Methods Article

Improving one-step scarless genome editing in Drosophila melanogaster by combining ovoD co-CRISPR selection with sgRNA target site masking

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Abstract

The precise and rapid construction of alleles through CRISPR/Cas9-mediated genome engineering renders Drosophila melanogaster a powerful animal system for molecular structure–function analyses and human disease models. Application of the ovoD co-selection method offers expedited generation and enrichment of scarlessly edited alleles without the need for linked transformation markers, which specifically in the case of exon editing can impact allele usability. However, we found that knockin procedures by homology-directed repair (HDR) under ovoD co-selection resulted in low transformation efficiency. This is likely due to repeated rounds of Cas9 cleavage of HDR donor and/or engineered genomic locus DNA, as noted for other CRISPR/Cas9 editing strategies before, impeding the recovery of correctly edited alleles. Here we provide a one-step protocol to improve the generation of scarless alleles by ovoD-co-selection with single-guide RNA (sgRNA) binding site masking. Using this workflow, we constructed human disease alleles for two Drosophila genes, unc-13/CG2999 and armadillo/CG11579. We show and quantify how a known countermeasure, the insertion of silent point mutations into protospacer

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adjacent motif (PAM) or sgRNA homology regions, can potently suppress unintended sequence modifications during CRISPR/Cas9 genome editing of D. melanogaster under ovoD0 co-selection. This strongly increased the recovery frequency of disease alleles.

Keywords: CRISPR, Cas9; genome engineering; Munc13; armadillo; neurodevelopmental disorder; synapse; cancer; Drosophila

Introduction

Based on the concerted human genome sequencing efforts of the past two decades, scientists and clinicians have access to detailed genetic information associated with a plethora of human diseases [1–3]. In model organisms with a suitably homologous gene set and amenability to gene targeting technologies, this information can ultimately be used to test for causality between mutation and disease state [4]. Such an approach provides a solid basis for defining the pathophysiological underpinnings of a human ailment and its genetic characteristics. The fashioning of RNA-guided Cas9 endonuclease activity selected positions in genomic DNA, now commonly referred to as CRISPR/Cas9 genome engineering, has expedited the generation of human disease models. The CRISPR/Cas9 methodology allows for precise and rapid genome editing in human cells [5–7] and a large array of model species including the fruit fly Drosophila melanogaster [8–12], which is successfully used as a model for human diseases [13–16].

Direct scarless insertion of human mutations into the Drosophila genome via CRISPR/Cas9-assisted homology-directed repair (HDR) affords the separation of the targeting template part, which carries the engineered disease mutation, from the selection marker required for transformant identification. A recently introduced method that utilizes a negative transformant selection strategy rests on co-editing of a female sterile ovoD0 allele and offers an elegant solution for this technical complication [17]. Using ovoD0 co-selection, the successful editing event at the target locus is identified by simultaneous correction of the HDR repair sequence from its flanking homology arms and thus impedes any PCR amplification from the ovoD1-inflicted sterility, thereby enriching for CRISPR/Cas9 events. However, when we applied ovoD0 co-selection for the generation of candidate, human pathogenic allele sets for two adjacent motif (PAM) sites for two gene targeting plasmids were successfully applied with single-stranded oligodeoxynucleotides as the donor template [22], quantitative assessment of such protective strategies for well-established CRISPR/Cas9 protocols using double-stranded donor templates [9, 10] in combination with ovoD0 co-selection is lacking. Here we provide such analysis and highlight guidelines for HDR plasmid construction to prevent undesired repeated target sequence cleavage. We show that this strategy ensures high success rates with enrichment of CRISPR/Cas9 editing events by ovoD0 co-selection, for example, in the construction of human disease models. Nonetheless, these findings are likely of general interest for CRISPR/Cas9 editing experiments and not limited to ovoD0-co-CRISPR approaches.

