Genome Engineering of *Drosophila* with the CRISPR RNA-Guided Cas9 Nuclease

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ABSTRACT We have adapted a bacterial CRISPR RNA/Cas9 system to precisely engineer the *Drosophila* genome and report that Cas9-mediated genomic modifications are efficiently transmitted through the germline. This RNA-guided Cas9 system can be rapidly programmed to generate targeted alleles for probing gene function in *Drosophila*.

GENETIC and molecular techniques to manipulate the genomes of model organisms are invaluable tools for understanding gene function. In *Drosophila*, chemical and insertional mutagenesis are powerful and widely utilized methods for disrupting gene function (St Johnston 2002; Venken and Bellen 2005). Imprecise excision of a transposable element inserted near a gene of interest can result in deletion of all or part of the locus. More recently, techniques that stimulate homologous recombination (HR) using an exogenous template have made precisely targeted genome modifications possible (Gloor *et al.* 1991; Banga and Boyd 1992; Nassif *et al.* 1994; Rong and Golic 2000; Gong and Golic 2003; Huang *et al.* 2009). The most widely used methods rely on double-strand breaks (DSBs) in the donor template to trigger HR, but these can be time consuming and labor intensive (Gao *et al.* 2008; Huang *et al.* 2009). Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been successfully employed in *Drosophila* to generate DSBs in genomic targets that trigger repair by either HR or error-prone nonhomologous end joining (NHEJ) (Bibikova *et al.* 2002; Liu *et al.* 2012). However, ZFNs and TALENs, which both comprise a nuclease joined to a site-specific DNA-binding domain, require the generation of a unique protein for each genomic manipulation (Wood *et al.* 2011). In the past few months, the simplified CRISPR RNA-guided Cas9 nuclease has shown broad potential for genome engineering in metazoans.

CRISPRs (clustered regularly interspaced short palindromic repeats) were first identified in *Escherichea coli* in 1987 and later shown to function as part of an adaptive immune system in bacteria and archaea (Ishino *et al.* 1987; Makarova *et al.* 2006; Barrangou *et al.* 2007). In type II CRISPR systems, a CRISPR RNA (crRNA), which contains sequence complementary to invading virus or plasmid DNA, and a *trans*-activating CRISPR RNA (tracrRNA) interact with a CRISPR-associated nuclease (Cas9) to direct sequence-specific cleavage of exogenous DNA. Recognizing the potential of harnessing this system for precise genome engineering in other organisms, Jinek and colleagues (Jinek *et al.* 2012) identified a minimal two-component system required for the site-specific cleavage of DNA—Cas9 and a chimeric RNA (chiRNA) comprising the crRNA and tracrRNA from *Streptococcus pyogenes*. Thus, in contrast to ZFNs and TALENs, this modified CRISPR RNA/Cas9 system directs a common nuclease to specific DNA sequences by a short, readily generated RNA. This obviates the need to create a unique chimeric nuclease for each genome manipulation and raises the possibility and promise of routine genome engineering.

In recent months, a number of laboratories have demonstrated that this heterologously expressed two-component CRISPR RNA/Cas9 system can induce site-specific DSBs in

doi: 10.1534/genetics.113.152710
Manuscript received May 9, 2013; accepted for publication May 23, 2013
Available freely online through the author-supported open access option.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.152710/-/DC1
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eukaryotic genomes, including cultured mammalian cells, human stem cells, and yeast (Cho *et al.* 2013; Cong *et al.* 2013; Dicarlo *et al.* 2013; Ding *et al.* 2013; Jinek *et al.* 2013; Mali *et al.* 2013). *In vivo* experiments in zebrafish and mice yielded mosaic animals, demonstrating that the system has the capacity to manipulate a variety of eukaryotic genomes (Chang *et al.* 2013; Hwang *et al.* 2013; Shen *et al.* 2013). Most recently, mice carrying homozygous mutations in two genes were generated through embryo injections (Wang *et al.* 2013). Nonetheless, to date the CRISPR RNA/Cas9 system has not been adapted for *Drosophila*, a key genetic model, and germline transmission of Cas9-induced changes has not been achieved in any organism. Here we demonstrate that the CRISPR RNA/Cas9 system can (1) mediate efficient genome engineering in *Drosophila* and (2) induce targeted genome modifications in the *Drosophila* germline that are transmitted to progeny.

