Supporting Information

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SI Materials and Methods

Cas9 Expression Plasmids. act-cas9*.* The plasmid *pUASTattB* (a gift from Konrad Basler, University of Zurich, Zurich) was cut with HindIII and EcoRI to remove the *UAST-hsp70* cassette. The *act5c* 5′ regulatory region was amplified with primers *act5c fwd/rev* from *pAct5c > CD2 > Gal4* (a gift from Silvia Aldaz, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom) and cloned into the open backbone from the previous step, creating *pAct5cattB*. *pAct5cattB* was cut with EcoRI and XhoI, followed by insertion of a human codon-optimized Cas9 coding sequence including a nuclear localization signal that was amplified from Addgene plasmid 41815 (1) using primers *hCas9Add41815* fwd/rev. The resulting plasmid was termed "*pAct5c-cas9*."

act-cas9^{D10A}. To create a nickase version of Cas9, aspartic acid 10 in one of the active sites was mutated to alanine (2). *Cas9*^{D10A} was amplified from *pAct5c-cas9* with primers *act-cas9nick* fwd/rev. The forward primer introduced the point mutation into the PCR product, which was inserted into *pAct5c-cas9* that had been digested with EcoRI.

nos-cas9*.* The plasmid *pUASTattB* was cut with BamHI to remove the *UAST-hsp70*-SV40 3′ UTR cassette. The *nos* regulatory regions were amplified with primers *nosProm fwd/rev* and *nos3′* fwd/rev from *pNos-PhiC31* (a gift from Konrad Basler). The Cas9 coding sequence with an SV40 nuclear localization signal was amplified from plasmid Addgene 41815 (1) using primers *hCas9Add41815* fwd2/rev2. The final plasmid was assembled in a four-fragment Gibson reaction to yield *pnos-cas9*.

nos-cas9:GFP. The plasmid was created in the same way as *pnos-cas9*, except that the Cas9 coding region fused to a sequence encoding GFP and two nuclear localization signals was amplified from Addgene plasmid 42234 [which contains a different human codon optimized version to plasmid 41815 (3)] with primers *hCas9Add42234* fwd/rev.

UAS-cas9 *(for* CFD3 *and* 4*).* The coding sequence of *Streptococcus pyogenes* Cas9 was codon-optimized for expression in *Drosophila* using the online tool from Integrated DNA Technologies (IDT), followed by addition of sequence coding for the nuclear localization signal of the *Drosophila* UDE1 protein (4) to the 3′ end. The DNA sequence was ordered as gBlocks from IDT with 30-bp overlapping homologies between fragments and was constructed by Gibson assembly. The *cas9* sequence was amplified from the assembled gBlocks using primers *DmCas9pJFRC81* fwd/rev and cloned into the KpnI/XbaI site of *pJFRC81* (Addgene 36432) (5) using conventional T4 DNA ligase-mediated cloning. The sequence provided by *pJFRC81* includes the 3′ UTR from the *Autographa californica* nuclear polyhedrosis virus *p10* gene, which acts as a strong translational enhancer.

UAS-cas9 *(for* CFD5 *and* 6*).* A *Drosophila* codon-optimized *cas9* with N- and C-terminal nuclear localization signal was cloned into pJFRC28 (Addgene 36431) (5), using the KpnI and XbaI restriction sites. The Cas9 cDNA was a gift from Justin Crocker (David Stern laboratory, Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA).

Guide RNA Expression Plasmids. The sequence encoding the three *Drosophila U6* genes (based on Flybase release FB2013_04) with the *U6* RNA sequences replaced by guide RNA (gRNA) core sequences (Table S5) was synthesized by IDT. The sequence starts with 5′-ATTTTCAACGTCCTCGATAG and ends with TTCGCTTAATGCGTATGCAT-3′. This construct is referred to as "*3xU6-gRNA*" and served as the PCR template for the cloning of the individual gRNA expression plasmids. The backbone of the individual *pCFD* gRNA vectors, containing an *attB* site, a *vermilion* eye pigmentation marker, and an Ampicillin resistance gene, was PCR amplified from *pValium22* (a gift from Norbert Perrimon, Harvard University, Cambridge, MA) using primers *pCFDbackbone* fwd/rev.

pCFD1, 2, *and* 3. *pCDF1*, *pCFD2*, and *pCFD3* contain, respectively, the *U6:1*, *U6:2*, and *U6:3* promoters. Each promoter was amplified from the *3xU6-gRNA* construct using a specific *U6 prom* fwd/rev primer pair. These promoter sequences were assembled together with the gRNA core and genomic 3′ region amplified from the *3xU6-gRNA* construct with *gRNAterm* fwd/rev and the *pCFD* backbone.

pCFD4. The double gRNA vector was produced by amplifying a *BbsI spacer-gRNA-U6-3 promoter* fragment from the *3xU6-gRNA* construct using *pCFD4* fwd/rev primers and cloning it into *pCFD1* that had been digested with BbsI.

pCFD1–3 allow cloning of annealed complementary oligonucleotides into the BbsI-digested backbone using standard procedures to produce the following 5′-to-3′ configuration: *U6* promoter-gRNA target sequence-gRNA core sequence. Two gRNA target sites can be introduced into *pCDF4* by a simple PCR-based method. Cloning procedures for *pCFD1–4* are documented in further detail in Fig. S3. The four gRNA expression vectors, together with their sequences, are available at www.addgene.org (*pCDF1,* Addgene no. 49408; *pCDF2,* Addgene no. 49409, *pCDF3,* Addgene no.49410; *pCDF4,* Addgene no. 49411). Most gRNA target sites used in this study were cloned into earlier versions of the pCFD backbone containing a *white* marker gene using Gibson assembly, except for *gRNA-e gRNA-cu* and *gRNA-y*^{offset}, which were generated in pCFD4. We confirmed that gRNA plasmids containing *white* or *vermilion* marker genes function with comparable efficiency.

