanking the first exon. Homology arms of ~1 kb flank the target sites. The donor vector also contains the first exon and is designed to incorporate an FRT site near the S1 target site as well as the 3xP3-DsRed marker and a second FRT sequence near the S3 target site. In the resulting *DSH3PX1*^{conditional} allele, the first exon and DsRed marker can be removed by Flp recombinase. (B) Flies in which the first exon of *DSH3PX1* has been removed can be identified by the loss of 3xP3-DsRed in the eye.

et al. 2013; Hsu *et al.* 2013; Mali *et al.* 2013b; Pattanayak *et al.* 2013; Cho *et al.* 2014) 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 carried out in cell lines (Cradick *et al.* 2013; Fu *et al.* 2013; Hsu *et al.* 2013; Mali *et al.* 2013b) and define potential off-target sites elsewhere in the genome as sites with (i) perfect matches (zero mismatches) to the seed sequence, (ii) one mismatch in the seed sequence and four or fewer mismatches in the distal sequence, and (iii) two mismatches in the seed sequence with a maximum of one mismatch in the distal sequence. High-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

Figure 4 CRISPR/Cas9-induced deletions can be identified through negative screening. (A) Schematic of the *DSH3PX1*^{EY08084} locus containing a *P* element marked by *w⁺mC*. Target sites for gRNAs S1 and S2 are indicated (red arrows). (B) Sequence alignment of the targeted deletion reveals breakpoints and imprecise repair at the predicted cut sites (red arrowheads), confirming a 14.2-kb deletion that includes the *P* element.

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 NAG 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 inviable. 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.

gRNA	High		Maximum		Independent F1s analyzed
	NGG	NAG	NGG	NAG	
R2	0	1	4	6	3/7
R3	0	0	2	4	6/21
S1	0	2	3	9	14/14
S2	0	1	5	9	12/12
S3	6	1	21	37	2/2

gRNA	off target #	off-target site sequence			# mismatches		indel frequency
		distal	proximal	PAM	proximal	distal	
R2	-	GATCCGCA	ACGTGCCTGTT	TGG	-	-	-
	1	cAttgtCA	AtGTCGCTGTT	TGG	1	4	0/3
	2	cAcCgGcg	ACGTGcCTGTT	TGG	1	4	0/3
	3	cAttcGtc	ACGTGCcATGTT	TGG	1	4	0/3
	4	GggCCaaa	ACGTGCCTGtC	GGG	1	4	0/3
R3	-	GACCGAAG	TGTCGCCTGATC	CGG	-	-	-
	1	aACtaAtG	TGcCGCTGATC	CGG	1	4	0/6
	2	cAcaAAAc	TGTCGCCTGgTC	TGG	1	4	0/6
S1	-	GAATTCTA	CACCTGGAACAG	AGG	-	-	-
	1	GAcCaACTA	CAaTTGGAACAG	CGG	1	3	0/14
	2	tAtTatTA	CACaTGAACAG	TGG	1	4	0/14
	3	GAtTgggA	CACCTTGGcACAG	TGG	1	4	0/14
	4	aggTgaac	CACCTTGAACAG	TAG	0	7	0/14
S2	-	GGAGCACA	AAGAAACCGCAC	CGG	-	-	-
	1	GgtGaAcg	AAaAAACCGCAC	AGG	1	3	0/12
	2	aGAcgACA	AAGcAACCGCAC	AGG	1	3	0/12
	3	atAGaAaA	AAGcAACCGCAC	TGG	1	4	0/12
	4	GgcGgcCc	AAGAAACCGtAC	AGG	1	4	0/12
	5	GGAGCcCA	AAGGAACCGcAG	GGG	2	1	0/12
S3	-	TCAACTCA	GTTGGAAATGT	TGG	-	-	-
	1	aaActTCA	GTTGGAAATGT	TGG	0	4	0/2
	2	TgAaaaat	GTTGGAAATGT	TGG	0	5	0/2
	3	ggAaaatg	GTTGGAAATGT	TGG	0	6	0/2
	4	ctgAaaat	GTTGGAAATGT	TGG	0	7	0/2
	5	aacttatt	GTTGGAAATGT	TGG	0	8	0/2
	6	gagggggg	GTTGGAAATGT	CGG	0	8	0/2
S3	7	cgggtgggA	GTTGGAAATGT	CAG	0	7	0/2

Figure 5 Off-target cleavage analysis. (A) Comparison of the off-target site profiles of the gRNAs used in this study. The numbers of NGG and NAG-adjacent off-target cleavage sites identified by CRISPR Optimal Target Finder using both high- and maximum-stringency criteria are represented as are the numbers of independent F1 flies investigated out of the total number of founders generated using each guide. The off-target sites analyzed in this study are indicated in green and listed in B. (B) All off-target sites surveyed in this study. The CRISPR target sequence is provided for each guide. Potential off-target sequences are listed with mismatches to the target sequence indicated by lowercase red letters. Noncanonical NAG PAM sites are underlined. The location (proximal or distal) and number of mismatches are also indicated. Note that for gRNA S3, a G nucleotide was added to the 5' end to improve transcription efficiency from the U6 promoter. The frequency of off-target indel formation is indicated as the number of independent events out of the number of independent lines analyzed. Note that for gRNA S3, a G nucleotide was added to the 5' end to improve transcription efficiency from the U6 promoter.