Materials and methods

Molecular reagents

All primer sequences used in this study are listed in Supplementary Table S1.

pU6-sgRNAs

CRISPR/Cas9 cutting sites 5’ and 3’ of the unc-13 and arm loci were identified by “CRISPR Optimal Target Finder” [10]. The genomic sequences of all CRISPR/Cas9 cleavage sites were confirmed by DNA sequencing of PCR fragments encompassing the suggested sites prior to cloning. Target-specific sequences for unc-13 sgRNAs were synthesized as 5’-phosphorylated oligonucleotides, annealed, and ligated into the BbsI sites of the pU6-BbsI-chiRNA vector [9], sgRNAs for arm targeting plasmids were synthesized by GenScript Biotech B.V. (The Netherlands) (Supplementary Table S2).

unc-13 HDR vectors

To generate mutation cluster 1 HDR vectors, a 4.3-kb product was PCR-amplified from w1118 genomic DNA using primers am_226F/am_223R and, after gel purification, was SacII/AvrII-digested pHD-DsRed-attP (pTL620), which gave rise to pAM66. For mutation cluster 2 HDR vectors, a 3.9-kb product was PCR-amplified from w1118 genomic DNA using primers am_227F and am_225R and, after gel purification, was SacII/AvrII cut and ligated into a 2.8-kb backbone fragment of SacII/AvrII-digested pHD-DsRed-attP (pTL620), which gave rise to pAM67. Quikchange mutagenesis to introduce the respective nucleotide exchanges was performed using Pfu DNA polymerase (Promega) in combination with DpnI digest to clear original bacterial plasmid background using primers optimized for Drosophila codon usage, carrying the mutated nucleotides contained by 12- to 21-bp flanking homologous sequences. Details are listed in Supplementary Table S3.

arm HDR vectors

To generate the arm HDR vector kit, a 1.5 kb fragment of w1118 genomic sequence was synthesized and cloned into pHD-DsRed-attP (pTL620) generating pTL947. All further mutations and modifications to the arm fragment were introduced into this plasmid as outlined in Supplementary Table S3.
Figure 1: ovoD-assisted CRISPR/Cas9 editing of unc-13 with masked proximal sgRNA binding sites. (A) Schematic of the domain structure of the human Munc13-3 and the Drosophila UNC-13 proteins. The human disease-associated mutations are organized into two clusters. Relative locations of the mutations in the proteins are indicated by downward triangles. (B) Schematic of the Drosophila unc-13 locus. Black boxes indicate exons, and light gray boxes indicate UTRs. (C and D) Enlarged view of the regions harboring the sgRNA binding site pairs used for Cas9 targeting to generate cluster 1 (C) and cluster 2 (D) mutations. Downward triangles mark the positions of the point mutations. (E) sgRNA sequences for cluster 1 (upper box) and cluster 2 (lower box) targeting. Off-target binding sites as predicted by FlyCRISPR optimal target finder are indicated in gray below the respective sgRNA binding site. Modified nucleotides used to mask sgRNA binding sites in the HDR plasmid for improved ovoD-assisted CRISPR/Cas9 targeting of cluster 1 are marked by lowercase letters in red. Note that the PAM sites of both modified sgRNA sites for cluster 1 maintain a NGG sequence and are thus, on their own, not suitable for Cas9 cleavage suppression. Cas9 cleavage site is indicated by blue triangles. Strand direction relative to genomic unc-13 sequence (+, forward strand; −, reverse strand).
sgRNA binding site modifications in HDR-fixed vectors

To prevent Cas9 cleavage of HDR vectors, silent mutations were introduced into sgRNA binding and PAM sites for unc-13 cluster 1: 6 nucleotides (nt) of the 5’-sgRNA binding site + 1 nt of its PAM site, and 4 nt of the 3’-sgRNA binding site + 1 nt of its PAM site were exchanged, respectively (Fig. 1B). For arm modifications, the 5’- and 3’-PAM sites were mutated by two and a single silent mutation (Fig. 4C), respectively. Modifications were performed at GenScript (Supplementary Table S3).

