A Versatile Two-Step CRISPR- and RMCE-Based Strategy for Efficient Genome Engineering in *Drosophila*

Xu Zhang, Wouter H. Koolhaas, and Frank Schnorrer[1]
Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
ORCID ID: 0000-0002-1628-9895 (X.Z.)

ABSTRACT The development of clustered, regularly interspaced, short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) technologies promises a quantum leap in genome engineering of model organisms. However, CRISPR-mediated gene targeting reports in *Drosophila melanogaster* are still restricted to a few genes, use variable experimental conditions, and vary in efficiency, questioning the universal applicability of the method. Here, we developed an efficient two-step strategy to flexibly engineer the fly genome by combining CRISPR with recombinase-mediated cassette exchange (RMCE). In the first step, two sgRNAs, whose activity had been tested in cell culture, were co-injected together with a donor plasmid into transgenic *Act5C-Cas9*, *Ligase4* mutant embryos and the homologous integration events were identified by eye fluorescence. In the second step, the eye marker was replaced with DNA sequences of choice using RMCE enabling flexible gene modification. We applied this strategy to engineer four different locations in the genome, including a gene on the fourth chromosome, at comparably high efficiencies. Our data suggest that any fly laboratory can engineer their favorite gene for a broad range of applications within approximately 3 months.

Reverse genetics is currently booming with the establishment of TALEN- and CRISPR-mediated genome engineering (Hsu *et al.* 2014; Sander and Joung 2014; Joung and Sander 2013). In particular, the CRISPR/Cas9 technology appears to efficiently and specifically introduce double strand DNA breaks in the genome of the organism, which can then be utilized to either introduce point mutations by error-prone nonhomologous end-joining (NHEJ) or integrate heterologous DNA into the chromosome using the homology-directed repair (HDR) pathway (Sander and Joung 2014). In *Drosophila*, CRISPR-induced NHEJ has mainly been utilized to mutate genes that result in a visible, easily scored phenotype, such as white eyes or yellow body color, or to mutate GFP transgenes (Gratz *et al.* 2013a; Sebo *et al.* 2014; Ren *et al.* 2013; Bassett *et al.* 2013). Mutants in genes with no visible phenotype required PCR screening for

their identification; therefore, high mutagenesis rates are important, which might be difficult to achieve at all positions in the fly genome (Kondo and Ueda 2013; Yu *et al.* 2013, 2014; Gokcezade *et al.* 2014; Port *et al.* 2014). Recently, this bottleneck was addressed by applying CRISPR-induced HDR to insert an attP-site together with a visible marker into the gene of interest (Baena-Lopez *et al.* 2013; Gratz *et al.* 2014; Xue *et al.* 2014). In some cases the visible marker was flanked by FRT or loxP sites allowing its excision to only leave one attP site (and one loxP or FRT site) within the gene. This attP site enables the introduction of any given DNA sequence into the gene of interest (Baena-Lopez *et al.* 2013; Gratz *et al.* 2014; Xue *et al.* 2014). However, the efficiency of reporter integration was rather low (Baena-Lopez *et al.* 2013) and only determined at a single genomic locus (Gratz *et al.* 2014; Xue *et al.* 2014), leaving the general applicability to the *Drosophila* genome an open question. Port *et al.* (2014) reported an alternative CRISPR-mediated HDR strategy by using a transgenic single guide RNA (sgRNA) source and crossing it to a transgenic Cas9 source. This method also enabled targeting of somatic cells in a tissue-specific manner, but it required the generation of new transgenic sgRNA lines for every locus (Port *et al.* 2014).

Here, we have developed a highly flexible two-step genome engineering platform that combines CRISPR-mediated HDR with ΦC31 recombinase-mediated cassette exchange (RMCE). In the first step, CRISPR is applied to integrate a splice acceptor and an SV40 terminator

together with a 3xP3-dsRed eye reporter. This enables both the efficient identification of the targeted event and the creation of a strong loss of function allele. In the second step, two flanking attP sites are utilized to replace the inserted DNA by any DNA of choice using RMCE, an established standard technology in *Drosophila* (Venken *et al.* 2011). Together, this allows flexible cassette exchange to freely manipulate the gene of interest. We successfully applied this method to four different locations in the genome and efficiently generated several allele variants, including a conditional allele, from a single targeting event. Our streamlined CRISPR/Cas9-based and RMCE-based strategies make it practical to flexibly engineer any *Drosophila* gene of choice for a broad range of applications within approximately 3 months.

MATERIALS AND METHODS

Fly strains and genetics
All fly work, unless otherwise stated, was performed at 25° under standard conditions. The *Lig4[169]* null allele (McVey *et al.* 2004) was obtained from the Bloomington *Drosophila* Stock Center, and *y[1], M(Act5c-Cas9, [w+])* in *M(3xP3-RFP.attP)ZH-2A, w[1118]* was a gift from Fillip Port and Simon Bullock before publication (Port *et al.* 2014). Both markers (w+ and 3xP3-RFP) were removed by crossing to heat-shock-Cre. The *y[1], M(Act5C-Cas9)ZH-2A, w[1118]* flies were recombined with *Lig4[169]* to obtain *y[1], M(Act5C-Cas9)ZH-2A, w[1118], Lig4[169]*.

Cell culture
Drosophila Schneider 2 (S2) cells stably expressing myc-Cas9 from a ubiquitin promoter were a gift from Klaus Förstemann before publication (Böttcher *et al.* 2014). S2 cells were cultured in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum (Life Technologies) and penicillin/streptomycin (GE Healthcare). sgRNA activities were tested by transfecting 1 μg sgRNA per 24 wells into the myc-Cas9 cells using Fugene HD (Promega), followed by DNA extraction and a T7-Endonuclease I assay (see supplied protocol for details).