gRNA Design. Target sites were designed so that they direct Cas9-mediated cleavage to the 5′ end of the coding sequence, except for *gRNA-wg*$^{P1-3}$, which targets the promoter. To reduce the risk of off-target cleavage, target sites were chosen that do not have highly homologous sites elsewhere in the genome. Off-target potential was assessed using CRISPR target finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) (6) or E-CRISPR (www.e-crisp.org/E-CRISP/) (7). Because a 5′ guanine is required for transcription from U6 promoters, target sites that lack this feature were extended in the 5′ direction by a single guanine. The 5′ extensions do not appear to affect gRNA function (8).

Wg::GFP Donor Plasmid Production. The 5′ and 3′ homology arms were PCR amplified from genomic DNA from *nos-cas9* flies using primers wgGFP5′fwd, wgGFP5′rev, wgGFP3′fwd, and wgGFP3′rev. The eGFP coding sequence flanked by sequences coding for short linker peptides from Ig G2 were amplified from an eGFP-containing plasmid (S.L.B. laboratory stock) using primers wgGFPGFPfwd and wgGFPGFPrev. The sequences of all primers as well as the sequence encoding the linkers can be found in Table S5. All fragments were assembled by Gibson assembly into pBluescript SK-(+) (Stratagene) that was digested with XhoI and NotI.

Fly Transgenesis and Culture. Transgenic lines were generated by standard PhiC31-integrase–mediated transformation using injected DNA constructs (9). The *attP* integration sites used for different experiments are documented in Tables S1 and S2.

Other stocks used and their sources are listed in Table S6. All crosses were performed at 25 °C with 50 ± 5% relative humidity and a 12-h light/dark cycle.

Embryo Injections. Embryos were collected on apple juice plates for 30 min at 25 °C, briefly rinsed with tap water, and dechorionated for 60 s in 6% sodium hypochlorite. After extensive washing with tap water, embryos were lined up on apple agar plates with a paintbrush, transferred to a coverslip coated in heptane glue, and desiccated for 5–8 min in a box containing silica gel. Embryos were covered in Voltalef 10S oil (VWR International) and transferred to a Nikon Eclipse microscope equipped with a manual micromanipulator (Narishige). DNA was microinjected in the proximity of the posterior pole of embryos using a heat-pulled glass needle (Microcaps, Drummond) attached to an air-filled 20-mL syringe. All injections were performed at 22 ± 1 °C, with typically 50–100 embryos injected 45–60 min after egg-laying. For the delivery of plasmid DNA for the production of transgenes, 150 ng/μL of DNA in sterile dH_2O was injected. Single-stranded oligonucleotides designed to modify the *wntless* (*wls*) or *ebony* (*e*) locus (ordered as 4-nM Ultramers from IDT) were injected into the posterior region of *nos-cas9*/+*; U6:3-gRNA-wls*/+ or *act-cas9*/+*; U6-3-gRNA-e*/+ embryos, respectively, as a 750-ng/μL or 500-ng/μL solution of DNA in dH_2O. Plasmid DNA that acts as a donor template for homologous recombination-mediated integration of eGFP into the *wg* locus was injected at a concentration of 750 ng/μL into *nos-cas9*/+*; U6:3-gRNA-wg*/+ embryos. After injection of plasmids or oligonucleotides, embryos were transferred on their coverslips to a plastic box containing wet paper towel at 25 °C until they hatched as larvae. Larvae were collected with forceps and transferred to a food vial with fresh yeast, followed by culture at 25 °C.

Immunohistochemistry and Visualization of GFP Fluorescence. Wing imaginal discs from third-instar larvae were dissected in chilled PBS. Fixation was performed in 4% paraformaldehyde in PBS containing 0.3% Triton-X100 (PBT) for 25 min at room temperature. After three washings with PBT, discs were incubated overnight at 4 °C with primary antibodies diluted in PBT. Imaginal discs subsequently were washed three times with PBT containing 1% heat-inactivated goat serum for at least 1 h, followed by incubation in secondary antibodies diluted in PBT for 2 h at room temperature. Samples were washed three times in PBT and mounted in Vectashield medium containing DAPI (Vector Laboratories). Antibodies used were polyclonal rabbit anti-Wntless (anti-Wls) (1:1,000) (10), monoclonal mouse anti-Wingless (anti-Wg) (4D4; 1:50; Developmental Studies Hybridoma Bank), Alexa Fluor 488 goat anti-mouse (1:400; Invitrogen) and Alexa Fluor 555 goat anti-rabbit (1:400; Invitrogen). Discs were prepared for visualization of Wg::GFP fluorescence by fixation in 4% paraformaldehyde for 25 min, followed by three washes for 5 min each in PBT and mounting in Vectashield.

Drosophila embryos were collected on apple agar plates and dechorionated for 90 s in 6% sodium hypochlorite. They were fixed for 20 min at room temperature on an orbital shaker in glass vials containing 2 mL n-heptane and 1 mL 4% paraformaldehyde (in PBS) that had been mixed and allowed to phase separate. The paraformaldehyde subsequently was replaced with 3 mL methanol, and the vitelline membrane was removed by vigorous shaking. Embryos were rinsed three times in methanol, resuspended in PBT, and immunostained following the protocol used for wing imaginal discs.