Genotyping of mutant fly strains

Genotyping was performed via Sanger sequencing. Primer pairs for each mutation and each sgRNA binding site, respectively, were designed: (unc−13#2: am−225F/am−256R; unc−13#3: am−245F/am−257R; unc−13#4: am−258F/am−259R; unc−13#5: am−242F/am−252R; unc−13#6: am−253F/am−219R; unc−13#7: am−254F/am−252R); clust 1—5’-sgRNA binding site: kg_14F/kg_15R; clust 1—3’-sgRNA binding site: kg_16F/kg_17R; clust 2—5’-sgRNA binding site: kg_20F/kg_21R; clust 2—3’-sgRNA binding site: kg_20F/kg_21R; arm: tl_911F/tl_914R).

Mutation Genotype Lines Stock ID
unc−13#2 unc−13#2[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0101–DL0103
unc−13#3 unc−13#3[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0104–DL0106
unc−13#4 unc−13#4[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0107–DL0109
unc−13#5 unc−13#5[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0092
unc−13#6 unc−13#6[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0110–DL0111
unc−13#7 unc−13#7[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0099
unc−13#2-med unc−13#2-med[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0112–DL0113
unc−13#3-med unc−13#3-med[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors] DL0097
arm WT arm WT[P(Tb1)FM7a, B sc v w y] DL0100
arm#2 arm#2[P(Tb1)FM7a, B sc v w y] DL0098

Fly strains

Generated in this work

AA numbering refers to D. melanogaster UNC-13A isofrom (Uniprot ID: Q8IM87).

CRISPR/Cas9 targeting

BDSC #56552, w1118, P{Bac(y−mDn2=vas-Cas9)1XKK00017/CyO, P{w−mTb1=cpp3904-A}};
BDSC #55821, y1 M[vas-Cas9.RFP]2H-2A w1118, (both a gift by Kate O’Connor-Giles and Jill Wildonger, University of Wisconsin, Madison, WI, USA)
BDSC #1309, ovoD2 v12/C[2]DX, y1 f1;
BDSC #78782, y1 sc v1 sev21[P(y1-v17.7 y1-v12.8 = nos-Cas9.R)mp18].

Other strains

BDSC #4759, w1118; P{w−mC=ActGFP}unc−13#2[5’-1-3 sgRNA binding site modifications in HDR-fixed vectors]/pan2
BDSC #24488, y1 M[RF3[3xP3.FB] GFP[E.3xP3]=vas-int.Dm]2H-2A
w1118 M[3xP3-RFP.attP]2H-102D

SGRN binding site errors, and mutation carriage was performed via Sanger sequencing at Microsynth AG (Switzerland). Genomic DNA was extracted with NucleoSpin Tissue kit (Machery-Nagel). With suitable primers, DNA fragments of interest less than 1000 bp were amplified via PCR. Gel electrophoresis was performed to separate the DNA bands. QIAEXII gel extraction kit (QIAGEN) was used to purify the DNA. In 1.5 ml tubes, the extracted DNA, water, and forward or reverse primer were mixed and sent to Microsynth AG or Eurofins. Sequencing results were analyzed with a plasmid editor.

Sanger sequencing

Sequencing of defined fragments of DNA, for example, for investigation of possible genomic off-target events, sgRNA binding site errors, and mutation carriage was performed via Sanger sequencing at Microsynth AG (Switzerland). Genomic DNA was extracted with NucleoSpin Tissue kit (Machery-Nagel). With suitable primers, DNA fragments of interest less than 1000 bp were amplified via PCR. Gel electrophoresis was performed to separate the DNA bands. QIAEXII gel extraction kit (QIAGEN) was used to purify the DNA. 1.5 ml tubes, the extracted DNA, water, and forward or reverse primer were mixed and sent to Microsynth AG or Eurofins. Sequencing results were analyzed with a plasmid editor.