Plasmids
CC6-U6-gRNA_hsp70-Cas9 plasmid was a gift from Peter Duchek before publication (Gokcezade *et al.* 2014). pJET1.2-STOP-dsRed: attP1 and splicing acceptor (SA) were amplified with primers XZ82 and XZ83, SV40 terminator with XZ84 and XZ85, and attP2 with XZ88 and XZ89 from DNA extracted from a MiMIC fly line (Venken *et al.* 2011); 3xP3-dsRed was amplified from a fosmid fly line (Langer *et al.* 2010) with primers XZ86 and XZ87. These fragments were cloned into pLR-HD plasmid by Golden Gate cloning (Cermak *et al.* 2011). This assembled attP1-SA-STOP-SV40-3xP3-dsRed-attP2 cassette was amplified with primers XZ195 and XZ196 and blunt cloned into pJET1.2 to generate pJET1.2-STOP-dsRed. Because this STOP-dsRed cassette is flanked by two BsmBI sites, it can be easily assembled with both homology arms (Figure 3A): each homology arm of approximately 1 kb was amplified from genomic DNA of the target genotype with Phusion polymerase (NEB) and blunt-end cloned into pJET1.2 (CloneJET PCR Cloning Kit, Thermo Scientific). Primers used to amplify the homology arms have a 5′ BsmBI site enabling Golden Gate assembly with the STOP-dsRed cassette. All primers used are listed in Supporting Information, Table S1. pBS-donor-backbones pBS-GGAC-TTCT, pBS-GGAC-ATGC, and pBS-CGGA-GTGC were constructed by linearizing pBluescript with *Kpn*I and *Sac*II, followed by amplification with primer pairs XZ150 and XZ151, XZ156 and XZ151, and XZ161 and XZ162, respectively, and re-ligation. The generated pBS-donor-backbones harbor two BsmBI sites for donor plasmid assembly.

pJET1.2-STOP-dsRed, pJET1.2-HA-left, pJET1.2-HA-right, and an appropriate pBS-backbone were assembled to the pBS-donor vector by Golden Gate cloning. attB plasmids FRT-2xTY1-FRT-V5 and 2xTY1-V5 fragments were synthesized as gBlocks (IDT) and cloned into the attB plasmid for all three reading frames. For construction of CC6-U6-gRNA_hsp70-Cas9-sgRNA1,3,4,7 and 9 the CC6-U6-gRNA_hsp70-Cas9 vector was cut with BbsI (NEB) and the annealed sgRNA targeting oligos were cloned into it. The vas-ΦC31(3xP3-EGFP.attB) plasmid was obtained from Johannes Bischof (Bischof *et al.* 2007). The attB site was removed by digestion with *Spe*I, followed by re-ligation.

All plasmids for embryo injections were purified with PureLink HiPure Plasmid Midiprep Kit (Life Technologies). Oligos are listed in Table S1.

sgRNA synthesis
The sgRNA dsDNA template was produced using overlap PCR with a small amount of a common sgRNA scaffold primer, a shorter sgRNA amplification primer, and a sgRNA gene-specific primer that includes the T7 promoter (Figure 3C) (Böttcher *et al.* 2014). All sgRNA primer sequences are listed in Table S1. The PCR product was cleaned by Qiagen MinElute kit (Qiagen). The sgRNAs were transcribed with T7-MEGAshortscript Kit (Life Technologies) and purified with MEGAclear Transcription Clean-Up Kit (Life Technologies).

Embryo injection
Preblastoderm embryos of the appropriate genotype were de-chorionated and injected with a FemtoJet apparatus (Eppendorf) using self-pulled glass needles (Harvard Apparatus) under standard conditions at room temperature. Injected embryos were kept for 2 d at 18° and the hatched larvae were collected and grown at 25°. For step 1 injections, pBS-donor plasmid, two sgRNAs, and (optionally) the CC6-U6-gRNA_hsp70-Cas9 plasmid were mixed and diluted in water. *Lig4[169]* embryos were injected with CC6-U6-gRNA_hsp70-Cas9 plasmid (100 ng/μl) and pBS-donor plasmid (500 ng/μl). *y[1], M(Act5C-Cas9)ZH-2A, w[1118], Lig4[169]* embryos were injected with both sgRNAs (60–70 ng/μl each) and pBS-donor plasmid (500 ng/μl). For step 2 injections, the attB plasmid (150 ng/μl) was mixed with vasa-ΦC31 plasmid (200 ng/μl).

Immunolabeling of IFMs: Hemi-thoraces of adult *Drosophila* were prepared and stained as described (Weitkunat and Schnorrer 2014). Rabbit anti-Salm was used at 1:50 (Kühnlein *et al.* 1994), mouse anti-Flag (Sigma), mouse anti-V5 (Abcam), and rhodamine phalloidin (Invitrogen) were all used at 1:500. Nuclei were visualized by embedding in Vectashield plus DAPI (Vector Laboratories) and images were acquired on a Zeiss LSM780 confocal and processed with FIJI and Photoshop.

Detailed *Drosophila* genome engineering protocol by CRISPR-RMCE

1. **CRISPR-sgRNA design and donor plasmid cloning ~10 d**
   1.1 Verify sequence of the planned targeting regions for the sgRNAs in the fly strain used and in the S2 cells by sequencing to identify potential polymorphisms compared with the published sequence.
   1.2 For designing the sgRNA targeting sites, choose one of the web tools (Beumer and Carroll 2014). We used an interface designed by the Zhang laboratory (http://crispr.mit.edu).
   1.3 sgRNA production
   1.3.1 Generate the dsDNA template for sgRNA *in vitro* transcription as described by Böttcher *et al.* (2014).

1.3.2 Transcribe sgRNA by T7-MEGAshortscript Kit (AM1354; Life Technologies). Use 150- to 250-ng template for a 20-μl reaction at 37° overnight.

1.3.3 Purify sgRNA by MEGAclear Transcription Clean-Up Kit (Life Technologies). Follow the manufacturer's protocol and, in step 3, add an equal volume of 100% ethanol to the sample.

1.3.4 Check the sgRNA integrity on a gel and measure the concentration using a photometer (Nanodrop, Thermo Scientific). The expected yield is 50–100 μg, which is enough for the S2 cell assay and the fly injections.

1.4 sgRNA activity assay in S2 cells

1.4.1 Grow S2 cells in Schneider medium with 10% FCS (Life Technologies) to $5–10 \times 10^6$ cells/ml at 25°.

1.4.2 Dilute cells to 0.7×10^6/ml and plate 1 ml cells in S2 medium with 10% FCS per well in a 24-well plate for each transfection.

1.4.3 Prepare the transfection mix by diluting 1 μg sgRNA in 50 μl serum free medium and 4 μl Fugene HD mix plus 46 μl serum free medium, mix both, and incubate for 45 min at room temperature.

1.4.4 Add the Fugene/RNA mix to each well and mix gently by pipetting.

1.4.5 After 48–60 hr at 25°, harvest the cells and extract the genomic DNA by QIAamp DNA mini kit (Qiagen).

1.4.6 For the T7 Endonuclease I assay, amplify an approximately 500-bp fragment, which harbors the sgRNA targeting site with Phusion polymerase (NEB), and denature and anneal the PCR product as described by Zhang et al. (2014).

