Materials and methods

Molecular reagents

phsp70-Cas9: Sequence encoding 3X Flag-NLS-Cas9-NLS was amplified from pX330 (CONG *et al.* 2013) and cloned as a ClaI/XbaI fragment between the Drosophila *hsp70* promoter and 3′ UTR in pHSS6hsILMi20 (a gift from Anastasios Pavlopoulos). For annotated sequence, see Supporting Information Figure S2A. Expression of Cas9 in embryos was confirmed by immunoblotting.

pU6-BbsI-chiRNA: The chiRNA sequence was placed under control of the Drosophila snRNA:U6:96Ab promoter for in vivo transcription (WAKIYAMA *et al.* 2005). The U6-chiRNA backbone was synthesized as a gBlock gene fragment (Integrated DNA Technologies) and blunt-end ligated into the EcoRV site of pBluescript SK(-). The resulting vector contains two BbsI cut sites to facilitate insertion of target-specific sequences (for annotated sequence, see Supporting Information Figure S2B). Target-specific sequences for *yellow* and *rosy* were synthesized as 5′-phosphorylated oligonucleotides, annealed and ligated into the BbsI sites of pU6-BbsI-chiRNA.

Donor template. The single-stranded DNA oligonucleotide (ssODN) donor template for homologous recombination was designed to contain 60-nt of homology directly adjacent to each Cas9-mediated DSB in the target locus flanking a 50-nt attP docking site (for annotated sequence, see Supporting Information Figure S2C). The ssODN was synthesized by Integrated DNA Technologies.

Embryo injections

w^{1118} preblastoderm embryos were injected through the chorion membrane using standard protocols. phsp70-Cas9 was injected at a concentration of 500 ng/μL. The pU6-chiRNA targeting constructs were injected at 500 ng/μL for single chiRNAs and 250 ng/μL each when two chiRNAs were injected. The donor template ssODN was diluted based on manufacturer's concentrations and injected at 100 ng/μL. All injection mixtures were prepared in water. Average embryonic survival following injection with Cas9 and a single chiRNA was 50%. Embryonic survival rates following multiplex injections of 2 chiRNAs with and without the ssODN donor were 68 and 69%, respectively. While this difference likely reflects improved quality of injections rather than the differences in the injection components, these rates indicate that expression of the components of the CRISPR RNA/Cas9 system does not significantly impair development.

SURVEYOR assay

Analysis of NHEJ products resulting from single chiRNA targeting was performed using the SURVEYOR Mutation Detection kit (Transgenomic). Briefly, genomic DNA was isolated from individual embryos 24 hours after injection. Approximately 500-bp flanking the targeted Cas9 cleavage sites in *yellow* and *rosy* were amplified using Herculase DNA polymerase (Agilent Technologies) according to the manufacturer's protocol. The resulting product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega). A total of 500 ng of purified PCR product was diluted in 1X Herculase reaction buffer to a final volume of 20 μL. Heteroduplexes were formed using the following parameters: 95°C for 10 min, 95°C to 85°C ramping at rate of -2.0°C/sec, 85°C for 1 min, 85°C to 25°C at a rate of -0.3°C/sec with 1 min holds at 75°C, 65°C, 55°C, 45°C, 35°C, and 25°C. Following duplex annealing, 16 μL of each sample (400 ng annealed duplexes) was mixed with 2 μL 0.15 M $MgCl_2$, 1 μL SURVEYOR Enhancer S, 1 μL SURVEYOR Nuclease S and incubated at 42°C for 60 min. The SURVEYOR reaction was stopped with 2 μL of Stop solution, and the products were separated by polyacrylamide gel electrophoresis. The gel was stained with SYBR Gold (Invitrogen), and visualized on a GE ImageQuant.

Molecular characterization of engineered loci

Genomic DNA was isolated from individual embryos 24 hours after injection. PCR was performed using primers flanking the targeted locus (Figure 1A, open arrows). Amplified products were purified and subcloned into pCR4-TOPO or pCRBluntII-TOPO (Invitrogen) prior to Sanger sequencing.