Genome sequencing

Genomic DNA was extracted from adult fly homogenate samples using a NucleoSpin Tissue kit (Machery-Nagel). 40 ng of the DNA was used to prepare paired-end libraries with the Nextera DNA Library Prep kit (Illumina, San Diego, USA). The barcoded libraries were purified and quantified using Qubit Fluorometric Quantification (ThermoFischer Scientific). Size distribution of the library DNA was analyzed employing the FragmentAnalyzer (Agilent). Sequencing of 2×150 bp was performed with a NovaSeq sequencer (Illumina). Demultiplexing of raw reads, adapter trimming, and quality filtering were performed according to Stokowy et al. [23]. Resulting read pairs were mapped to the Drosophila r6 genome using the Burrows-Wheeler aligner [24] and visualized using the Integrative Genomics Viewer v2.9.4 [25]. Freebayes v1.3.5 ([https://arxiv.org/abs/1207.3907](https://arxiv.org/abs/1207.3907)) was employed for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Furthermore, CNVkit was used for variant calling and SnpEff v5.0e for variant annotation [26]. Only variants with an allele frequency of ≥20, sequencing depth of ≥30, and genotype quality of ≥20 were considered in our analysis. Further...
times independently (n = 9). In all experiments, the results from the three independent crosses were very similar, which allowed pooling the results. Twenty days after the transferral to a new vial, the adult F1 generation was sorted and the number of individuals was counted based on their phenotype. The results were evaluated by calculating the mean fraction of each phenotype.

Results

Inadvertent sequence alterations at Cas9 cleavage sites during HDR genome editing with ovoD co-selection

In order to study synaptic release in the context of neurodevelopmental disorders and active zone (AZ) dysfunction, we set...
out to construct Drosophila alleles containing human missense mutations of the AZ component Munc13-3/UNC-13. Munc13 homologs are multi-domain proteins (Fig. 1A) and exert evolutionarily highly conserved steps in synaptic vesicle priming [28–30]. In addition, Munc13 proteins govern the nanoarchitecture of AZs by positioning synaptic vesicles at defined coupling distances to the release triggering calcium channel complex [31].

To generate point mutated unc-13 alleles, we first determined the position of the amino acid exchanges by aligning the human Munc13-3 gene product, in which the mutations were originally identified, and isoforms of the Munc13-3 homolog UNC-13 of D. melanogaster. The location of three of these human missense mutations (#2, #3, #4; cluster 1) was mapped to an N-terminal region of the UNC-13A isoform, which shows low structural complexity without known protein domains (Fig. 1A). Cluster 1 mutations are located close to each other in a large exon spanning 4890 bp (Fig. 1B and C), which is exclusively spliced into mRNA species encoding the fly UNC-13A isoform (Fig. 1A). Each Munc13-3 missense mutation was then individually introduced into the respective HDR vector to generate two sets of plasmids (cluster 1: pHDR-unc-13#2, pHDR-unc-13#3, pHDR-unc-13#4; cluster 2: pHDR-unc-13#5, pHDR-unc-13#6, pHDR-unc-13#7) for transgenesis. Next, for the two sgRNA plasmids to release the unc-13 target exon cluster, the respective HDR plasmid for DNA double-strand break (DSB) repair with the mutated genome fragment, and a single sgRNA for ovoD editing were co-injected into ovoD embryos with constitutive germline expression of Cas9 from a nos-Cas9 transgene [53]. We recovered 8–48 founder animals per each Munc13-3 mutation (129 stocks in total), crossed them with a suitable Chr4-marker, and expanded the stocks.

All clonal fly strains, that is individual F1 progeny of each founder, proved fertile, demonstrating permanent correction of the ovoD allele in their genetic background. PCR-based genotyping confirmed successful integration of the individual missense mutations in unc-13 in 34/129 (26%) ovoD-corrected lines cumulatively for all point mutations (see Table 2 for details). We crossed offspring from three independently recovered founder animals per human mutation over an embryonic lethal unc-13KO null allele [29] in order to determine their genetic behavior (Fig. 2A). As each clonal population per individual Munc13-3 mutation insertion was derived from founder animals, which received the same missense codon, we assumed that their offspring would show comparable quantitative outcome in this simple phenotypic assay. In contrast, transheterozygous unc-13#3/unc-13#5 offspring displayed pronounced differences in lethality. For example, when we analyzed unc-13#3/unc-13#5 transheterozygotes, two of the three analyzed lines showed Mendelian ratios that indicated no loss of UNC-13#4 function, while one displayed complete lethality (Fig. 2B). Similarly, also individual fly strains for mutations #3, #5, #6 and #7 exhibited differences in viability when the engineered unc-13 mutation was uncovered by the unc-13KO null allele. These results alerted us to a general problem regarding the targeting fidelity of our ovoD co-selection approach.