Results

Site-directed cleavage of Drosophila genes

To test whether the CRISPR RNA/Cas9 system could induce site-specific DSBs in *Drosophila*, we targeted the *yellow* gene, which is on the X chromosome and commonly used for genome engineering studies (Rong and Golic 2000; Bibikova *et al.* 2002; Liu *et al.* 2012). Efficient target recognition by the CRISPR RNA/Cas9 system requires 20 nucleotides (nt) of homology between the chiRNA and its genomic target. Cleavage also requires that the 3′ end of the genomic target sequence contains a 3-basepair (bp) proto-spacer adjacent motif (PAM) sequence, NGG, which differentiates self from invading DNA in the endogenous system (Jinek *et al.* 2012). Thus, selection of a 20-nt target sequence is limited only by the requirement for an adjacent PAM sequence. In our plasmid-based system, the ideal target sequence also begins with a G that allows for precise transcriptional initiation of the chiRNA from the U6 promoter (Wakiyama *et al.* 2005, Materials and Methods).

Plasmids encoding Cas9 and a chiRNA targeting the first exon of *yellow* (YE1), disruption of which is expected to abolish *yellow* function, were co-injected into preblastoderm embryos (Figures 1A and Supporting Information, Figure S1, Figure S2, and Table S1. To assess Cas9-mediated cleavage, we isolated DNA from individual embryos 24-hr post-injection. Following PCR amplification of the targeted genomic site, we used the SURVEYOR mutation detection assay, which employs an endonuclease that recognizes and cuts at the site of mismatches, to detect small insertions or deletions (indels) that result when DSBs are imperfectly repaired by NHEJ. DSBs that are repaired by HR using either the sister chromatid or homologous chromosome as a template do not result in an altered locus and are not detectable. The SURVEYOR assay indicated site-specific indels in one of 13 embryos injected with Cas9 and the YE1 chiRNA (Figure 1B). Consistent with the loss of *yellow* in a subset of somatic cells, we observed mosaic yellow patches in adult male cuticles after YE1 injection (6%, $n = 67$). Based on the size of the cleavage products produced in the SURVEYOR assay, the induced cleavage occurred at the YE1 target site indicating that the chiRNA successfully directed Cas9 to the *yellow* locus where it induced DSBs in the targeted DNA.

To determine if co-injection of Cas9 and a chiRNA could generate indels at other loci, we designed two chiRNAs to target the *rosy* gene (Figure 1C, R1 and R2). Again, the SURVEYOR assay identified Cas9-induced site-specific cleavage at the targeted sites in a subset of embryos (Figure 1D, 3/4 embryos for each chiRNA). Thus, the RNA-guided Cas9 system can be readily programmed to induce DSBs in multiple *Drosophila* genomic targets.

Engineering defined deletions

Encouraged that the CRISPR RNA/Cas9 system functioned in *Drosophila*, we next asked whether Cas9-mediated DSBs could be harnessed to generate defined deletions. We reasoned that introducing two chiRNAs, Y5′ targeting the 5′ end of *yellow* and Y3′ targeting the 3′ end, might guide Cas9 to precisely delete the 4.6-kilobase (kb) *yellow* locus (Figure 1A). We isolated DNA from embryos 24 hr after co-injection of Cas9, Y5′ chiRNA, and Y3′ chiRNA and performed PCR to determine whether *yellow* was deleted. Using primers flanking the targeted sites (Figure 1A, open arrows), a 650-bp product was amplified in 13/16 embryos, as expected if a targeted deletion occurred. (Figure 1E). Moreover, as adults, 66% of injected males ($n = 52$) displayed yellow mosaicism, consistent with a high level of targeted deletion in somatic cells.

To investigate individual repair events, the DNA amplified from the targeted locus was pooled, subcloned, and sequenced. In nine of nine clones analyzed, the 4.6-kb *yellow* locus was deleted and the junction created by repair of the two cut sites showed the small indels typical of NHEJ (Figure 1F). These results extend findings from mammalian cell culture in which 19- and 118-bp sequences were deleted by cotransfection of Cas9 and two chiRNAs (Cong *et al.* 2013; Mali *et al.* 2013) and demonstrate that such "multiplex" genome editing is an effective tool for precisely deleting large genomic regions in organisms.

Cas9-mediated homologous recombination

The ability to edit genomic sequence by introducing specific mutations or exogenous sequences to monitor or manipulate protein activity provides a critical tool for dissecting gene function. One means of achieving this goal is to replace a gene with a sequence that enables site-specific integration of engineered genes into the targeted locus, such as the attP ΦC31 phage recombination site. Such gene replacement can be accomplished by stimulating HR in the presence of a donor template that includes the site-specific integration sequence (Gao *et al.* 2008; Huang *et al.* 2009). To this end, we designed a single-strand oligodeoxynucleotide (ssODN) repair template that includes a 50-nt attP site flanked by