Cuticle Preparations. *Drosophila* embryos were dechorionated as describe above and transferred to glass vials containing 2 mL n-heptane and 3 mL methanol that had been mixed and allowed to phase separate. The vitelline membrane then was removed by vigorous shaking. Embryos were washed with methanol and transferred to microscope slides. Excess methanol was removed, and embryos were covered in Hoyer's solution. Slides were incubated for at least 2 h at 60 °C before imaging.

Image Acquisition. Images of whole flies were captured on a Canon 550D digital camera equipped with a Canon 50-mm f1.8 lens mounted on a Leica MZFLIII stereomicroscope. Manual settings were used throughout, and the lighting was kept constant during image acquisition. Flies presented within the same figure were imaged on the same day. Before imaging, flies were incubated overnight in 20% glycerol/80% ethanol and on the next day were mounted in 100% glycerol. Adult wings were mounted in 100% glycerol and imaged on a Zeiss Axioplan microscope equipped with a CoolSnap HQ2 camera (Photometrics). Fluorescent images of wing imaginal discs and embryos were acquired on a Zeiss LSM710 or LSM780 confocal microscope using the sequential scanning mode and a 40×/1.3 NA oil or 20×/0.5 NA air objective.

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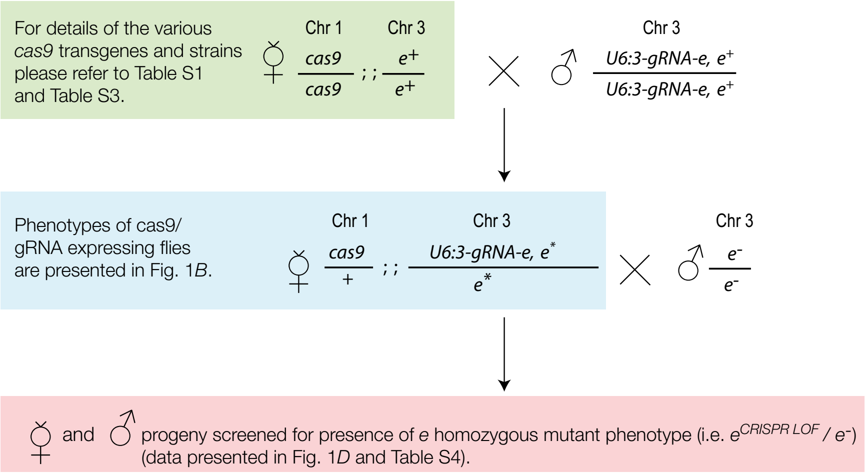

Fig. S1. General crossing scheme used to compare the efficiency of various *cas9* strains in the soma and germ line. Only relevant genetic elements are shown (full genotypes of *cas9* and *gRNA* strains are listed in Table S3). Note that some *cas9* transgenes are located on the third chromosome along with a wild-type *e* allele. A similar crossing scheme was followed to assess targeting of the *y* gene (i.e., flies containing the *cas9* and *U6:3-gRNA-y* transgenes were crossed to a *y* mutant strain). Chr, chromosome; *CRISPR-LOF*, loss-of-function CRISPR allele.

Fig. S2. Further evidence that the *nos-cas9* transgene lacks detectable activity when crossed to *U6:3-gRNA-y*. A *nos-cas9 U6:3-gRNA-y* male is shown that inherited a single, wild-type *y* allele. No yellow cuticle is observed, indicating that this allele is not mutated in cells giving rise to cuticle.