In order to evaluate possible sequence errors introduced during the targeting and DSB repair procedures, we inspected the regions flanking the Cas9 cutting sites by Sanger sequencing. We found various nucleotide insertions or deletions at one or both sgRNA positions in almost each clonal unc-13# strain leading to loss or gain of nucleotides, which resulted in additional missense or frame-shift mutations of the unc-13 open-reading frame (ORF) (Fig. 2C). In order to test for additional undesired sequence modifications within the unc-13 locus regions that are unrelated to the genomic sgRNA target positions, we sequenced the genomes of four clonal fly strains (Fig. 2D). This confirmed the presence of the sequence modifications at the Cas9 cleavage sites but did not reveal additional sequence errors that may account for the diverse genetic behavior of the individual unc-13# alleles we constructed.

We concluded that the inadvertent genomic sequence errors at the sgRNA binding sites in targeted founders were locally confined due to the CRISPR/Cas9 targeting procedure. As the original sgRNA binding sites were reconstituted through the HDR of the engineered unc-13 locus, we surmised that the indels were likely caused by repeated rounds of Cas9 cleavage of the already edited genomic DNA and its subsequent DSB repair. Ultimately, this likely resulted in erroneous deletion or incorporation of nucleotides rendering the targeting round futile.

**Reduction of sgRNA binding site homology in HDR plasmids potently suppresses errors at Cas9 cleavage positions**

In order to test this assumption and to recover incontestable unc-13 alleles without inadvertent sequence abnormalities, we constructed a new set of HDR plasmids for cluster 1 mutations #2 and #3 (pHDR-unc-13#2, pHDR-unc-13#3). To prevent Cas9 processing of the successfully engineered locus DNA, we

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**Table 1: Human Munc13-3 and CTPNB1 alleles**

| Allele description (shorthand) | Human mutation | Fly mutation |
|-------------------------------|----------------|--------------|
| unc-13#2                      | C69F           | V675F        |
| unc-13#3                      | A319E          | D923E        |
| unc-13#4                      | R548C          | D1136C       |
| unc-13#5                      | T1104M         | T1729M       |
| unc-13#6                      | T1053I         | A1679I       |
| unc-13#7                      | I1189T         | I1814T       |
| arm#1                         | delW25-I35     | delW35-I46   |
| arm#2                         | S37C           | S48C         |
| arm#3                         | T41A           | T52A         |
| arm#4                         | S45F           | S56F         |

Amino acid numbering to human and fly homologs. Reference protein sequences: MUNC13-3 (#Q8NB66), UNC-13A (#Q8IM87), CTPNB1 (#P35222), and ARM (#P18824).
modified both original sgRNA binding site sequences in the HDR plasmid by nucleotide exchanges yielding silent mutations, which would not cause amino acid changes in the gene products [9, 20, 21, 34]. Due to the positions of the PAM sequence of sgRNA binding sites 1 and 2 within the unc-13 ORF, we could not simply inactivate the PAMs through point mutations of the two 3’-GG PAM-nucleobases without impacting amino acid coding. Instead, we exchanged 7 nt and 5 nt of the 23-bp spanning selected transformants.

We then repeated ovoD1-assisted CRISPR/Cas9 editing for unc-13#2 and unc-13#3 mutations using the modified HDR plasmids. After recovery of transformants and the generation of stably balanced stocks, we determined the presence of the Munc13-3 missense mutations and noted that 11/20 lines for unc-13#3 and 10/20 lines for unc-13#4 targeting (in total 53%) contained the edited codons (Table 2). This indicated that the HDR plasmid sequence modifications effectively suppressed all events that caused inadvertent sequence changes in the edited locus, for example, by quelling repeated rounds of endonuclease cleavage followed by DSB repair, and allowed for the successful recovery of ovoD1 co-selected transformants.

