

## 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 Clal/XbaI fragment between the *Drosophila hsp70* promoter and 3' UTR in pHSS6hslMi20 (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*<sup>1118</sup> preblastoderm embryos were injected through the chorion membrane using standard protocols. phsp70-Cas9 was injected at a concentration of 500 ng/ $\mu$ L. The pU6-chiRNA targeting constructs were injected at 500 ng/ $\mu$ L for single chiRNAs and 250 ng/ $\mu$ L each when two chiRNAs were injected. The donor template ssODN was diluted based on manufacturer's concentrations and injected at 100 ng/ $\mu$ L. 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  $\mu$ L. 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  $\mu$ L of each sample (400 ng annealed duplexes) was mixed with 2  $\mu$ L 0.15 M MgCl<sub>2</sub>, 1  $\mu$ L SURVEYOR Enhancer S, 1  $\mu$ L SURVEYOR Nuclease S and incubated at 42°C for 60 min. The SURVEYOR reaction was stopped with 2  $\mu$ L 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.