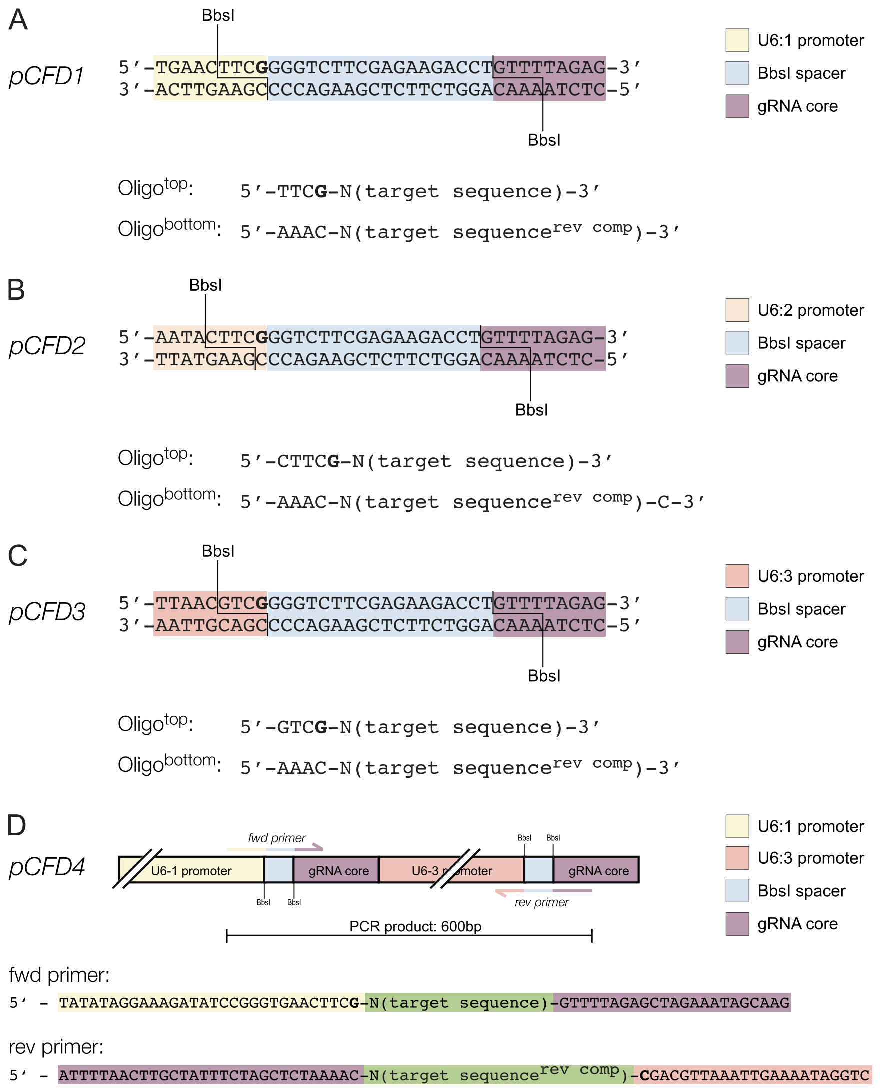

Fig. S3. Cloning strategies to introduce gRNA target sites into *pCFD* vectors. (*A*–*C*) Plasmids *pCFD1* (*A*), *pCFD2* (*B*), and *pCFD3* (*C*) contain two inverted type-IIS BbsI restriction sites to allow seamless cloning of target sites. The BbsI cassette can be replaced by the desired target sequence by digesting the plasmid backbone with BbsI and ligating the linear backbone to annealed oligonucleotides with compatible ends (schematized below each cloning site). The G in bold is the first nucleotide that is transcribed and is necessary for transcription from *U6* promoters. If the target site has a 5′ G, then the target sequence to be introduced will be 19 nt long; otherwise, the target sequence will be 20 nt long. There is evidence from mammalian cells that shorter target sequences can be tolerated and can increase the specificity of CRISPR/Cas (11). Note that the BbsI cassette in *pCFD2* (*B*) is truncated by 1 bp, making it necessary to add a 3′ cytosine to the bottom-strand oligonucleotide. (*D*) Cloning strategy to introduce two target sites into the tandem gRNA vector *pCFD4*. Target sites are incorporated into the forward and reverse primers, which also contain 3′ homology to the *pCFD4* backbone to allow PCR amplification, and 5′ homology to the *pCFD4* backbone to allow homology-directed cloning. PCR products are cloned into the BbsI-digested *pCFD4* backbone by homology-directed cloning (e.g., Gibson assembly).

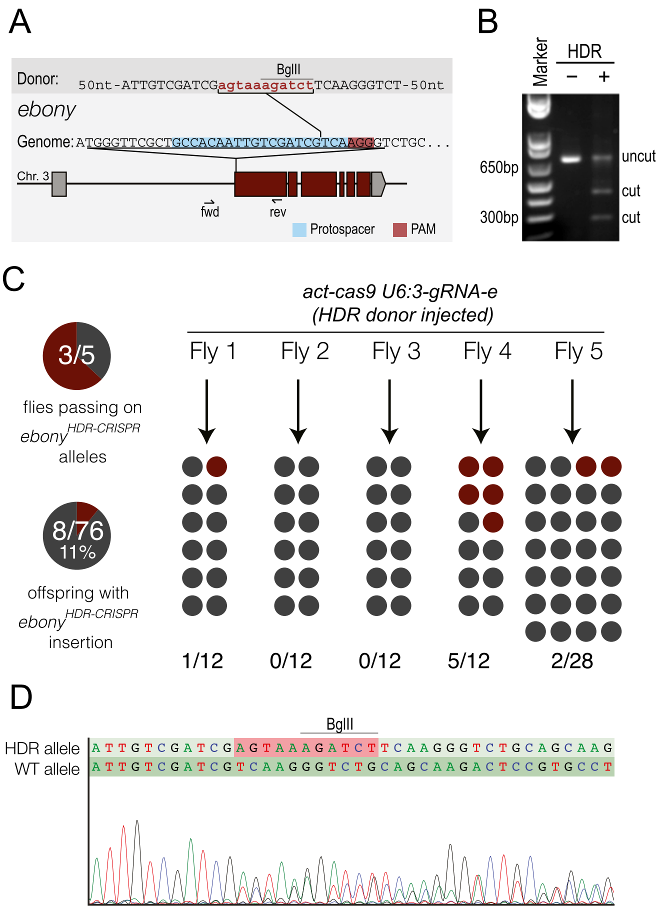

Fig. S4. Efficient incorporation of an exogenous sequence by homology-directed repair (HDR) into the *e* locus following Cas9-induced DSBs. (*A*) Schematic of the donor DNA in relation to the gRNA target site at the *e* locus. The donor was a single-stranded oligonucleotide with 60-nt homology to the target locus at either side of the Cas9 cut site and an 11-nt insert (lowercase). This insert introduces an in-frame stop codon (TAA) and a BglII restriction site. The locations of the primers used for the genotyping PCR in *B* are shown below the schematic of the genomic locus. Donor DNA was injected into embryos that were the progeny of *act-cas9* females and *U6:3-gRNA-e* males. (*B*) Successful integration of the donor construct could be detected in the offspring of injected embryos by BglII digestion of PCR products. Agarose gel showing pattern observed in the absence (–) and presence (+) of the HDR. The 700-nt fragment present in both samples is derived from the wild-type e locus transmitted by the other parent. (*C*) Summary of results from screening flies for HDR events by PCR and restriction digest. Note that flies that developed from injected embryos were selected at random, i.e., without consideration for their pigmentation phenotype. (*D*) Sequence verification of the precise integration of the donor DNA in the *e* locus by direct sequencing of a PCR product amplified from a heterozygous fly that tested positive by BglII restriction digest. Double peaks in the chromatogram represent an overlay of the sequence of the mutant and wild-type *e* alleles.