1.4.7 Mix on ice 10 μl annealed PCR product with 10 μl T7 Endonuclease I master mix [2 μl T7 endonuclease I buffer, 0.5 μl T7 endonuclease I (5 units, NEB) and 7.5 μl water].

1.4.8 Digest at 37° for 15–20 min using a PCR machine and load on 1.5% agarose gel immediately.

1.4.9 Estimate the efficiency of different sgRNAs by comparing the band intensities of the digested and nondigested bands.

1.5 Generation of donor plasmid (can be performed in parallel with steps 1.3 and 1.4 to save time)

1.5.1 Amplify left and right homology arms (approximately 1 kb, start as close to the sgRNA cutting site as possible) with Phusion polymerase (NEB) from the fly strain that is used for HDR and clone them into pJET-1.2 according to the CloneJET PCR Cloning Kit (Thermo Scientific).

1.5.2 Assemble the Golden Gate Cloning reaction [50 ng pBS-backbone, 80 ng pJET1.2-HA-left, 80 ng pJET1.2-HA-right, 80 ng pJET1.2-STOP-dsRed, 1.5 μl 10x T4 ligation buffer, 1 μl BsmBI (NEB, R0580), 1 μl T4 ligase (NEB, M0202) add water to 15 μl].

1.5.3 Ligate in PCR machine using the following cycles: 15 cycles of 37°, 5 min/16°, 10 min/37°, 15 min/50°, 5 min/80°, and 5 min/4°.

1.5.4 Assemble the Plasmid-safe nuclease reaction (15 μl ligation reaction 3 μl 10× Plasmid-safe buffer 1.2 μl 25 mM ATP 1 μl Plasmid-safe nuclease (Epicentre) 9.8 μl water).

1.5.5 Incubate at 37° for 60 min in PCR machine and transform 5–10 μl in bacteria. Most growing colonies will be correct.

2. **Fly step 1 - CRISPR-mediated HDR ~6 wk**

2.1 Inject 600–800 Act5C-Cas9, lig4[169] embryos with pBS-donor (500 ng / μl) and two sgRNAs (each 60–70 ng/μl, targeting close to the chosen homology arms). Collect at least 50 fertile mosaic G_0 flies.

2.2 Cross G_0 flies individually (at least 50 vials) either to yw flies or appropriate balancer flies and screen all the F_1 progeny for fluorescent red eyes using a fluorescent binocular (Leica MZ16-FA). Keep track of how many independent G_0 founders lead to how many F_1 carrier flies.

2.3 Generate stocks from an individual F1 carrier by crossing to balancer flies resulting in an isogenized stock for the engineered chromosome. Verify the targeting event by PCR and sequencing.

3. **Fly step 2: ΦC31-mediated RMCE ~6 wk**

3.1 Inject a "generic" plasmid generated by Venken et al. (2011) or this study or your own custom-made gene-specific attB plasmid (150 ng/μl) mixed with vasa-ΦC31 plasmid (200 ng/μl) into approximately 200 embryos from an amplified stock generated at 2.3.

3.2 Cross G_0 flies individually to an appropriate balancer and screen all F_1 progeny for nonfluorescent eyes using a fluorescent binocular (Leica MZ16-FA).

3.3 Generate stocks from an individual F1 carrier by crossing to balancer flies, resulting in an isogenized stock for the engineered chromosome. Verify the correct orientation of the RMCE by PCR (will be ~50%).

RESULTS

Strategy overview

We aimed to develop a versatile and efficient strategy to modify the Drosophila genome that would allow various genome modifications such as the introduction of single point mutations, protein tags, exon deletions, or other desired changes in the gene of choice. Despite the suggested higher efficiency of CRISPR/Cas9-induced HDR as compared with Zn-finger–induced or TALEN-induced HDR, the identification of successfully targeted carrier flies is still a limiting step in the process. PCR-based screening or melting curve analysis methods require DNA extraction (Beumer et al. 2013a), which can be inconvenient for efficient stock generation. Therefore, we decided to develop a two-step strategy as illustrated in Figure 1, which enables efficient identification of the carrier flies and allows entirely flexible genome engineering. In the first step, we insert a 3xP3-dsRed marker enabling easy identification of the HDR event. A strong splice acceptor, followed by STOP codons and an SV40 polyA terminator, precedes the dsRed cassette. The inserted DNA is flanked by two attP sites in opposite orientations, a strategy that we adapted from the popular MiMIC system (Venken et al. 2011). If this cassette is inserted into an intron or replaces an endogenous exon, then it results in truncated mRNA of the targeted gene. Thus, step 1 can be used to create a loss-of-function allele (Figure 1).

In the second step, RMCE is applied to replace the DNA between both attP sites by any DNA of choice, leaving a minimal scar of two attR sites, preferably in introns. RMCE has been used very efficiently in the MiMIC system, demonstrating that attR sites in introns generally do not interfere with gene function (Venken et al. 2011). Hence, our strategy enables the generation of various alleles, like a defined point mutation, a tagged allele, an exon replaced by a tag, or a conditional allele, from a single HDR carrier (Figure 1). This strategy should allow flexible editing of any Drosophila gene within approximately 3 months (Figure 2).

