

A simplified and efficient germline-specific CRISPR/Cas9 system for *Drosophila* genomic engineering

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Abbreviations: CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated; gRNA, guide RNA; crRNA, CRISPR repeat RNA; tracrRNA, trans-activating CRISPR RNA; ZFN, zinc finger nuclease; TALENs, transcription activator-like effector nucleases; PAM, protospacer adjacent motif; NHEJ, non-homologous end joining

The type II CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/CRISPR-associated) has recently emerged as an efficient and simple tool for site-specific engineering of eukaryotic genomes. To improve its applications in *Drosophila* genome engineering, we simplified the standard 2-component CRISPR/Cas9 system by generating a stable transgenic fly line expressing the Cas9 endonuclease in the germline (Vasa-Cas9 line). By injecting vectors expressing engineered target-specific guide RNAs into Vasa-Cas9 fly embryos, mutations were generated from site-specific DNA cleavages and efficiently transmitted into progenies. Because Cas9 endonuclease is the universal component of the type II CRISPR/Cas9 system, site-specific genomic engineering based on this improved platform can be achieved with lower complexity and toxicity, greater consistency, and excellent versatility.

Introduction

Site-specific endonucleases are recent additions to the geneticist's toolbox that have shown to be powerful and promising, enabling sequence-specific targeted mutagenesis of any given locus within the genome.¹ ZFNs (Zinc Finger Nucleases) and TALENs (Transcription Activator-Like Effector Nucleases) are composed of programmable, sequence-specific DNA-binding modules linked to a nonspecific endonuclease domain for DNA cleavage.^{2,3} CRISPR/Cas9 systems, which are exemplified by the best studied type II system from *Streptococcus pyogenes*, employ a short RNA mediated target recognition mechanism using the crRNA (CRISPR repeat RNA):tracrRNA (trans-activating CRISPR RNA) loaded Cas9 endonuclease to achieve sequence-specific DNA cleavage.^{4,5} Target site recognition of the type II CRISPR/Cas9 system relies solely on the Watson–Crick base pairing between a short stretch of crRNA (spacer) and one strand of target DNA (protospacer), which is immediately followed by a “NGG” tri-nucleotide protospacer adjacent motif (PAM) on the opposite strand (Fig. 1A). The type II CRISPR/Cas9 system was engineered to a simplified 2-component system by using a single hybrid hairpin guide RNA (gRNA) mimicking the crRNA:tracrRNA complex to load Cas9 for sequence-specific

DNA cleavage.^{4,6,7} Using these engineered gRNAs circumvents the requirement for small RNA processing without compromising targeting efficiency and specificity. Without the need to design and construct new targeting proteins as in the case of ZFNs and TALENs, the simplicity and ease to make short gRNAs approximately 100 nucleotides long capable of targeting virtually any position in a gene makes the CRISPR/Cas9 system an attractive tool for genomic engineering applications.^{6,7} Indeed, during the past several months we have witnessed the sudden explosion of successful applications of the CRISPR/Cas9 system in almost all eukaryotic models, including efficient multiplexed genomic modifications in mice and human cells.⁶⁻⁹

Such booms of “CRISPR CRAZE” include 3 recently published reports demonstrating successful applications of the 2-component CRISPR/Cas9 system in *Drosophila*,¹⁰⁻¹² suggesting it to be a new Swiss army knife available to fly geneticists for routine and rapid modifications of their favorite genes. Despite its great promise and advantages, the CRISPR/Cas9 system still needs substantial optimization before it can be considered reliable enough for robust routine genome engineering applications. Recent reports showing significant off-target effects of gRNA-loaded Cas9¹³⁻¹⁷ warrant precaution and further improvement of targeting fidelity. In addition, reported targeting efficiency varies greatly, and often