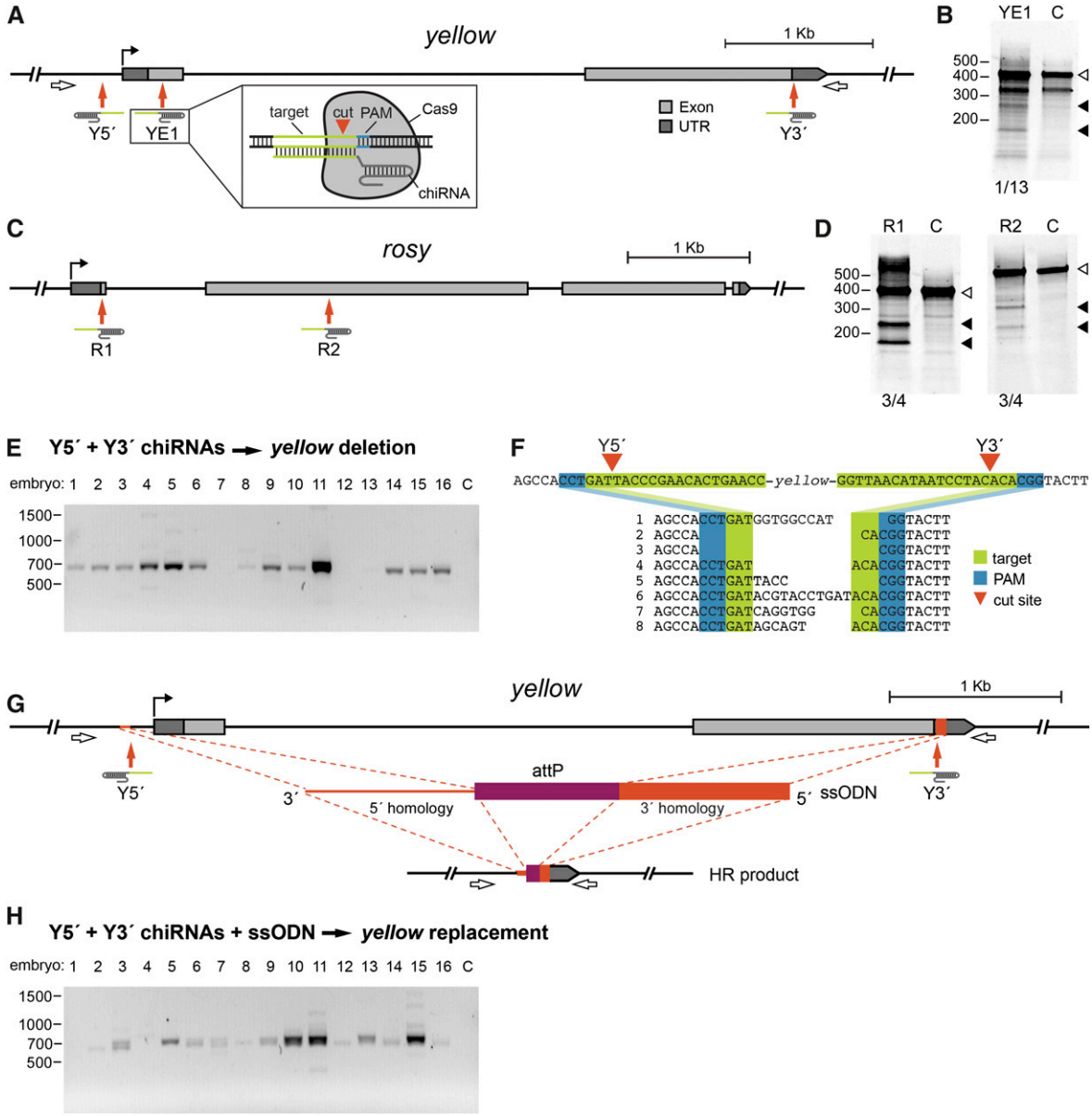

Figure 1 Cas9-generated DSBs are repaired by NHEJ and HR in *Drosophila* embryos. (A) Schematic of the *yellow* locus indicating YE1, Y5′, and Y3′ chiRNA target sites. The predicted cut site for each chiRNA is indicated (red arrows). Inset shows schematic of CRISPR RNA/Cas9 system components interacting with target DNA. For additional detail, see Figure S1A. (B) Results of the SURVEYOR assay indicate that targeted cleavage occurred in *yellow* at the predicted YE1 cut site (left, embryos injected with YE1 chiRNA; right, control, C, embryos). Duplexes lacking indels were uncut (open arrowhead), whereas duplexes containing indels were cut asymmetrically at the targeted site (solid arrowheads). One of 13 embryos injected with YE1 chiRNA produced duplexes with indels. (C) Schematic of the *rosy* locus indicating R1 and R2 chiRNA target sites (red arrows). (D) Results from SURVEYOR assays indicate R1 and R2 chiRNAs generated indels at the targeted cut sites (embryos injected with R1 chiRNA, R2 chiRNA, or control, C, are as indicated). Uncut duplexes (open arrowhead) and cut duplexes (solid arrowhead) are indicated. Three of four embryos injected with either R1 chiRNA or R2 chiRNA produced duplexes with indels. (E) The *yellow* locus was amplified from embryos injected with Cas9, Y5′ chiRNA, and Y3′ chiRNA using the primers indicated in A (open arrows). Targeted deletions result in a 650-bp PCR product (C, uninjected control embryo). (F) Products from nine PCRs were pooled for sequencing. Sequence alignments reveal breakpoints and imprecise repair between predicted cut sites (red arrowheads). One repair event that resulted in the 4.6-kb deletion of *yellow* plus an indel deleting an additional 27 bp and inserting 93 bp is not shown. (G) Schematic of the *yellow* ssODN design. The ssODN includes 60-nt homology arms (red) flanking a 50-nt attP docking site (purple). The homology arms correspond to the sequences immediately adjacent to the predicted cut sites (red arrows). For additional detail see Figure S1C. (H) The *yellow* locus was amplified from embryos injected with Cas9, Y5′ chiRNA, Y3′ chiRNA, and ssODN using the primers indicated in A. HR events in which the locus is deleted and replaced with attP will yield a 700-bp product. Products from three reactions were pooled for sequence analysis, which confirmed the replacement of *yellow* with attP in five of eight clones.

Table 1 Germline transmission rates

		Injected males		Injected females			
chiRNA(s)	ssODN donor	% (no.) founders	% (no.) progeny	% (no.) founders	% (no.) progeny	% (no.) founders yielding targeted event	% (no.) overall germline transmission
YE1	—	6.4 (3/47)	0.23 (5/2128)	4.8 (1/21)	0.26 (4/1525)	100 (4/4)	5.9 (4/68)