CRISPR design and cloning

In step 1, we aimed to insert a STOP-3xP3-dsRed cassette flanked by two attP sites using a donor plasmid (Figure 1). Because the same strategy should be applicable to any gene, we established a single-step
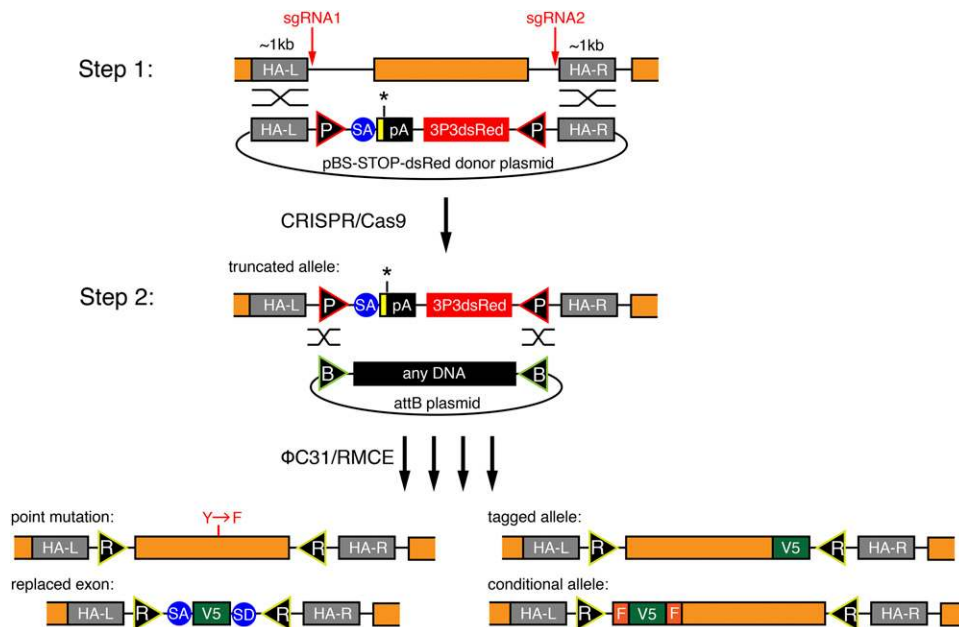
Figure 1 A two-step method to flexibly engineer the fly genome. Overview of the two-step procedure. In step 1, a donor vector consisting of two attP sites (P), a splice acceptor (SA), and STOP codons (yellow box, black asterisk) followed by an SV40 polyadenylation signal (pA) and a 3xP3dsRed marker are inserted on Cas9 cleavage with two sgRNAs. The orange coding exon is excised. In step 2, ΦC31-mediated RMCE inserts any DNA sequence between the two attB sites (B). Examples for various engineered exons are given, resulting in attR sites (R) in introns. F stands for FRT.

Golden Gate protocol to assemble the STOP-dsRed donor plasmid containing approximately 1-kb homology arms on each side, which has been shown to be of sufficient length for efficient HDR (Beumer *et al.* 2013b) and can be easily amplified by PCR. Cloning of the homology arms into the donor vector is thus very straightforward and takes only a few days for the gene of choice (Figure 3A, see *Materials and Methods*). This donor vector is the template for the HDR in step 1.

In step 2, RMCE exchanges the STOP-dsRed by any sequence located between two attB-sites in a provided donor plasmid (Figure 1). RMCE works very reliably and a large collection of plasmids to tag genes or insert reporters for various applications is available (Venken *et al.* 2011). These plasmids are fully compatible with our step 2 design. We have generated additional "generic" attB plasmids that can be used to tag any gene with a 2xTY1-V5 tag or to engineer a conditional allele using an FRT flanked 2xTY1 cassette followed by a V5 tag (Figure 3B). Flp-mediated deletion of the 2xTY1 cassette will lead to a frame shift and thus can be used to create loss-of-function clones at very high efficiency, as the flip-out will occur *in cis* (Hadjieconomou *et al.* 2011). The TY1 tag is a convenient affinity tag (Sarov *et al.* 2012). We have generated both constructs in all three reading frames.

Figure 2 Two-step genome engineering timeline. Schematic overview of the major steps of the genome engineering procedure. Details are provided in *Materials and Methods*.
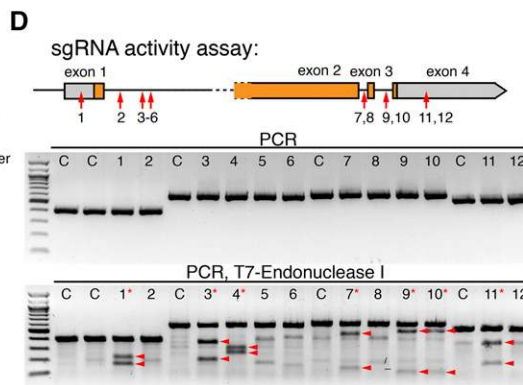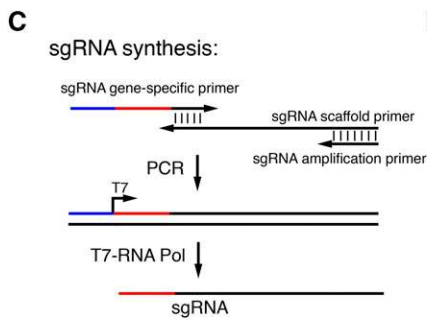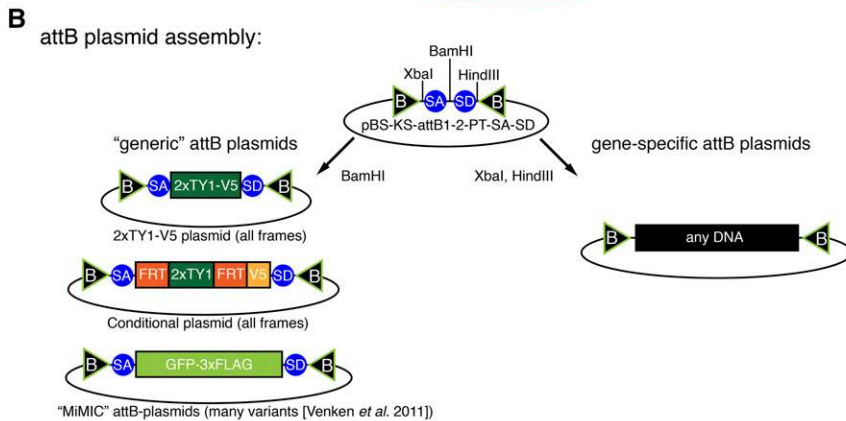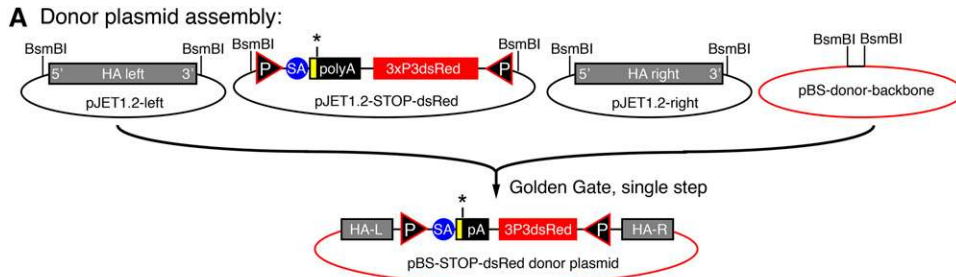
Figure 3 Cloning scheme and sgRNA activity tests. (**A**) Single-step Golden Gate assembly scheme of the STOP-dsRed donor vector cloned into a modified pBluescript backbone. (**B**) Scheme of the "generic" attB plasmids used in our study. A simple cloning step is sufficient to generate any gene-specific attB plasmid that can be used to replace an excised exon. (**C**) sgRNA synthesis scheme. (**D**) sgRNA activity assay of 12 different sgRNAs in S2 cells. PCR result with (bottom) or without (middle) T7-Endonuclease I treatment are shown. Digested products are marked by arrow heads and effective sgRNAs are marked by a red asterisk. C are controls without sgRNA.