Screening

To assess germline transmission of targeted genome modifications, adults that developed from injected embryos were individually crossed to y^1, w^1. The offspring of crosses were screened for 10 days after the first flies emerged for progeny with yellow cuticles. Transmission rates were calculated both as a percentage of parental crosses that produced one or more yellow progeny and as a percentage of total progeny. The total number of progeny screened was calculated by weight. Specifically, for each genome manipulation we pooled progeny daily and weighed 100 flies. The remaining flies were weighed and their number calculated based on the weight of the hand-counted flies. Transmission of expected events was confirmed by sequence analysis.

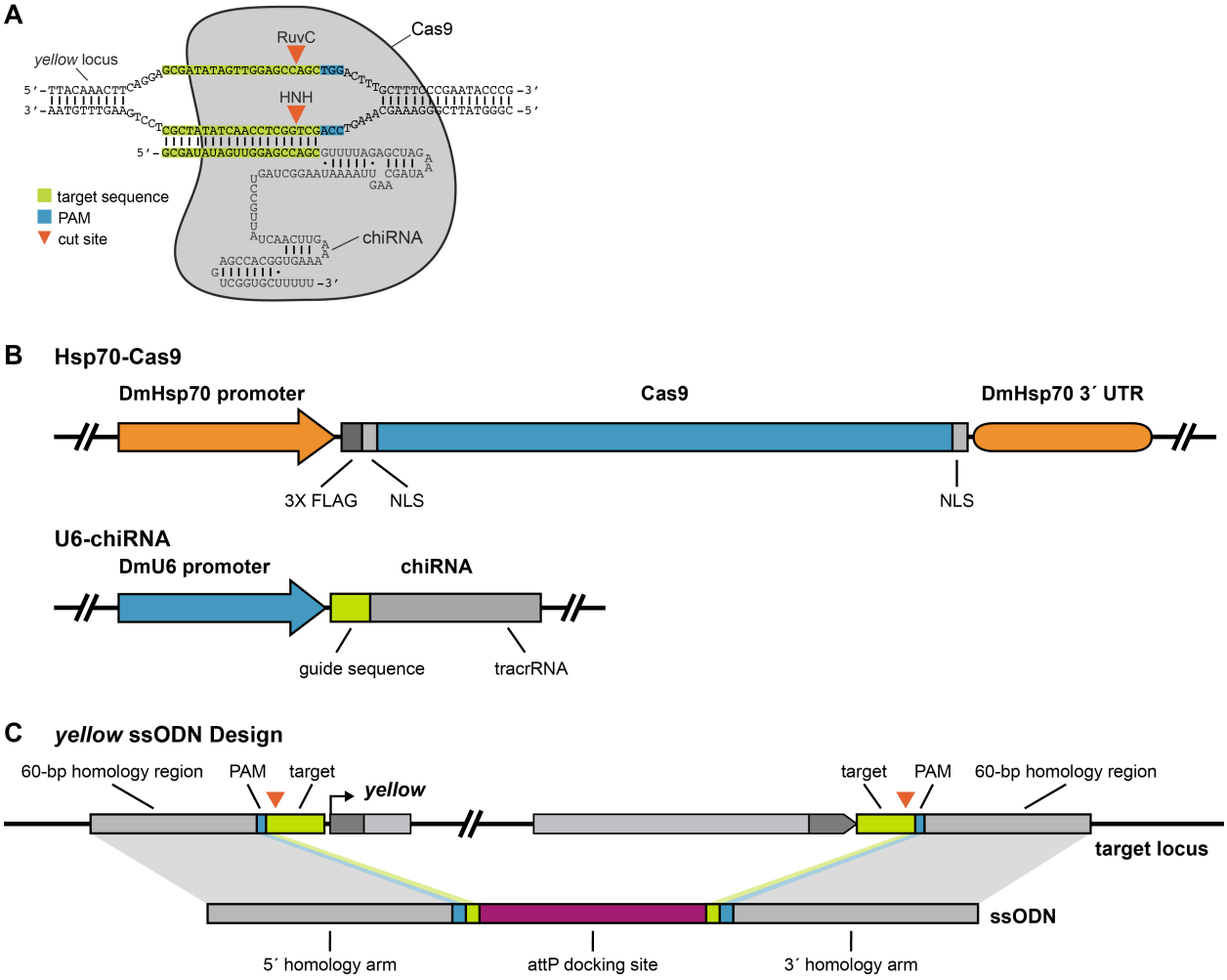

Figure S1 CRISPR RNA/Cas9 molecular reagents used in this study

(A) Schematic of the modified CRISPR RNA/Cas9 system (based on Jinek et al., 2012) used in this study (YE1 is shown as an example). A chiRNA guides Cas9 to complementary target DNA. Target recognition requires 20-nt of homology and a 3-bp PAM sequence, NGG, at the 3′ end of the genomic target sequence. Cas9 cleaves the complementary DNA strand via its HNH endonuclease domain, while its RuvC-like endonuclease domain cleaves the noncomplementary strand (red arrowheads), resulting in a DSB at the targeted site. (B) Cas9, derived from *Streptococcus pyogenes* and codon optimized for eukaryotic expression (Cong *et al.* 2013), was expressed under the control of the Drosophila *hsp70* promoter and 3′ UTR. The chiRNAs utilized in this study consist of 20-nt of target-specific guide sequence followed by 76-nt of tracrRNA sequence as this chiRNA exhibited maximal activity in mammalian cells (Patrick Hsu and Feng Zhang, personal communication). The chiRNA is transcribed from the Drosophila snRNA:U6:96Ab promoter (Wakiyama *et al.* 2005). (C) ssODN donor templates contain 60-nt homology arms matching genomic sequence immediately adjacent to each site-specific cut flanking a 50-nt attP docking site for efficient subsequent manipulation of the targeted locus. Red arrowheads mark the predicted Cas9 cleavage sites. Note that the ssODN is designed such that neither it nor the modified locus will be CRISPR targets. Not to scale. More detailed information and protocols can be found at FlyCRISPR.molbio.wisc.edu.