Y5′, Y3′	—	25 (13/52)	1.3 (61/4608)	14.3 (5/35)	1.5 (35/2421)	5.6 (1/18)	1.1 (1/87)
Y5′, Y3′	+	8.6 (3/35)	1.0 (24/2336)	23.1 (6/26)	1.3 (34/2655)	22.2 (2/9)	3.3 (2/61)

Flies injected with Cas9 and the indicated chiRNAs and ssODN template were crossed to *yellow* (y^1) and progeny screened for yellow cuticles. The percentage of injected flies producing one or more yellow progeny (founders) is indicated along with the percentage of total progeny exhibiting yellow cuticle. At least one progeny per founder was sequenced to determine if the targeted event had occurred. The percentage of founders in which the targeted event occurred in one or more progeny is reported, as is the overall germline transmission rate (percentage injected flies yielding expected event).

60-nt homology arms corresponding to sequences 5′ and 3′ of *yellow* (Figure 1G and Figure S1C). We co-injected Cas9 and the Y5′ and Y3′ chiRNAs with this ssODN, which we predicted would serve as a template for repair of the deleted locus by HR resulting in the replacement of *yellow* with attP. Using the same primers as above (Figure 1A, open arrows), we performed PCR on DNA extracted from single injected embryos. In 13/16 embryos, we detected the 700-bp product that is expected to result from replacement of *yellow* with attP (Figure 1H). In agreement with highly efficient disruption of *yellow*, 62% of injected males exhibited a mosaic cuticle as adults ($n = 35$).

To confirm that the ssODN donor served as a template for repair by HR, the PCR products amplified from injected embryos were pooled and subcloned. Sequence analysis of individual repair events revealed that the *yellow* gene was replaced by attP from the ssODN template in five of eight clones. Two clones contained the 4.6-kb deletion but lacked the attP sequence, indicating site-specific induction of DSBs repaired by NHEJ rather than HR. This mirrors our PCR results, in which doublets of ~700 and 650 bp were detected, likely representing the inclusion or lack of the 50-bp attP sequence (Figure 1H, lanes 3, 10 and 11). The higher-molecular-weight bands faintly visible in some lanes may reflect aberrant repair events (Figure 1H, lanes 11 and 15). We observed such an event in one of eight clones. In this clone, the 4.6-kb *yellow* locus was deleted and part of the attP sequence was incorporated along with 40 bp of novel sequence. This unusual event may reflect repair via a synthesis-dependent strand annealing (SDSA) mechanism in which the two broken DNA strands independently select repair templates (Nassif *et al.* 1994). Previous studies of HR induced by DSBs in genomic targets have also identified SDSA as a primary mechanism of repair and reported similar events (Nassif *et al.* 1994; Bozas *et al.* 2009).