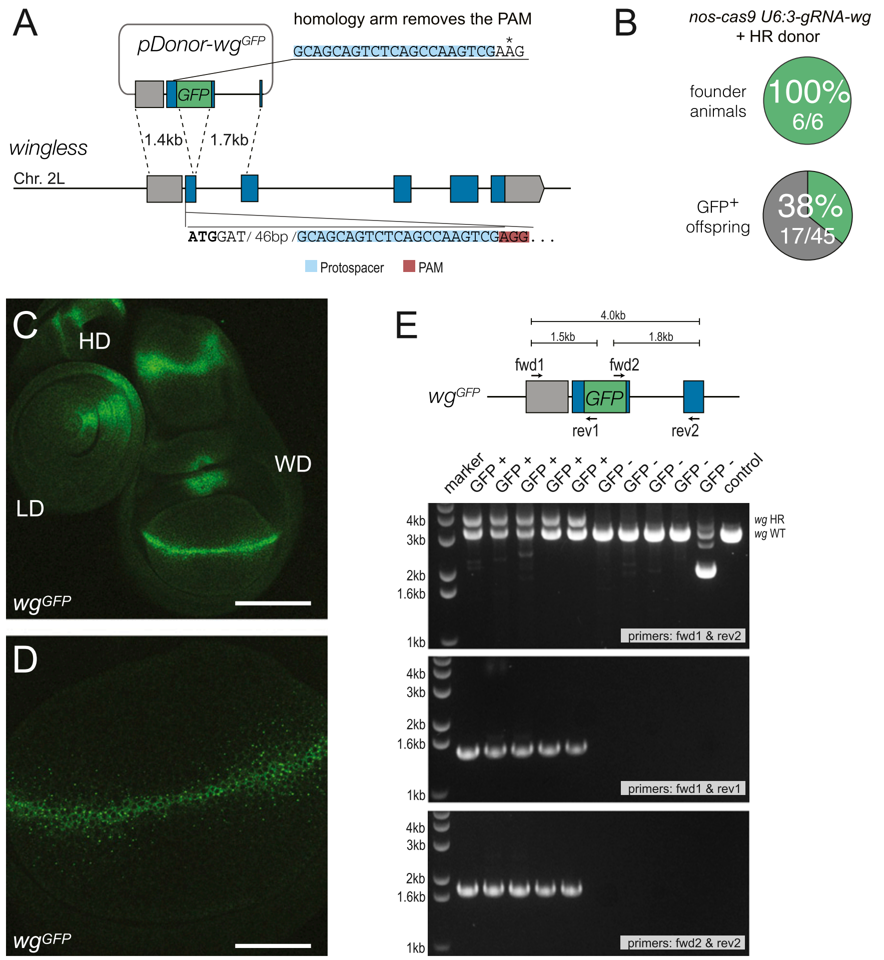

Fig. S5. Efficient integration of a GFP tag into the endogenous *wg* locus by homologous recombination (HR) using *nos-cas9* and *U6:3-gRNA-wg*. (*A*) Schematic of the donor plasmid, *wg* locus, and *gRNA-wg* target sequence. The donor is designed to introduce an eGFP-coding sequence flanked on either side by sequences coding for a short linker peptide from IgG into the first coding exon of *wg*. The exogenous sequence is flanked by homology arms of 1.4 kb (5′ homology) and 1.7 kb (3′ homology). The 5′ homology arm contains a synonymous mutation that removes the protospacer-adjacent motif (PAM) sequence for *gRNA-wg* to prevent mutagenesis after the integration of donor-derived sequences. The circular donor plasmid was injected into *nos-cas9 U6:3-gRNA-wg* embryos. (*B*) Injected animals were crossed to a balancer strain, and offspring were screened at the third-instar larval stage for the appearance of green fluorescence in dissected imaginal discs. All six injected animals tested gave rise to GFP$^+$ offspring, with 17 of the 45 larvae examined showing GFP expression. In all cases GFP expression was restricted to the Wg-expression domain. In the six crosses, two of nine larvae, six of 11 larvae, three of five larvae, one of five larvae, three of eight larvae, and two of seven larvae were GFP$^+$. (*C* and *D*) Images showing examples of GFP$^+$ imaginal discs. Each image is a single confocal section. (*C*) Low-magnification image showing GFP fluorescence in the Wg expression domains of a wing (WD), leg (LD), and part of a haltere (HD) imaginal disc. (Scale bar: 150 μm.) (*D*) A high-magnification view of GFP fluorescence at the dorsal–ventral boundary of a wing imaginal disc. In addition to strong signal from a stripe of three to four cells, punctate signal is found more distally from this site. This pattern is reminiscent of that observed when endogenous Wg protein is detected using a specific anti-Wg antibody (12). (Scale bar: 50 μm.) (*E*) Diagnostic PCR to test for ends-out HR of the donor plasmid in offspring of *nos-cas9 U6:3-gRNA-wg* embryos injected with the Wg::GFP donor plasmid. After examination of GFP fluorescence in imaginal discs, genomic DNA was extracted from the remaining material of 10 of the dissected larvae. Five of these larvae had GFP$^+$ imaginal discs, and five had GFP$^-$ discs. DNA also was extracted from a *nos-cas9* larva, which served as a negative control. PCRs were performed using the primers indicated in the schematic. Note that primers rev1 and fwd2 do not anneal to the wild-type *wg* locus. Primers fwd1 and rev2 are located outside the homology arms and thus do not anneal to sequences in the donor plasmid. All larva with GFP$^+$ discs tested positive for integration of the GFP sequence at the *wg* locus; the presence of the 4-kb band from all these larvae using fwd1 and rev2 primers demonstrates ends-out targeting (i.e., in which the plasmid backbone is not incorporated) of one of the alleles. One of the GFP$^-$ larvae yielded a much shorter band with primers fwd1 and rev2, suggesting a large (∼1 kb) CRISPR/Cas-induced deletion. This product presumably amplified more efficiently than the product from the wild-type allele because of its relatively small size.