A. pHsp70-Cas9

DmHsp70 promoter and 3'UTR
3x Flag
NLS
Cas9

ATCCCCCTAGAATCCCAAAACAAACTGGTTATTGTGGTAGGTCATTTGTTTGGCAGAAAGAAAA
CTCGAGAAATTTCTCTGGCCGTTATTCGTTATTCTCTCTTTTCTTTTTGGGTCTCTCCCTCTCT
GCACTAATGCTCTCTCACTCTGTCACACAGTAAACGGCATACTGCTCTCGTTGGTTCGAGAGAG
CGCGCCTCGAATGTTCGCGAAAAGAGCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCG
ACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC
GCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAATTAAAAGTAAC
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GCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACAC
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GAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGCT
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CGGCGAGAAGAAGAATGGCCTGTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAAC
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ACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAA
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CCCCTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAG
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CCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAA
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CATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGG
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GAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAG
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AGTACGGCCTTTGACCCGTGTGCAATCGCATGTGTCGCGCCAGCTTGTTCTGCGAAATAAACTA
ACGGGAATTCCTGCAGCCCGGGGGATCCGCGGCCGC

B. chiRNA expression vector pU6-BbsI-chiRNA

Dm snRNA:U6:96Ab promoter
BbsI sites for inserting guide sequence
crRNA repeat-derived sequence
tracrRNA
U6 terminator

GTTCGACTTGCAGCCTGAAATACGGCACGAGTAGGAAAAGCCGAGTCAAATGCCGAATGCAGAG
TCTCATTACAGCACAATCAACTCAAGAAAAACTCGACACTTTTTTACCATTTGCACTTAAATCC
TTTTTTATTCGTTATGTATACTTTTTTTGGTCCCTAACCAAAACAAAACCAAACTCTCTTAGTC
GTGCCTCTATATTTAAAACTATCAATTTATTATAGTCAATAAATCGAACTGTGTTTTCAACAAA
CGAACAATAGGACACTTTGATTCTAAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTAAGA
CCATTTGCCAATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGTATG
TTTTCCTCAATACTTCGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT

C. *yellow* ssODN

5' and 3' homology arms
PAM
Target sequence corresponding to the nt that remain after cleavage
attP

GTTGTCTTTAAATATTCTTTACATCAATCGAGTGTGTGAGAATATACCCAAGTACCGTGTCTAC
GCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACTACATCAGGTGGCTTATGCTG
TTCCCATAGATCGGCCAACTTTGCGTTTTGTCTTCCATGATT

Figure S2 Sequences of CRISPR RNA/Cas9 constructs

Annotated sequences of (A) codon-optimized Cas9 (Cong *et al.* 2013) under the control of the Drosophila *hsp70* promoter and 3' UTR. (B) chiRNA expression vector for generating site-specific, U6-driven chiRNAs (based on Cong et al., 2013), and (C) the ssODN donor used to replace *yellow* with attP.

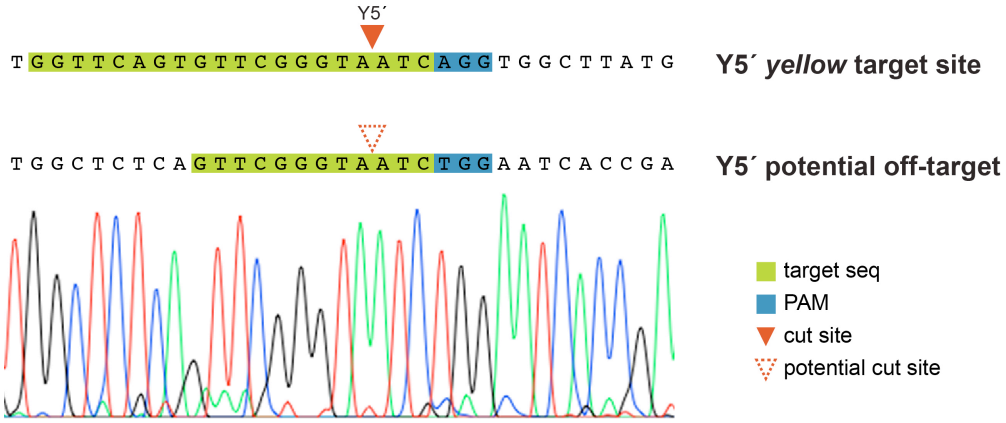

Figure S3 Off-target analysis of germline transformants

The potential off-target site of Y5′, based on the criteria established by Cong et al., 2013, is shown with a representative sequence trace indicating the presence of wild-type sequence at this site in germline transformants generated with the Y5′ chiRNA. Note that Y3′ and YE1 have no potential off-target sites that meet these criteria.

Table S1 chiRNA sequences used to target *yellow* and *rosy*

Name	Target Sequence + PAM	Strand	Cleavage site
YE1	GCGATATAGTTGGAGCCAGCTGG	Sense	+ 97 bp
Y5′	GGTTCAGTGTTCGGGTAATCAGG	Antisense	- 307 bp
Y3′	GGTTAACATAATCCTACACACGG	Sense	+ 4,336 bp
R1	GCACTTCACGATGTCTAACTCGG	Sense	+ 8 bp
R2	GATCCGCAACGTCGCCTGTTTGG	Sense	+ 1,843 bp

Sequences of the five different chiRNAs used to disrupt *yellow* and *rosy*. Target strand orientation is relative to the targeted gene. The site of the Cas9-generated DSB is indicated relative to the ATG translational start site of *yellow* or *rosy* (both genes have a single translational start site).

Supporting references

Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto *et al.*, 2013 Multiplex genome engineering using CRISPR/Cas systems. Science 339**:** 819-823.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna *et al.*, 2012 A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337**:** 816-821.

Wakiyama, M., T. Matsumoto and S. Yokoyama, 2005 Drosophila U6 promoter-driven short hairpin RNAs effectively induce RNA interference in Schneider 2 cells. Biochem Biophys Res Commun 331**:** 1163-1170.