Germline transmission of Cas9-induced genome modifications

Our results demonstrate that the readily programmed CRISPR RNA/Cas9 system can be employed to precisely mutate, delete, or replace genes *in vivo*, so we next sought to determine whether Cas9-induced mutations could be transmitted through the germline to create stable mutant strains. We crossed adult flies injected as embryos with Cas9 and YE1 chiRNA to flies carrying a null mutation in *yellow* (y^1), allowing us to phenotypically screen female progeny of injected males and all progeny of injected females. A total of 6.4% of injected males and 4.8% of injected females yielded at least one offspring exhibiting the *yellow* null phenotype (Table 1, injected flies that yield yellow offspring are referred to as "founders"). To determine if yellow progeny resulted from Cas9-induced mutations, the target locus was amplified and sequenced from at least one yellow progeny per founder. Indels were present at the predicted Cas9 cut site in each case (Figure 2A). Interestingly, we recovered a 4-bp deletion in the progeny of multiple founders. This is likely due to preferential repair by microhomology-mediated end joining (MMEJ) facilitated by the presence of a short repeat adjacent to the YE1 cut site (Figure 2A, underlined in wild-type sequence) (McVey and Lee 2008). Thus, the rate of mutations induced by Cas9 and a single chiRNA is comparable to that obtained with a pair of ZFNs targeting the *yellow* locus (6% transmission for males and 0% for females) and lower than that reported for two TALENs (17% for males and 45% for females) (Bibikova *et al.* 2002; Liu *et al.* 2012).

We also investigated whether multiplex-mediated genome editing was transmitted through the germline. We first analyzed the progeny of flies that had been injected with Cas9, Y5′ chiRNA, and Y3′ chiRNA to precisely delete *yellow*. A total of 25% of injected males and 14.3% of injected females produced yellow progeny (Table 1, founders). PCR and sequence analysis of mutant progeny identified the precise deletion of *yellow* in the progeny of one founder (Figure 2B and Table 1). Partial deletions of *yellow*, up to ~3.7 kb in size, were also detected by PCR and sequence analysis, consistent with imperfect repair of Cas9-induced deletions (Figure 2B).

We next analyzed flies that had been injected with Cas9, Y5′ chiRNA, Y3′ chiRNA, and the ssODN donor template to determine if the replacement of *yellow* with attP was also transmitted to progeny. A total of 8.6% of injected males and 23.1% of injected females produced one or more yellow offspring (Table 1, founders). Sequence analysis showed that two (22%) of the founders transmitted the precisely engineered replacement of *yellow* with attP (Figure 2C and Table 1). By comparison, depending on the specific donor, 1–19% of mutant progeny of flies injected with ZFNs and

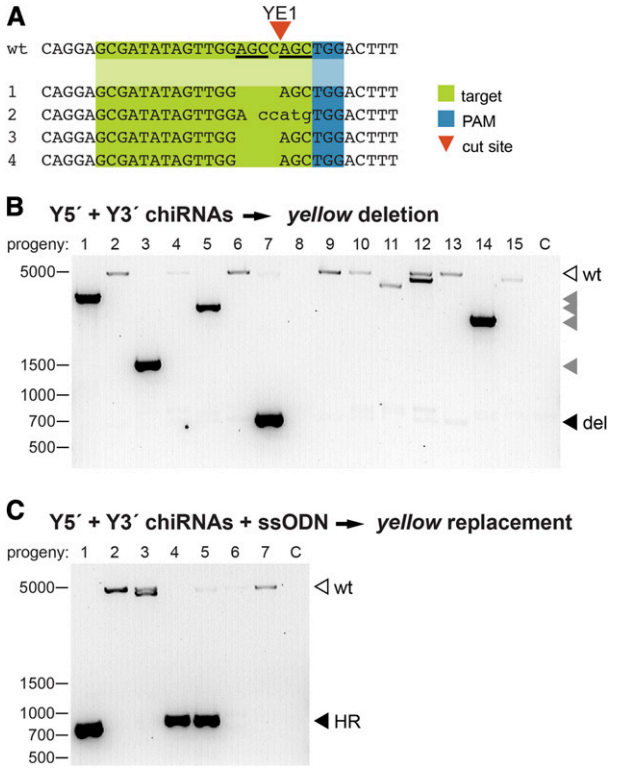

Figure 2 CRISPR-induced modifications are efficiently transmitted through the germline. (A) Representative sequences of independent *yellow* mutants generated by Y1E-guided Cas9-induced DSBs. The predicted YE1 cleavage site is indicated with a red arrowhead. Short sequence repeats flanking the cleavage site (underlined in the wild-type sequence) may have facilitated the preferential generation of the same 4-bp deletion in three independent founders by MMEJ. (B) The *yellow* locus was amplified from yellow progeny of males and females injected with Cas9, Y5′ chiRNA, and Y3′ chiRNA using the primers indicated in Figure 1A. Fifteen independent progeny are represented (C, no template control). A 650-bp PCR product is expected (solid arrowhead) if a targeted deletion of *yellow* has been transmitted. PCR products corresponding to partial deletions (shaded arrowheads), and wild-type (open arrowhead) *yellow* are indicated. (C) The *yellow* locus was amplified from yellow progeny of flies injected with Cas9, Y5′ chiRNA, Y3′ chiRNA, and ssODN using the primers indicated in Figure 1A. Seven independent progeny are represented (C, no template control). A 700-bp product is expected following the targeted replacement of *yellow* with attP. PCR products corresponding to correct HR events are indicated (solid arrowheads) and were confirmed by Sanger sequencing.

ssODN donor templates designed to introduce stop mutations in a *rosy* exon reflected HR-mediated repair (Beumer *et al.* 2013). These data demonstrate that Cas9-mediated HR events are effectively transmitted through the germline.