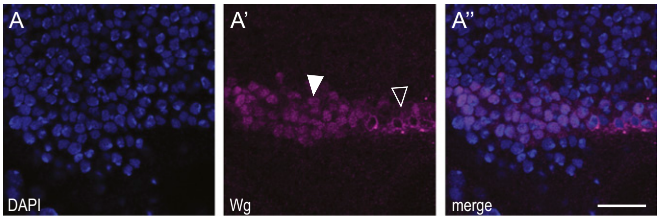

Fig. S6. Mutations at the *gRNA-wg* target site can relocate Wg to the nucleus. A high-magnification view of a third-instar wing imaginal disc from a *cas9 gRNA-wg*–expressing animal is shown. A presumptive clone of cells with Wg (Wg protein in magenta in *A′* and *A″*) mislocalized to the nucleus (DNA in blue in *A* and *A″*) is shown (solid arrowhead) next to tissue that retains wild-type Wg localization (open arrowhead). This image is from a different animal from the one shown in Fig. 5*C*. (Scale bar: 20 μm.)

Table S1. Transgenic Cas9 lines used in this study

Name	Promoter/3′ UTR	Cas9 coding sequence[*,†]	Integration site of *cas9* construct (chromosome)	Stock numbers
Stocks generated in this study. (www.crisprflydesign.org)				
act-cas9	*act5C/SV40*	Hs_Cas9 (Addgene: 41815)	attP-ZH2a (X)	CFD1 BL:54590 VDRC: 300000
act-cas9^{D10A}	*act5C/SV40*	Hs_Cas9 (Addgene: 41815)	attP2 (3L)	VDRC: 300008
nos-cas9:GFP	*nos/nos*[‡]	Hs_Cas9:GFP (Addgene: 42234)	attP-ZH2a (X)	N/A
nosG4VP16 UAS-cas9[§]	*nos(Gal4/UAS)/p10*	Dm-Cas9	attP2 (3L)	CFD3_nos BL: 54593 VDRC: 300003
nos-cas9	*nos/nos*[‡]	Hs_Cas9 (Addgene:41815)	attP-ZH2a (X)	CFD2 BL:54591 VDRC: 300001
UAS-cas9	*UAS/p10*	Dm_Cas9[¶]	attP2 (3L)	CFD3 BL: 54592 VDRC: 300002
UAS-cas9	*UAS/p10*	Dm_Cas9[¶]	attP2 (3L)	CFD5 BL: 54595 VDRC: 300005
Stocks generated by O'Connor-Giles, Harrison, and Wildonger laboratories (www.flycrispr.molbio.wisc.edu) (6)				
vasa-cas9	*vasa/vasa*[‡]	Hs_Cas9 (Addgene:42230)	attP-ZH2a (X)	BL:51323
vasa-cas9	*vasa/vasa*[‡]	Hs_Cas9 (Addgene:42230)	attP-VK00027 (3R)	BL:51324

act, *actin5C*; BL, Bloomington *Drosophila* Stock Center; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; N/A, not applicable; *nos*, *nanos*; VDRC, Vienna *Drosophila* RNAi Center.

[*] Species refers to codon optimization; all constructs express *Streptococcus pyogenes* Cas9 protein.

[†] *cas9* constructs contain different nuclear localization signals (NLS): Hs_Cas9 (Addgene: 41815) contains a single SV40 NLS; Hs_Cas9 (Addgene: 42234) contains two NLS based on the SV40 NLS; Dm_Cas9 has one Ude1 NLS (4).

[‡] *nos* and *vasa* 3′ UTRs are designed to target protein synthesis to the germ cells.

[§] *UAS-cas9* from strain CFD3.

[¶] Lines CFD3 and CFD5 have different codon optimization.

Table S2. Transgenic gRNA lines used in this study

gRNA line	Integration site
U6:3-gRNA-y[*]	attP2
U6:1-gRNA-y[†]	attP40
U6:2-gRNA-y[†]	attP40
U6:3-gRNA-y[†]	attP40
U6:3-gRNA-e	attP2
U6:3-gRNA-wls	attP40
U6:3-gRNA-wg	attP40
U6:1-U6:2-U6:3-gRNA-wg$^{P1–3}$	attP2
U6:1-gRNA-e U6:3-gRNA-cu	attP2

[*] Used to target *y* in Fig. 1.

[†] Used to target *y* in Fig. 2.

Table S3. Crosses comparing various Cas9 lines in Fig. 1

Virgin females	Males*	WT *y* alleles in F1 females/ males	WT *e* alleles in F1 females/ males	Expected frequency of *y[-]* flies in F2[†], %
y[1] act-cas9$^{ZH\text{-}2A}$	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	2/1	2/2	25
y[1] vasa-cas9$^{ZH\text{-}2A}$	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	2/1	2/2	25
w;; vasa-cas9$^{VK00027,\ y+}$	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	4/3	2/2	0
y[1] nos-cas9:GFP$^{ZH\text{-}2A}$	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	2/1	2/2	25
y[1];; UAS-cas9$^{attP2,\ y+}$ *nos-Gal4::VP16*	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	3/2	2/2	0
y[1] nos-cas9$^{ZH\text{-}2A}$	*w f;;U6:3-gRNA*$^{attP2,\ y+}$	2/1	2/2	25

*gRNA transgenes contain either the *y* or *e* target site (see Table S5 for sequences).