CRISPR activity assay in cell culture

Many search algorithms exist to predict sgRNA target sequences for a given gene region (Beumer and Carroll 2014). However, to date there is no simple way of confirming if any of the predicted sgRNAs work efficiently. We developed such a selection assay to be able to only inject effective sgRNAs into fly embryos. We designed 12 different sgRNAs targeting different regions in the *salm* gene and synthesized the sgRNAs by a standard PCR and *in vitro* transcription reaction (Figure 3C). These sgRNAs were then individually transfected into Cas9 expressing S2 cells (Böttcher *et al.* 2014), and their cleavage efficiency was determined with a simple T7-Endonuclease I assay (see *Materials and Methods*) (Zhang *et al.* 2014). On average, approximately half of the tested sgRNAs work efficiently in this assay (Figure 3D), strongly suggesting that such a preselection test is useful to improve the *in vivo* success rates.

Step 1: HDR in *Lig4* mutant embryos

To test the efficiency of inserting our STOP-dsRed cassette, we designed three donor constructs targeting different regions in the *salm* gene (Figure 4A): the first, deleting parts of exon 1; the second, inserting the cassette into intron 1; and the third, deleting exon 3. For each construct approximately 1-kb homology arms were cloned in the

STOP-dsRed donor vector. We injected the STOP-dsRed donor as circular plasmid together with two plasmids each containing a U6 promoter-driven sgRNA verified in S2 cells and a hsp70-Cas9 source (see *Materials and Methods*). We injected into *Ligase4* mutant embryos, which were reported to exhibit a higher rate of HDR than wild-type embryos (Beumer *et al.* 2013a,b). We injected between 700 and 1500 embryos for each of the three constructs and were able to recover 11 red-eyed F1 carriers from two independent founders for the first intron construct and 72 red-eyed F1s from four independent founders for the third exon deletion construct (Table 1). This demonstrated that our strategy works in principle, but because we failed to recover the first exon deletion allele, we wanted to further improve the efficiency by using a different Cas9 source.

Step 1: Transgenic Cas9 improves HDR efficiency

A number of transgenic Cas9 flies have been generated recently, and some of which have been used successfully (Ren *et al.* 2013; Xue *et al.* 2014; Gratz *et al.* 2014; Port *et al.* 2014). To test whether a transgenic Cas9 source is more efficient for HDR than a source from an injected plasmid, we targeted the same positions as above, but now using *Act5C-Cas9, Lig4* flies. For this, we recombined an *Act5C-Cas9* transgene
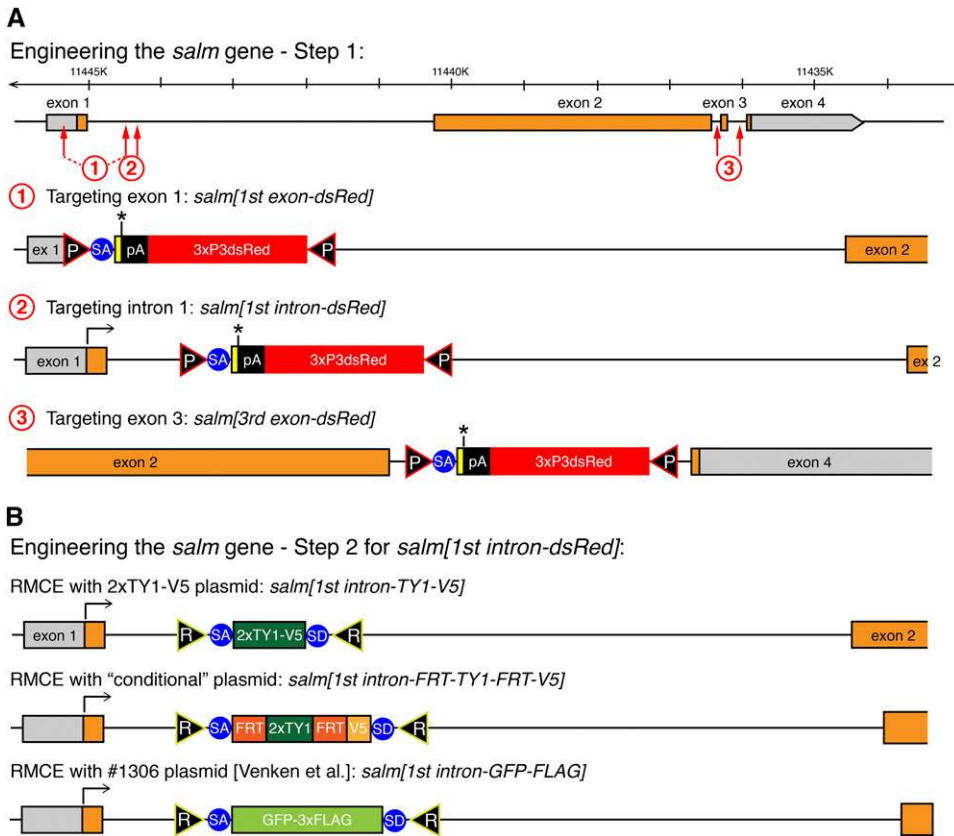
Figure 4 Engineering of the *salm* gene. (**A**) Step 1 engineering of the *salm* gene. The genomic *salm* organization is depicted with coding exons in orange. The sgRNA targeting sites are indicated by red arrows and the resulting *salm[1st exon-dsRed]*, *[1st intron-dsRed]* and *[3rd exon-dsRed]* alleles are shown. (**B**) Step 2 engineering of the *salm* gene. RMCE products of the *salm[1st intron-dsRed]* with three different exon cassettes are shown.

expressing Cas9 ubiquitously, including maternally in the germline (Port *et al.* 2014), with the *Lig4[169]* null allele. Additionally, we removed the *white* and *3xP3dsRed* markers from the *Act5C-Cas9* transgene to obtain a *Act5C-Cas9, Lig4[169]* chromosome that is useful for the injection of our donor plasmids (see *Materials and Methods*). We injected 700 *Act5C-Cas9, Lig4[169]* embryos with two *in vitro* transcribed sgRNAs targeting either the first exon, the first intron, or the third exon of *salm* (using the same sgRNA target sequences as used above). We obtained one, nine, and four independent founders producing 13, 54, and 59 F1 carriers, respectively, demonstrating that all three locations were targeted successfully with frequencies between 3% and 14% per fertile G_0 (Table 1). To verify that the targeted insertion occurred correctly, we tested a total of nine independent carriers from the three locations by PCR and sequencing. We were able to confirm that all of these targeted correctly by "ends-out" homologous recombination. We did not detect any "ends-in" insertions, which were reported to occur occasionally (Yu *et al.* 2014) (Figure S1 shows *salm[1st intron-dsRed]* as an example).