Bi-directional integration of sgRNA masking mutations 5’ to Cas9 cleavage sites suggests multiple repair mechanisms including synthesis-dependent strand annealing-aided repair

In addition, we observed that the sgRNA binding site masking mutations encoded on the HDR plasmids, which are largely located 5’ of the DSB generated by Cas9 cleavage, were introduced into the genomic DNA of engineered fly stocks with high efficiency (unc-13#3mod, 5’-Cas9 cut: 11/11 lines; 3’-Cas9 cut: 10/11 lines; unc-13#4mod, 5’-Cas9 cut: 10/10 lines; 3’-Cas9 cut: 10/10 lines; Fig. 3A and B). Those mutations appear inaccessible for repair mechanisms that involve only DNA synthesis in 5’- to 3’-direction at the Cas9 cleavage points followed by ligation to restore duplex DNA. This result thus suggests that HDR during the employed CRISPR/Cas9 editing procedures utilized synthesis-dependent strand annealing (SDSA) as a principal repair mechanism (Fig. 3C) [35].

### Table 2: Overview of ovoD1-assisted gene targeting efficiency and precision of the unc-13 locus without and with the use of modified sgRNA sites

| Allele | With unmodified sgRNA sites in HDR plasmid, n/N (%) | With modified sgRNA sites in HDR plasmid, n/N (%) |
|--------|-----------------------------------------------|-----------------------------------------------|
| unc-13#2 | 21/48 (44) | 20/48 (42) |
| No. of clonal F1 offspring analyzed | 3/21 (14) | 11/20 (55) |
| With edited missense mutation | 1/5 (33) | 11/11 (100) |
| With correct unmodified/modified 5’-gRNA site sequence | 2/3 (67) | 10/11 (91) |
| unc-13#3 | 14/24 (58) | 20/24 (83) |
| No. of clonal F1 offspring analyzed | 12/24 (50) | 10/20 (50) |
| With edited missense mutation | 0/8 (0) | 10/10 (100) |
| With correct unmodified/modified 3’-gRNA site sequence | 0/8 (0) | |
| unc-13#4 | 8/16 (50) | Not applicable |
| No. of clonal F1 offspring analyzed | 5/8 (63) | Not applicable |
| With edited missense mutation | 0/4 (0) | |
| With correct unmodified/modified 5’-gRNA site sequence | 0/4 (0) | |
| unc-13#5 | 48/96 (50) | Not applicable |
| No. of clonal F1 offspring analyzed | 8/16 (50) | Not applicable |
| With edited missense mutation | 2/4 (50) | |
| With correct unmodified/modified 3’-gRNA site sequence | 2/4 (50) | |

Notes: For experimental sets using unmodified sgRNA sites, “correct” refers to their wild-type sequence, and for experiments using modified sgRNA sites, “correct” refers to the modified sequence.
Mutagenesis of PAM sites in HDR plasmids improves targeting success under ovoD co-selection

Finally, we tested the applicability of our modified targeting workflow with another independent Drosophila locus. We selected the human β-catenin 1 homolog CTNNB1, which is encoded by the armadillo/arm locus in the fly [36] (Fig. 4A) and which is not genetically linked to unc-13. Mutations of the β-catenin 1 gene are notorious for their roles in a broad spectrum of human neoplasms such as tumors of the brain, the skin, or the intestine [37–39]. However, detailed analysis of the molecular effects caused by CTNNB1 mutations is hampered by the lack of in vivo models that can aid in establishing causality in β-catenin structure–function relationships [40, 41].

We constructed and injected two sets of sgRNA and HDR plasmids to place four clinically relevant CTNNB1 mutations (Table 1), some of which affect β-catenin phosphorylation [42, 43], in the arm locus through CRISPR/Cas9 editing under ovoD co-selection (Fig. 4B). Similar to our initial unc-13 strategy, the sgRNA binding site sequences in the HDR plasmids were left unchanged in the first arm transgenesis set so they remained homologous to the genomic sequence of the target arm locus. For the second set of injections, the PAMs of the 5' and 3' sgRNA binding site sequences in the HDR plasmids were disabled by one or two innocuous point mutations, respectively (Fig. 4C).