A common concern with sequence-guided nucleases is the potential for off-target cleavage. Cong *et al.* (2013) demonstrated that cleavage by Cas9 requires a PAM and a perfect match of at least 12 nt at the 3′ end of the targeting sequence, while single mismatches in the remaining 8 nt of targeting sequence are tolerated. Given the high frequency at which PAM sites occur in the *Drosophila* genome, we were able to readily select targets with minimal potential for off-target cleavage. Of the three chiRNAs used to generate germline transformants, only the Y5′ chiRNA has a 12-nt match to sequence adjacent to a PAM elsewhere in the genome. PCR and sequence analysis of this site in flies carrying Y5′-mediated deletion or replacement of *yellow* demonstrated the presence of wild-type sequence, consistent with the absence of off-target cleavage (Figure S3). Moreover, engineered flies developed into viable and fertile adults. The lack of detectable off-target effects is in accordance with findings in other systems and suggests high specificity of targeting by the CRISPR RNA/Cas9 system (Wang *et al.* 2013).

In summary, overall germline transmission rates, defined as the percentage of injected flies that yielded at least one offspring carrying the targeted event, ranged from 1.1% for multiplex-mediated deletion of *yellow* to 5.9% for single chiRNA-induced NHEJ. Perfect replacement of *yellow* with attP was transmitted through the germline of 3.3% of injected flies (2/61). Importantly, transformed flies were healthy and fertile and showed no evidence of off-target cleavage.

Discussion

Our results demonstrate the efficient generation of genome modifications via the CRISPR RNA/Cas9 system in *Drosophila* and for the first time show germline transmission of Cas9-mediated modifications. A single chiRNA can guide Cas9 to a specific genomic sequence to induce DSBs that are imperfectly repaired by NHEJ, while multiplex targeting can be used to generate large defined deletions. By combining multiplex targeting of *yellow* with the introduction of an ssODN donor template containing exogenous sequence, we successfully replaced *yellow* with an attP docking site. Interestingly, while germline transmission rates for each of these manipulations were similar, injection of two chi-RNAs (with or without the ssODN donor) yielded ~10 times more mosaic males than a single chiRNA. This may be due to the fact that multiplex injections provide multiple routes for mutating the target locus, including single DSBs induced by either chiRNA and repaired by NHEJ. The lower percentage of founders yielding the precise targeted event in the multiplex injection paradigms supports this interpretation. Our results also suggest that the frequency of events in somatic tissue does not directly correlate with the frequency of events in the germline, consistent with previous observations with ZFNs and TALENs in *Drosophila* (Bibikova *et al.* 2002; Liu *et al.* 2012).

It is likely that the CRISPR RNA/Cas9 system can be further optimized. Studies in vertebrates suggest that expression levels of CRISPR RNAs are a key determinant of efficiency (Jinek *et al.* 2013). Transgenic lines expressing Cas9 and tracrRNA under the control of endogenous germline-specific promoters might yield increased efficiency. Moreover, it may be possible to bias repair to HR over NHEJ by exploiting a Cas9 mutant that induces single-strand DNA breaks (Jinek *et al.* 2012; Cong *et al.* 2013; Mali *et al.* 2013) or conducting Cas9-mediated genome engineering in a genetic

background that reduces NHEJ (Beumer *et al.* 2008). Because the components of the CRISPR RNA/Cas9 system can be injected into embryos of any genotype, it should also be possible to use a marked transposable element residing in the targeted locus to identify defined deletions or replacements by loss of the positive marker, obviating the need for molecular screening when screening by mutant phenotype is not possible. Finally, combining the CRISPR RNA/Cas9 system with dsDNA donors containing larger homology regions may also increase efficiency (Nassif *et al.* 1994; Beumer *et al.* 2013). While the generation of such templates is more labor intensive, the ability to incorporate a selectable marker would add to the utility of the CRISPR RNA/Cas9 system for some applications.