The first step of our gene-targeting strategy inserts a strong splice acceptor followed by a STOP cassette into the gene and thus should terminate transcription at this position. By design, the *salm[1st exon-dsRed]* allele additionally has a deleted ATG. As expected, the *salm[1st exon-dsRed]* allele is homozygous lethal, as well as lethal *in trans* to *salm[1]*, demonstrating that we created a strong *salm* loss-of-function allele (Figure 5A). The *salm[1st intron-dsRed]* allele harbors an insertion in the first intron (Figure 4A). This allele is also homozygous lethal, and lethal *in trans* to *salm[1]*, suggesting that the splice acceptor and STOP cassette are used efficiently to create a strong loss-of-function allele (Figure 5A). The *salm[3rd exon-dsRed]* allele only deletes the last 36 amino acids of the long SalmPA isoform, including 10 amino acids of the last zinc finger (Figure 4A). This allele is homozygous viable (Figure 5A). Taken together, these data suggest that our CRISPR-mediated step 1 strategy works efficiently to isolate targeted carrier flies at a practical frequency for routine use. Conveniently, these step 1 alleles are generally loss-of-function alleles if the insertion is located within the gene.

■ **Table 1 Summary of the transformation efficiencies for the four different genomic locations modified in this study**

Location	Injected Genotype	sgRNAs	Injected Embryos	Larvae	G0 Adults	Fertile G0 Adults	Independent G0 Founders	Total Red-Eyed F1	Independent Founders Per Fertile G0s
salm[1st exon-dsRed]	*Lig4*^{−/−}	sgRNA1 + sgRNA3	1500	396	166	ND	**0**	0	ND
salm[1st intron-dsRed]	*Lig4*^{−/−}	sgRNA3	700	171	149	ND	**2**	11	ND
salm[3rd exon-dsRed]	*Lig4*^{−/−}	sgRNA7 + sgRNA9	1500	312	150	ND	**4**	72	ND
salm[1st exon-dsRed]	*Act5C-Cas9, Lig4*^{−/−}	sgRNA1 + sgRNA3	700	124	48	30	**1**	13	3.3%
salm[1st intron-dsRed]	*Act5C-Cas9, Lig4*^{−/−}	sgRNA3 + sgRNA4	700	200	122	64	**9**	54	14.1%
salm[3rd exon-dsRed]	*Act5C-Cas9, Lig4*^{−/−}	sgRNA7 + sgRNA9	700	291	150	99	**4**	59	4.0%
bent	*Act5C-Cas9, Lig4*^{−/−}	*bent*-sg1 + *bent*-sg3	700	204	118	56	**1**	5	1.8%

Step 2: Flexible gene editing by RMCE

A major benefit of our editing strategy is the flexible step 2 that enables the near-seamless insertion of any DNA sequence with only two remaining attR sites (Figure 1). To test the feasibility of step 2, we chose the *salm[1st intron-dsRed]* allele. We exchanged the STOP-dsRed cassette with a short 2xTY1-V5 exon, a FRT-2xTY1-FRT-V5 conditional exon, and a large GFP-3xFLAG exon from Venken *et al.* (2011) (Figure 4B). As expected, in all three cases the cassette exchange worked routinely and, typically, injection of approximately 200 embryos is sufficient to obtain two or more RMCE events in the correct orientation (see *Materials and Methods*). Importantly, the *salm[1st intron-dsRed]* lethality was reverted by RMCE in all three cases (Figure 5B). This demonstrates that our editing protocol generally does not result in any unwanted lethal mutations on the edited chromosome.

Salm protein is expressed in indirect flight muscles (IFMs) and is essential for fibrillar IFM fate specification (Schönbauer *et al.* 2011). Thus, we should detect the tagged Salm protein versions in the IFM nuclei of adult flies. Tagged protein from all three alleles, *salm[1st intron-TY1-V5]*, *salm[1st intron-FRT-TY1-FRT-V5]*, and *salm[1st intron-GFP-*

FLAG] is expressed in IFMs. Salm-TY1-V5 and Salm-GFP-FLAG are readily detected in the IFM nuclei, and Salm-FRT-TY1-FRT-V5 shows an additional dotty pattern in the cytosol, which might be caused by the FRT sequence translated into protein (Figure 5C–G). The fibrillar IFM morphology is normal in all three homozygous *salm* alleles, showing that the tagged Salm proteins are indeed functional. Each IFM fiber contains several hundred nuclei. The conditional *salm[1st intron-FRT-TY1-FRT-V5]* should now enable a clonal loss-of-function analysis of *salm* in muscle only, as flip-out *in cis* is highly efficient (Hadjieconomou *et al.* 2011). Thus, this strategy should generally be versatile for the genetic analysis of muscle in the future.