After injection of the first plasmid set, emerging founder females were recovered and balanced over an X-chromosomal balancer. Through PCR-based sequencing of the targeted CTNNB1 mutations, we could not recover a single ovoD1-rescued animal with a desired arm mutation (0/38) (for details, see Table 3). This suggested that similar problems as in the initial unc-13 targeting attempt occurred also during the arm targeting procedure, for example, that unabated Cas9 activity of the engineered locus resulted in detrimental genome alterations, which ultimately precluded the development of founder animals. In contrast, after transgenesis using the modified HDR plasmids for two arm alleles, we established 32 clonal founder strains, of which 15 contained the inserted point mutation indicating an ovoD co-selection efficiency of 47 %. None of those arm edited founders exhibited additional inadvertent sequence problems at or adjacent to the Cas9 cleavage sites as shown by PCR-based sequencing (Table 3).

We conclude that, as an alternative or in addition to reducing the homology of sgRNA binding sites, also mutagenesis of the PAM sequences in HDR plasmids for Drosophila genome editing can protect engineered loci from sequence errors at the sites of DSB repair.

Discussion

Here, we provide an optimized protocol for efficient and expedient use of ovoD-assisted CRISPR/Cas9-mediated mutagenesis. ovoD-assisted CRISPR/Cas9-mediated genome engineering is an elegant strategy [17], which provides an effective approach to scarlessly engineer models of human disease-related point...
mutations at large scale and study their consequences at the molecular, tissue, organ, and organism level. Our initial attempts to use this technique for direct exon editing without incorporation of a selection marker were, however, hampered by a low transgenesis efficiency (26% for *unc-13*, 0% for *arm*-engineering) and by the incorporation of unwanted indels at Cas9 targeting sites due to intact sgRNA binding motifs in HDR donor plasmid constructs. This likely led to multiple rounds of Cas9 cleavage of the donor DNA and/or re-cleavage of the engineered locus and introduction of sequence errors by nonhomologous end joining rather than the desired HDR.

Earlier protocols proposed to prevent the potential re-cleavage of the exchanged DNA fragment by masking the sgRNA binding sites in the donor plasmid products [9, 20, 21, 34]. However, a quantitative assessment of such interventions to improve genomic engineering precision and efficiency in *Drosophila* – specifically in combination with *ovoD*-co-editing [17] – is lacking thus far. In the present study, we employed...
ovoD engineering (with and without suitable HDR plasmids for CRISPR/Cas9-mediated genome engineering), our protocol, further simplifying the construction of binding site mutations may thus be reduced in future applications. Nonetheless, previous reports indicated that three mismatches suffice to prevent Cas9 cleavage [44]. The number of sgRNA variants inserted four to six non-PAM donor mutations to achieve potent suppression of sgRNA annealing to HDR plasmid DNA. Insertion of correctly engineered loci increased to 50%. Cas9 targeting can, however, be profoundly hampered by unwanted re-cleavage and indel incorporation. Here we have re-assessed the technical means to circumnavigate these in the context of ovoD mutagenesis masking of the PAM proximal region (see unc-13 mutagenesis) or of the PAM site itself (see arm mutagenesis) and could demonstrate that the number of founder animals with correctly engineered loci increased to ~50%.