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/ μ l) with *in vitro* transcribed gRNAs at concentrations >25 ng/ μ l 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 (Cradick *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; 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). It is possible to mutate one nuclease 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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Supporting Information

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

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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

```
GGATCTAATTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCTGAACCCCTCGACCGCCGGA
GTATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAAGCTAAGCAAA
TAAACAAGCGCAGCTGAACAAGCTAAACAATCGGCTCGAGACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTTACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA
CGGCAAGCTGACCCGTGAAGTTTATCTGCACCACCGGCAAGCTGCCCGTGGCCCGACCCCTCGTGACCACCCGTGACCTACGGCG
TGCAGTGCTTCAGCCGCTACCCCGACCATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCGC
ACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCT
GAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATGG
CCGACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTAC
CAGCAGAACACCCCATCGGCGCAGGGCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAAGACCC
CAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAA
GCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGA
ACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACA
AATTTCAAAATAAAGCATTTTTTTCACTGCATTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAGCTAGCATAAATTCG
TATAATGTATGCTATACGAAGTTATGCTAGCGGGCCCCCCTCGATCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTG
CAGCTGGTTGTAGGTGCAGTTGCGCTTCTTGCACTTGGCGCACTTGAGGAGATCGGTCTTGGTGCCCTGCACGGTGGCCAGCTGGG
CATCGTTGATGGCTCCTTGACGAACCTTCTCGCGCAGCTTCTTCATCTCGTCGCTGGCCATCTCCTCCGGCGTCATTTTGGCCAGC
TGCTTGGCAGTGACGGCGCCGCACATAAAGTTGCCGCGCAATCCAGGATTCTTGGGGTCTTTCAGATTGGCCACGCGTGACCTAAT
GCGATTCTTGTACTTCATATCCGTGTTATTGAACTCGGAGTAAATGGCATCCTCCAGTTTCGGCGGCCATTTCTCCGGCTCACCGC
ATCCTTCGGGCACCTTCACCAATCTTCAGTGCGGTGGCCAGCATTTTCGCGGCATTTGATGCGGACCGCATCTGTATGCCGCCGGAG
GGAACGAGGTCTGTGACGAGGATGTCGAGCCTTCTTCTCTTTATCCTTGGATGAGCTGGATGAACTGGACTTATCCTTGCCGGA
GATTGACGACGACGACTTGGCGGCACTGGTGGACTTCGAGGCACTGCTGTTGTTGGAGGAGCCCTCCTTAGCGGAGCTGTTGTTTCG
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D. *DSH3PX1* conditional allele donor vector

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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) pHD-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	<i>y[1] M{vas-Cas9}ZH-2A w[1118]/FM7c</i>	51323
vasa-Cas9 on 3	<i>w[1118]; PBac{y[+mDint2]=vas-Cas9}VK00027</i>	51324
vasa-Cas9, U6-tracrRNA on X	<i>y[1] M{vas-Cas9,U6-tracrRNA}ZH-2A w[1118]</i>	51326
vasa-Cas9, U6-tracrRNA on 3	<i>w[1118]; PBac{y[+mDint2]=vas-Cas9,U6-tracrRNA}VK00027</i>	51325
U6-tracrRNA on X	<i>y[1] M{w[GMR.PHb]=U6-tracrRNA}ZH-2A w[1118]/FM7c, P{w[+mC]=GAL4-Kr.C}DC1, P{w[+mC]=UAS-GFP.S65T}DC5, sn[+]</i>	51322
U6-tracr RNA on 2	<i>w[1118]; PBac{y[+mDint2] w[GMR.PHb]=U6-tracrRNA}VK00037/CyO, P{Wee-P.ph0}2</i>	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	GGTACACCTGCGCAGTCGCTATGGTAATTAGGACGTGACG	DsRed-attP
SH3PX1 5' Arm AarI R	GGACCACCTGCCCTCCTACCAGAGGCACTCTCTAATTTGATAAAT	DsRed-attP
SH3PX1 3' Arm SapI F	GAGTGCTCTTCTTATCACCAGGGCACACACTTCCACAC	DsRed-attP
SH3PX1 3' Arm SapI R	GGCTGCTCTTCGGACTGGTAAACACAAGCAGAGAGC	DsRed-attP
SH3PX1 5' Arm F SpeI	GGACTAGTGTTCAACAACGACTCTTATTGATCG	DsRed-FRT
SH3PX1 5' Arm R XbaI	TTTCTAGAGAATAGGAACTTCAGAGGCACTCTCTAATTTGA	DsRed-FRT
SH3PX1 3' F Arm XbaI	TCTCTAGAAAGTATAGGAACTTCTCCAAGGTAGAAATTCAGGT	DsRed-FRT
SH3PX1 3' Arm R HindIII	CCAAGCTTCTTATAGAGACGTCAACCCACCTGT	DsRed-FRT
3xP3 DsRed NgoMIV F	ATTGCCGGCGAATTCGAGCTCGCCCGGGGA	DsRed-FRT
3xP3 DsRed NgoMIV R	GGCCGGCGAAGTTCCTATACTTTCTAGAG	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.