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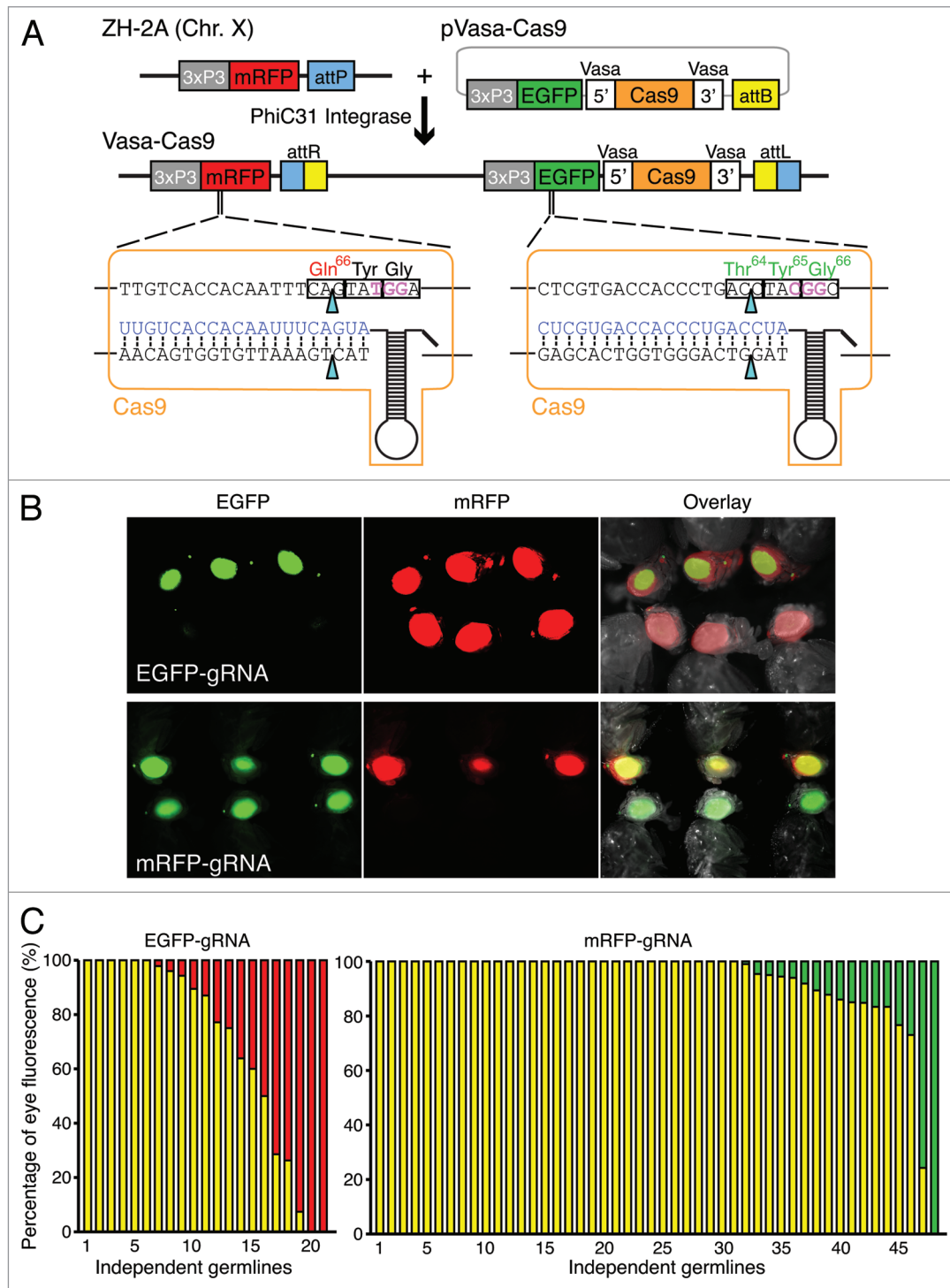


Figure 1. For figure legend, see page 3.

the germline transmission rate of acquired mutation is low.¹⁰⁻¹² For example, 3 recent reports of the CRISPR/Cas9 system targeting the *Drosophila yellow* gene have an apparent 10-fold difference in efficiency.¹⁰⁻¹² Such discrepancies might arise from different methods to introduce gRNAs and Cas9 into fly embryos, varying choices of gRNAs against different positions in the *yellow* gene, and variability unavoidable from independent injection practices.