[†] In the absence of CRISPR/Cas mutagenesis. Note that no *e* mutant animals are expected in the *gRNA-e* experiments without CRISPR/Cas mutagenesis.

Table S4. Collated results of experiments assessing germ-line transmission of loss-of-function mutations in *e* and *y* using fully transgenic CRISPR/Cas

Cas9 line	% founders* (#)	% phenotypically mutant offspring per cross, mean ± SEM	No. of phenotypically mutant offspring (total)
Targeting e with *cas9* and *U6:3-gRNA-e* (data presented in Fig. 1*D*)			
act-cas9	100 (10/10)	54 ± 3[†]	613 (1,116)[†]
vasa-cas9 on X	100 (9/9)	52 ± 4[†]	534 (1,002)[†]
vasa-cas9 on 3	100 (9/9)	48 ± 3[†]	627 (1,379)[†]
nos-cas9:GFP	100 (10/10)	42 ± 3[†]	611 (1,409)[†]
nosG4VP16 UAS-cas9	100 (9/9)	46 ± 6[†]	426 (887)[†]
nos-cas9	100 (8/8)	26 ± 3[†]	290 (1,109)[†]
Targeting *y* with *cas9* and *U6:3-gRNA-y* (data presented in Fig. 1*E*)			
act-cas9	100 (10/10)	99 ± 0.4[‡]	762 (767)
vasa-cas9 on X	100 (5/5)	100 ± 0[‡]	149 (149)
vasa-cas9 on 3	100 (10/10)	86 ± 2[‡]	670 (779)
nos-cas9:GFP	100 (10/10)	71 ± 3[‡]	612 (781)
nosG4VP16 UAS-cas9	100 (4/4)	83 ± 7[‡]	244 (315)
nos-cas9	100 (7/7)	76 ± 6[‡]	299 (368)
Comparing *U6* promoters driving *gRNA-y* with *nos-cas9* (data presented in Fig. 2*C*)			
U6 promoter[§]			
U6:1	100 (3/3)	69 ± 1[‡]	278 (359)
U6:2	100 (3/3)	41 ± 3[‡]	351 (595)
U6:3	100 (3/3)	99 ± 0.3[‡]	410 (412)

*Founders are defined as those flies that transmitted nonfunctional alleles to the next generation.

[†] Phenotypic screening for *e* mutant alleles does not account for in-frame mutations at the *gRNA-e* target site that do not disrupt gene function.

[‡] Data are normalized to account for phenotypically yellow mutant offspring that arise because of the genetic background (*Materials and Methods*).

[§] *U6-gRNA-y* constructs are inserted at attP40 in this experiment; *U6:3-gRNA-y* is inserted at attP2 in the data presented in Fig. 1*E*.