Gene editing on the fourth chromosome

To demonstrate the general applicability of our gene editing strategy, we decided to apply it to an additional locus. We chose the *bent* gene, located on chromosome four, which is highly heterochromatic and thus difficult to manipulate by standard genetic tools. To our knowledge, there is only a single case reported in the literature that targeted a gene located on the fourth chromosome by classical ends-out mediated
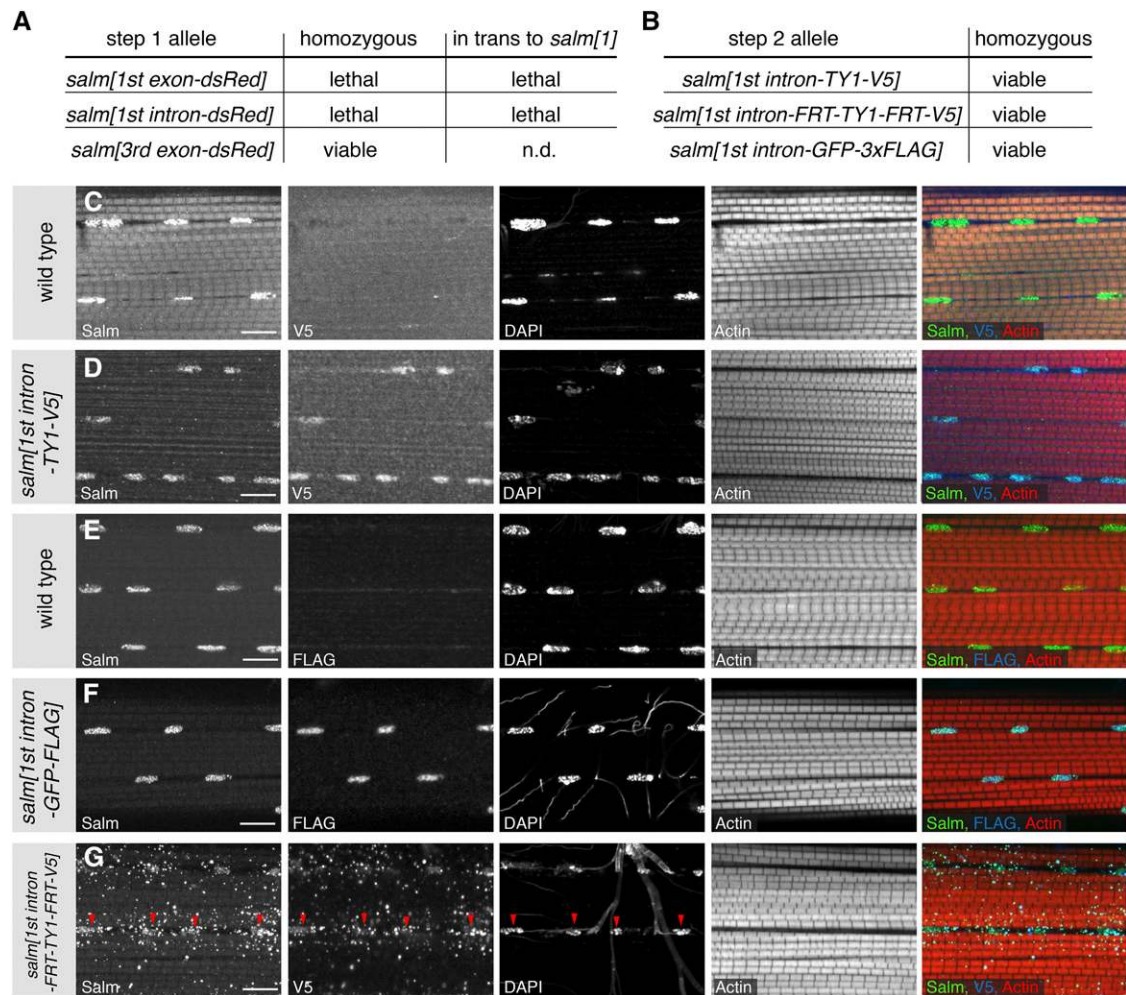

Figure 5 Phenotypic analysis of the engineered *salm* alleles. (A) Lethality assay of the *salm-dsRed* alleles as homozygous or in trans to *salm[1]*. (B) All tagged *salm[1st intron]* alleles regain homozygous viability after step 2. (C–G) Localization of the tagged Salm proteins. Untagged Salm is located in the nucleus of wild-type IFMs (C, E), whereas V5 tagged Salm is only detected in the *salm[1st intron-TY1-V5]* and the *salm[1st intron-FRT-TY1-FRT-V5]* alleles (D, G). FLAG is found in the IFM nuclei of *salm[1st intron-GFP-FLAG]* adults (F). The Salm-FRT-TY1-FRT-V5 protein is found in the nuclei (red arrow heads) and also located in dots in the cytoplasm (G). Note the normal fibrillar morphology of the myofibrils in all the homozygous *salm[1st intron]* alleles (D, F, G). Actin was stained with phalloidin and the scale bars are 10 μm.

homologous recombination using long homology arms (Rodriguez-Jato *et al.* 2011). *bent* is a very large gene composed of at least 46 exons that are spread across more than 51 kb of genomic DNA (Figure 6). *bent* encodes for Projectin, a titin-like protein that is specifically expressed in muscles and essential for correct sarcomeric organization (Fyrberg *et al.* 1992; Ayme-Southgate *et al.* 1995; Schnorrer *et al.* 2010). It is supposedly silent in germ cells, in which the targeting event must happen. We chose to delete exon 11, an exon at the beginning of the PEVK domain of Projectin (Ayme-Southgate and Southgate 2006), using two sgRNAs flanking the exon. Both sgRNAs tested positively in the S2 cell assay (data not shown). We again used approximately 1 kb homology arms and injected the donor vector into 700 *Act5C-Cas9, Lig4[169]* embryos. We isolated five carriers from 1 founder out of a total of 56 fertile G_0 flies, resulting in an HDR efficiency of 1.8%. We confirmed the *bt[11th intron-dsRed]* allele by sequencing of the locus. As expected, *bt[11th intron-dsRed]* is homozygous lethal and also lethal *in trans* to *bt[I-b]*, a strong bent allele (Ayme-Southgate *et al.* 1995), again suggesting that the inserted splice acceptor is used effectively and transcription is prematurely terminated. Together, these results demonstrate that our CRISPR-mediated targeting strategy also works efficiently on the fourth chromosome, suggesting it can be generally applied to any locus of choice in the fly genome.

DISCUSSION

CRISPR/Cas9 has been used successfully in many model organisms to generate mutants or to introduce targeted changes by HDR (Hsu *et al.* 2014). In *Drosophila*, there has been no general agreement regarding which strategy works most effectively to engineer the genome. To simply mutate a gene by CRISPR/Cas9-induced NHEJ, Cas9 was either injected as mRNA (Bassett *et al.* 2013; Yu *et al.* 2013), provided from an injected plasmid (Gratz *et al.* 2013a; Baena-Lopez *et al.* 2013), or provided from a transgenic source (Kondo and Ueda 2013; Ren *et al.* 2013; Sebo *et al.* 2014). Similarly, the sgRNA was either injected as *in vitro* transcribed sgRNA or provided by an injected plasmid or a transgenic source. A standard protocol has not yet emerged, although several genes have been mutated.