The ease of producing sequence-specific chiRNAs makes the CRISPR RNA/Cas9 system an appealing method for genome editing. From the initial cloning steps to identifying transformants, stable lines with targeted genome alterations can be generated within a month. Our demonstration that the CRISPR RNA/Cas9 system can be used to create and transmit precise genomic modifications in *Drosophila* opens the door to rapid engineering of the genome to investigate gene function and regulation.

Acknowledgments

We thank the Bloomington Drosophila Stock Center for fly stocks and Michael Cox, Carlos Flores, Judith Kimble, and Andrew Mehle for invaluable discussions and critical comments on the manuscript. We are grateful to Feng Zhang for sharing reagents and an unpublished manuscript with us, Kevin Eliceiri for sharing injection equipment, and Kathy Vaccaro for sharing her expertise on embryo injections. This work was funded by startup funds from the University of Wisconsin to M.M.H., J.W., and K.O.C.G. and grants from the National Institute of Neurological Disorders and Stroke, National Institutes of Health to J.W. (R00 NS072252) and K.O.C.G. (R00 NS060985). All plasmids used in this study will be made available to academic laboratories through the nonprofit distributor Addgene along with detailed guidance at http://FlyCRISPR.molbio.wisc.edu.

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Communicating editor: J. Sekelsky

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.152710/-/DC1

Genome Engineering of *Drosophila* with the CRISPR RNA-Guided Cas9 Nuclease

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DOI: 10.1534/genetics.113.152710

File S1

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.