Table S5. Oligonucleotides and gRNA target sites used in this study

Primers used for cloning (5′–3′)	
act5c fwd	ATACGAAGTTATGCTAGCGGATCCAAGCTTGCGGCCGCAATTCTATATTC
act5c rev	AGCCGCGGCCGCAGATCTGTTAACGAATTCCGGGGATCGATCCTGTAAGC
hCas9Add41815fwd	CAGCTTACAGGATCGATCCCCGGGAATTCaccATGGACAAGAAGTACTC
hCas9Add41815rev	TCACAAAGATCCTCTAGAGGTACCCTCGAGTCACACCTTCCTCTTCTTCT
act-cas9nickfwd	GCAGCTTACAGGATCGATCCCCGGGAATTCACCATGGACAAGAAGTACTCCATTGGGCTCGCTATCGGCACAAACAG
act-cas9nickrev	TTAGCGTCGGCGAGGATCACTC
nosPromfwd	TATGCTATACGAAGTTATGCTAGCGGATCCAAGCTTCGACCGTTTTAACC
nosPromrev	GAGTACTTCTTGTCCATGGCGAAAATCCGGGTCGAAA
nos3′fwd	GAAGAGGAAGGTGTGAGCGAATCCAGCTCTGGAGCA
nos3′rev	GTGACCTACATCGTCGACACTAGTGGATCCTTCCTGGCCCTTTTCGAGAA
hCas9Add41815fwd2	GACCCGGATTTTCGCCATGGACAAGAAGTACTCCAT
hCas9Add41815rev2	CCAGAGCTGGATTCGCTCACACCTTCCTCTTCTTCT
hCas9Add42234fwd	GACCCGGATTTTCGCCATGGACAAGAAGTACAGCAT
hCas9Add42234rev	CCAGAGCTGGATTCGCCTACTTGTACAGCTCGTCCA
DmCas9pJFRC81fwd	GAGGGTACCAACTTAAAAAAAAAAATCAAAATGGATAAGAAGTATAGCAT
DmCas9pJFRC81rev	GTATCTAGATTACTCCTGCTTGCGCTTCT
gRNA core	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
pCFDbackbonefwd	ATAAAATACATTGCATAGATCTGAATTCTATCTAGACTCCCTCGTGCGCTCTCCTG
pCFDbackbonerev	GATAGAATTCAGATCTATGCAATGTATTTTATTAAAAACGG
U61promfwd	CGTTTTTAATAAAATACATTGCATAGATCTGAATTCATTTTCAACGTCCTCGATAGTA
U61promrev	TATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTCTTCTCGAAGACCCCGAAGTTCACCCGGATATCTT
U62promfwd	CGTTTTTAATAAAATACATTGCATAGATCTGAATTCGTTCGACTTGCAGCCTGAAA
U62promrev	TTTCTAGCTCTAAAACAGGTCTTCTCGAAGACCCGAAGTATTGAGGAAAACATAC
U63promfwd	CGTTTTTAATAAAATACATTGCATAGATCTGAATTCTTTTTTGCTCACCTGTGATTGCTC
U63promrev	TATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTCTTCTCGAAGACCCCGACGTTAAATTGAAAATAGGTCT
gRNAtermfwd	GGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCA
gRNAtermrev	ACACCACAAATATACTGTTGCCGAGCACAATTGTCTAGAATGCATACGCATTAAGCGAAC
pCFD4fwd	ATATATAGGAAAGATATCCGGGTGAACTTCGGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAG
pCFD4rev	TATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTCTTCTCGAAGACCCCGACGTTAAATTGAAAATAGGTCT
gRNA target sites	
gRNA-y target	GCGATATAGTTGGAGCCAGC
gRNA-e target	GCCACAATTGTCGATCGTCA
gRNA-y offset top	CAAGGATCCACCCTTTGTCC
gRNA-y offset bottom	TCACCTTGGTGACGCCGTCT
gRNA-wg	GCAGCAGTCTCAGCCAAGTCG
gRNA-wls	GACCATACTGGAGAACCTGAG
gRNA-wgP$^{1-3}$	GCGATCGGATCGGGGATCTC
gRNA-wgP$^{1-3}$	GCTGCTGACAAACGCAGAGT
gRNA-wgP$^{1-3}$	GCAGCTGCAATGCAGGAGTC
gRNA-cu	AGCCGGGGTGCTTAGACCGG
HDR donor	
Ebony HDR donor	AAAGTATTCCCCACAGTTAATATATCTTCAAGATGGGTTCGCTGCCACAATTGTCGATCGagtaaagatctTCAAGGGTCTGCAGCAAGACTTCGTGCCTAGAGCTCTGCACCGCATCTTCGAGGAGCAGC
Wls HDR donor	AAACCAGTGAGCTCCAAACCTTCGAGCAACCAAGATGTCGGGCACCATACTGGAGAACCTGAGTGCCCGCAAGCTGTCCATATTGGTGGCCACTTTGCTGCTCTGCCAGGTGTTGTGCTT
HR donor plasmid and HR diagnosis	
wgGFP5′fwd	AGGTCGACGGTATCGATAAGCTTGATATCGCGGAATGCCAAAGTGTGTGGTAAC
wgGFP5′rev	CCTTCCGGATTTCTGTTTGCCTTCGACTTGGCTGAGACT
wgGFP3′fwd	ATCGCCTCGAAGCCAAAGGGCGCCTCGGTTCGTGCATCCATGTGGTGGTAAGTTCATTGAA
wgGFP3′rev	GGAGCTCCACCGCGGTGGCGGCCGCTCTAGGGGCGGTATACGTCAATTGCCGGC
wgGFPGFPfwd	CAAGTCGAAGGCAAACAGAAATCCGGAAGGATCGCCTCGAAGCCAAAGGG
wgGFPGFPrev	TGCACGAACCGAGGCGCCCTTTGGCTTCGAGGCGATCTTGTACAGCTCGTCCATGCCGAGA
IgG2 linker	ATCGCCTCGAAGCCAAAGGGCGCCTCGGTTCGTGCA
wg_genotyping_fwd1	GAAGCGGCCAAGCAATGGATGAGG
wg_genotyping_rev1	CTCGCCCTTGCTCACCATAGATC
wg_genotyping_fwd2	CTCGGCATGGACGAGCTGTACAAG
wg_genotyping_rev2	TCGCTGGGTCCATGTACATGATGGG

Table S6. Additional fly stocks used in this study

Stock	Source
Stocks used to generate *cas9* lines	
y[1] w[67c23] P{y[+t7.7]=nos-phiC31int.NLS}X; P{y[+t7.7]=CaryP}attP2	Gift from Simon Collier, University of Cambridge, Cambridge, UK
y[1] M{3xP3-RFP.attP}ZH-2A w[*]; M{vas-int.Dm}ZH-102D	Bloomington (BL: 24480)
Stocks used to generate gRNA lines	
y[1] v[1] P{y[+t7.7]=nos-phiC31int.NLS}X; P{y[+t7.7]=CaryP}attP40	Bloomington (BL: 24709)
y[1] sc[1] v[1] P{y[+t7.7]=nos-phiC31int.NLS}X; P{y[+t7.7]=CaryP}attP2	Bloomington (BL: 25710)
Balancer and marker stocks:	
P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; Sp/CyO	S.L.B. laboratory stock
P{ry[+t7.2]=hsFLP}1, y[1] w[1118];; MKRS/TM6b e	S.L.B. laboratory stock
w[1118];; TM3 e/TM6b e	S.L.B. laboratory stock
w[1118], f[1]	S.L.B. laboratory stock
Gal4 lines	
P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; engrailed-Gal4 tubulin-Gal80^{ts} *UAS-CD8:GFP/CyO; MKRS/TM6b*	Gift from Ryohei Yagi and Konrad Basler, University of Zurich, Zurich
w[1118];; P{GAL4::VP16-nos.UTR}CG6325^{MVD1}	Gift from Katja Röper, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK
Cas9 lines from flyCRISPR	
y[1] M{vas-cas9}ZH-2A w[1118]/FM7c	Bloomington (BL: 51323)
w[1118]; PBac{y[+mDint2]=vas-cas9}VK00027	Bloomington (BL: 51423)
wg loss-of-function stock used for genetic complementation	
w[1118]; wg$^{TX\text{-}Cherry}$*/CyO*	Gift from Jean-Paul Vincent, Medical Research Council National Institute for Medical Research, London (13)