NHEJ can only induce small insertions or deletions. In contrast, HDR allows the defined engineering of a given gene and thus is suitable for a much wider range of applications. CRISPR/Cas9-mediated HDR has been used in *Drosophila* to insert short attP or tag sequences from single-strand oligonucleotides as donors (Gratz *et al.* 2013a) or larger cassettes including a dsRed marker cassette from a plasmid donor (Baena-Lopez *et al.* 2013; Yu *et al.* 2014; Gratz *et al.* 2014; Xue *et al.* 2014), again using various ways of injected or transgenic sources of

sgRNAs or Cas9. The injected genotype was variable; sometimes *Lig4* mutants were used (Baena-Lopez *et al.* 2013; Yu *et al.* 2014; Gratz *et al.* 2014; Xue *et al.* 2014), sometimes they were not used (Baena-Lopez *et al.* 2013; Yu *et al.* 2014; Gratz *et al.* 2014; Xue *et al.* 2014; Gokcezade *et al.* 2014; Port *et al.* 2014). Often the detection of the targeted event required laborious fly screening by PCR (Gratz *et al.* 2013b; Yu *et al.* 2014; Gokcezade *et al.* 2014).

Here we aimed to develop a universal and efficient CRISPR-based strategy that enables flexible genome engineering, including the insertion of large tags into the coding region of a gene or the generation of conditional alleles. This strategy should be generally applicable to most *Drosophila* genes. Our results confirmed that approximately 1-kb homology arms are of sufficient length to insert a large marker cassette, as has been suggested before for other loci (Gratz *et al.* 2014; Yu *et al.* 2014; Xue *et al.* 2014; Port *et al.* 2014). Thus, we could develop an efficient donor plasmid assembly protocol that facilitates cloning of the donor vector for any gene within a few days. Additionally, our data support the value of a quick pretesting strategy of predicted sgRNAs in S2 cells to eliminate inefficient sgRNAs, which would likely reduce targeting efficiency *in vivo*. However, we have not tested how well sgRNA efficiencies in S2 cells correlate with efficiencies *in vivo*. Conveniently, the same *in vitro* transcribed RNAs can be used for both S2 cell transfections and embryo injections. Our results suggest that a transgenic Cas9 source mediates HDR effectively in *Ligase4* mutant germline cells. Although *Act5C-Cas9* expression is not restricted to the germline, injections of the donor vector together with two verified sgRNAs led to a targeting efficiency of 2%–14% of fertile G_0 flies for the incorporation of the large STOP-dsRed cassette, even for the *bent* locus on the heterochromatic fourth chromosome. This suggests that approximately 50–100 fertile G_0 flies should be sufficient in most cases to identify positive carriers. We and others (Gokcezade *et al.* 2014) have observed relatively high developmental lethality of the injected G_0 flies, which might be caused by somatic knock-out of the targeted gene. Thus, survival rate of the injected embryos and larvae might be increased when using a germline-restricted Cas9 source, such as *nos-Cas9*. However, *nos-Cas9* was reported to be less efficient in germline transmission compared with *Act5C-Cas9* (Port *et al.* 2014). We thus far have deleted up to approximately 1 kb of genomic sequence by HDR. Larger deletions would likely occur at reduced efficiencies; however, the dsRed marker should still make it practical to find them. The straightforward identification of carriers together with our simple cloning scheme should easily facilitate the insertion of the STOP-dsRed cassette into the gene of choice.

Recent reports using a transgenic sgRNA source (Port *et al.* 2014) or an injected sgRNA source (Ren *et al.* 2014) report very effective
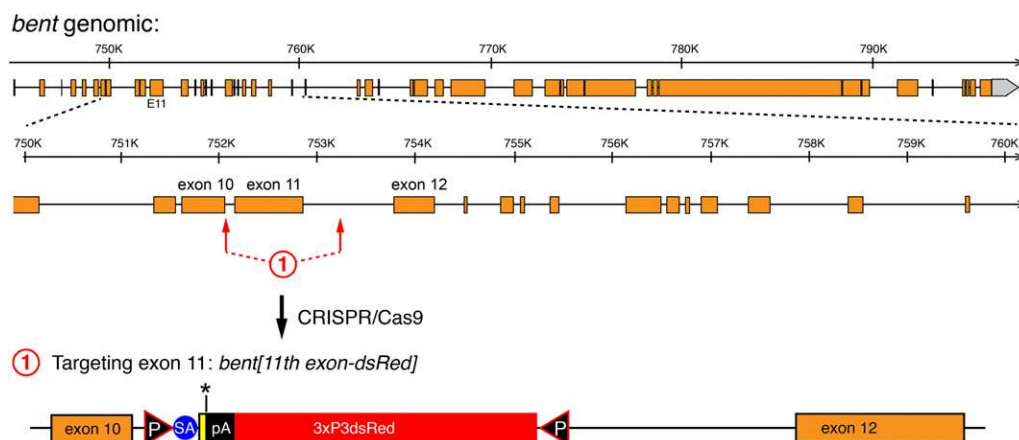
Figure 6 Engineering of the *bent* gene. The entire genomic *bent* organization is shown at the top with a 10-kb zoom-in below. Coding exons are in orange. The sgRNA targeting sites flanking exon 11 are indicated by red arrows and the resulting *bent* [11th exon-dsRed] allele is shown at the bottom.

HDR rates with more than 50% of the fertile G_0 flies being positive founders and, thus, screening by PCR-based methods were practical in these cases. However, both studies used only a single locus to insert the GFP tagging cassette; hence, a direct comparison with the efficiencies that we report here is difficult.

Our two-step strategy combines the advantages of both CRISPR and RMCE, thus allowing very flexible modifications of a particular gene region with minimal effort. Multiple fluorescent and affinity tags can be easily inserted or a deleted exon can effectively be replaced by various engineered exon versions. In principle, larger gene parts consisting of multiple exons can also be deleted and replaced by modified versions. This method is particularly valuable for genes that harbor complex transcriptional control and function in many tissues such as *salm* or for genes that are exceptionally large and exhibit complex alternative splicing patterns such as *bent*. The two-step strategy allows structure–function analysis at the endogenous locus without interfering with the regulatory regions included in introns, which cannot be achieved by simply inserting a cDNA at the transcriptional start site. The functionality of our method was verified by the reversion of the lethality for the step 2 alleles in the first intron of *salm*. This furthermore suggests that both steps do not generate additional unintended changes on the chromosome. Therefore, we hope that our strategy will promote the wide application of CRISPR-mediated HDR in *Drosophila*, making it a routine tool used in every fly laboratory like EMS mutagenesis or *P*-element–mediated transformation was in the past century.

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