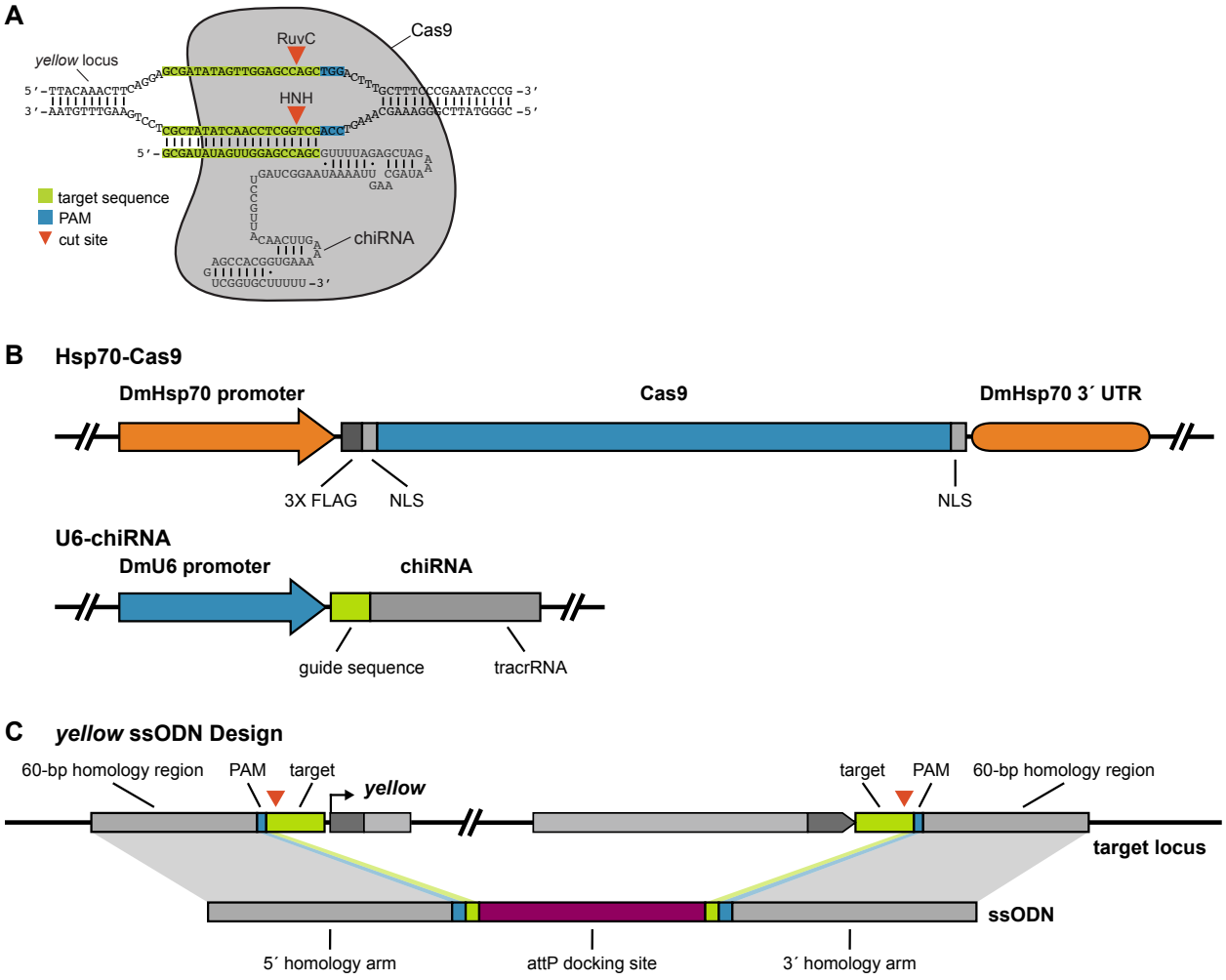

Figure S1 CRISPR RNA/Cas9 molecular reagents used in this study

(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
CAGCAACCAAGTAAATCAACTGCAACTACTGAAATCTGCCAAGAAGTAATTATTGAATACAAGA
AGAGAACTCTGGGGGATCTGATCGATATGGACTATAAGGACCACGACGGAGACTACAAGGATCA
TGATATTGATTACAAAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATC
CACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGG
GCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACAC
CGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCC
GAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGCT
ATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGA
AGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGTG
GACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAACTGGTGGACA
GCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGATCAAGTTCCGGGG
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CTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCA
AGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCC
CGGCGAGAAGAAGAATGGCCTGTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAAC
TTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGGACACCTACGACG
ACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAA
GAACCTGTCCGACGCCATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCC
CCCCTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAG
CTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGG
CTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATC
CTGGAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGA
AGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCAT
TCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATC
CTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGA
TGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAAGTGGTGGACAAGGGCGC
TTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTG
CTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAAT
ACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTGGA
CCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAA
ATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCA
CATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGA
CATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGG
CTGAAAACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACA
CCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGAC
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GGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTGATCGAAATG
GCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCG
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GCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAA
CTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTTCTGAAGG
ACGACTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGACAACGT
GCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGAACGCCAAGCTG
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CGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCT
TCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTG
GGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCC
AAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATCA
TGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGT
GAAAAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGCTGGAAAACGGCCGGAAG
AGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATG
TGAACTTCCTGTACCTGGCCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCA
GAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAG
TTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGC
ACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCT
GGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACC
AAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCG
ACCTGTCTCAGCTGGGAGGCGACAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAA
GAAAAAGTAATCTAGAGATCTTCCATACCTACCAGTTCTGCGCCTGCAGCAATGGCAACAACGT
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CCGAACGGATTTTCGTAGACCCTTTCGATCTCATAATGGCTCATTTTATTGCGATGGACGGTCA
GGAGAGCTCCACTTTTGAATTTCTGTTCGCAGACACCGCATTTGTAGCACATAGCCGGGACATC
CGGTTTGGGGAGATTTTCCAGTCTCTGTTGCAATTGGTTTTCGGGAATGCGTTGCAGGCGCATA
CGCTCTATATCCTCCGAACGGCGCTGGTTGACCCTAGCATTTACATAAGGATCAGCAGCAAAAT
TTGCCTCTACTTCATTGCCCGGAATCACAGCAATCAGATGTCCCTTTCGGTTACGATGGATATT
CAGGTGCGAACCGCACACAAAGCTCTCGCCGCACACTCCACACTGATATGGTCGCTCGCCCGTG
TGGCGCCGCATATGGATCTTAAGGTCGTTGGACTGCACAAAGCTCTTGCTGCACATTTTGCAGG
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.

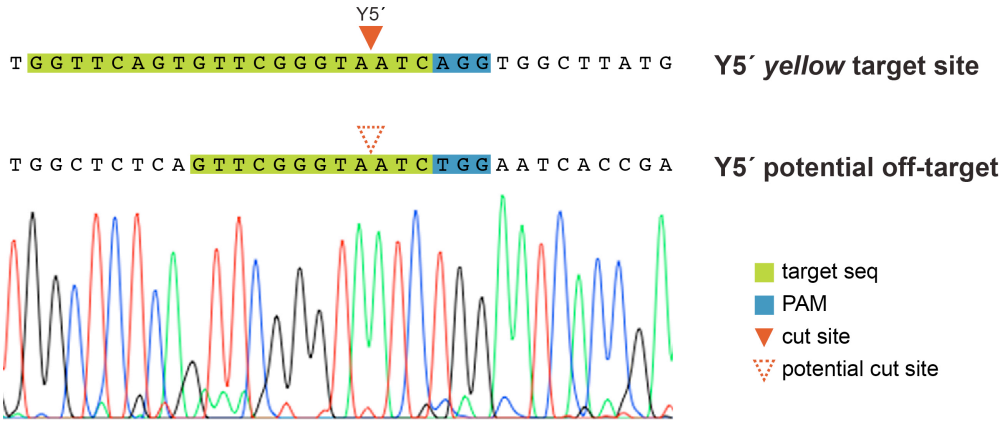

Figure S3 Off-target analysis of germline transformants

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

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