For efficient and precise unc-13 genome engineering, we inserted four to six non-PAM donor mutations to achieve potent suppression of sgRNA annealing to HDR plasmid DNA. Nonetheless, previous reports indicated that three mismatches suffice to prevent Cas9 cleavage [44]. The number of sgRNA binding site mutations may thus be reduced in future applications of our protocol, further simplifying the construction of suitable HDR plasmids for CRISPR/Cas9-mediated genome engineering (with and without ovoD co-selection). Interestingly, the SDSA pathway is likely active in repairing DSBs during HDR gene editing events. Thus, it has been proposed to be the primary mechanism for integration of large insertions during genome editing with CRISPR/Cas9 [35, 45]. After annealing to the donor sequence, both 3'-ends are elongated and complementary strands are synthesized. These strands eventually hybridize to a double-strand accomplishing DSB repair. Elongation can exceed 4500 bp [35]. Our results are compatible with this assumption. As silent mutations located up- and downstream of the Cas9 cutting sites were incorporated into the target genome, a bidirectional repair mechanism such as SDSA is likely responsible for our observation. Genome sequencing showed no further alteration in the unc-13 gene apart from single-nucleotide polymorphisms. Most deviations occurred in more than one clonal line and were unrelated to the mutation cluster.

CRISPR/Cas9 constitutes a valuable gene editing tool for Drosophila and other model species presenting a highly valuable basis for the investigation of human pathogenic gene sequence variants. Combined with a selection protocol based on ovoD co-editing, rapid scarless editing is feasible even of exonic gene regions. Precision and efficiency of a HDR-mediated CRISPR/Cas9 targeting can, however, be profoundly hampered by unwanted re-cleavage and indel incorporation. Here we have re-assessed the technical means to circumnavigate these in the context of ovoD co-editing by introducing silent sgRNA binding site mutations during HDR vector design problems [9, 20, 21, 34], which efficiently suppress undesired Cas9 processing of the HDR plasmid before or of the engineered locus after transgenesis.

### Supplementary data

**Supplementary data** are available at Biology Methods and Protocols online.

### Data availability

Data are available in supplementary material. Plasmids, primers, genetic data and flies described in this article are also available upon request.

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### Author contributions

K.J.G. and A.M. performed the experiments, analyzed the data, and wrote the manuscript. P.B. performed the experiments. K.K., D.L.D., A.V., M.A.B., R.A.J., N.S. performed the

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**Table 3: Overview of ovoD-assisted gene targeting efficiency and precision of the arm locus without and with the use of modified PAM sites**

| Allele | With unmodified PAM site in HDR plasmid, n/N (%) | With modified PAM site in HDR plasmid, n/N (%) |
|--------|-----------------------------------------------|-----------------------------------------------|
| arm\(^{WT}\) | No. of clonal F\(_1\) offspring analyzed 8 | 14 |
| | With edited missense mutation 0/8 (0) | 3/14 (21) |
| | With correct unmodified/modifed 5'-PAM site sequence | |
| | With correct unmodified/modifed 3'-PAM site sequence | |
| arm\(^{\#1}\) | No. of clonal F\(_1\) offspring analyzed 13 | NA |
| | With edited missense mutation 0/13 (0) | |
| | With correct unmodified/modifed 5'-PAM site sequence | |
| | With correct unmodified/modifed 3'-PAM site sequence | |
| arm\(^{\#2}\) | No. of clonal F\(_1\) offspring analyzed 2 | 18 |
| | With edited missense mutation 0/2 (0) | 12/18 (67) |
| | With correct unmodified/modifed 5'-PAM site sequence | |
| | With correct unmodified/modifed 3'-PAM site sequence | |
| arm\(^{\#3}\) | No. of clonal F\(_1\) offspring analyzed 8 | NA |
| | With edited missense mutation 0/8 (0) | |
| | With correct unmodified/modifed 5'-PAM site sequence | |
| | With correct unmodified/modifed 3'-PAM site sequence | |
| arm\(^{\#4}\) | No. of clonal F\(_1\) offspring analyzed 7 | NA |
| | With edited missense mutation 0/7 (0) | |
| | With correct unmodified/modifed 5'-PAM site sequence | |
| | With correct unmodified/modifed 3'-PAM site sequence | |

Notes: For experimental sets using unmodified PAM sites, “correct” refers to their wild-type sequence, and for experiments using modified PAM sites, “correct” refers to the modified sequence.
experiments and analyzed the data. M.H., J.R.L. and H.B. analyzed the data. D.L. and T.L. initiated the study, designed the experiments, analyzed the data, and wrote the manuscript.

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