To systematically improve the performance of the CRISPR/Cas9 targeting system, it would be best to reduce system complexity and further standardize and control the introduced variables. Another significant drawback associated with co-injection of gRNAs and Cas9 into embryos is exemplified by the alarmingly high lethality rates (up to ~90%) necessary to achieve high mutagenesis rates.¹¹ Because it is known that off-target cleavages by gRNA-loaded Cas9

Figure 1 (see previous page). Disrupting EGFP and mRFP genes using a germline-specific CRISPR/Cas9 system. **(A)** Scheme for generating Vasa-Cas9 transgenic flies and designing EGFP and mRFP gRNAs. The germline-specific Cas9 expressing vector, pVasa-Cas9, was integrated into the ZH-2A site on chromosome X mediated by the phiC31 integrase/attP/attB integration system. The resulting Vasa-Cas9 strain contains the germline-specific Vasa-driven Cas9 sequence and the eye promoter 3xP3-driven EGFP and mRFP sequences. Two gRNAs were designed to target mRFP and EGFP, respectively. Blue letters indicate the gRNA spacer sequences. The 3' common regions of the gRNAs are represented with identical hairpin structures. Bold magenta labels the PAM motifs of the DNA substrates. Cyan triangles indicate the Cas9 cut sites. Key chromogenic residues are labeled red and green respectively. **(B)** EGFP and mRFP expression in G1 adult eyes. Representative images of G1 adult eyes from Vasa-Cas9 G0 flies injected with EGFP (top panels) or mRFP (bottom panels) gRNA-expressing vectors. Six flies are shown in each condition: those that retain fluorescent signals in top rows, and those showing a loss of fluorescent signals in bottom rows. EGFP signals are shown on the left, mRFP in the middle, and their overlays with bright-field signals on the right. **(C)** Scoring of EGFP and mRFP mutation germline transmission rates. EGFP (left panel) and mRFP (right panel) gRNA-induced mutation germline transmission rates are sorted from lowest (100% wild type) to highest (100% mutant). Each bar represents G1 scoring of an individual cross from a single injected G0 Vasa-Cas9 fly. Twenty-one G0 crosses (total 465 G1 flies) were scored for EGFP and 48 G0 crosses (total 1418 G1 flies) were scored for mRFP gRNA-induced G1 mutations. Yellow portions indicate the percentage of G1 flies expressing both EGFP and mRFP (wild type), red portions indicate mRFP only expression (EGFP mutant), and green portions indicate EGFP only expression (mRFP mutant). Fifteen out of 21 (71%) G0 EGFP-gRNA vector injected adults and 17 out of 48 (35%) G0 mRFP-gRNA vector injected adults harbor transmittable G1 germline mutations. Two EGFP- and 1 mRFP-gRNA vector injected G0 adults produced 100 percent G1 mutant progenies.

can cause chromosomal abnormalities,¹³⁻¹⁷ it is reasonable to postulate that widespread cytotoxicity might be causing the difficulty of obtaining viable and fertile animals that pass on the intended mutations. This inconvenience also demands injection of a significant number of embryos in order to generate flies with germline incorporation of mutations.

To improve the CRISPR/Cas9 system for more robust and widespread *Drosophila* genome engineering applications, we aspired to reduce the complexity of the current 2-component injection scheme to 1 component. To achieve this goal, we generated a transgenic fly line expressing the Cas9 endonuclease exclusively in germline cells (Vasa-Cas9 line) (Fig. 1A). The unloaded Cas9 is known to exhibit no DNA cleavage activity^{4,6,7} and is universally required for any CRISPR/Cas9 mediated-genomic engineering application. The germline-specific expression of Cas9 aims to improve the rate of germline transmission of CRISPR/Cas9 generated mutations and limit widespread toxicity introduced to the embryo by nonspecific Cas9 endonuclease activity. To further reduce the variability caused by potential degradation of injected gRNAs, we injected DNA vectors expressing gRNA sequences under the control of a RNA Polymerase III-dependent U6 promoter using standard embryonic germline injection. Here we demonstrate consistent and efficient targeting of 2 independent genes using this simple and streamlined CRISPR/Cas9 genome editing system.

Results

Inspired by the widely used germline-specific Vasa-phiC31 integrase and nanos-phiC31 integrase transgenic strains with highly efficient site-specific transgenesis,¹⁸ we made an equivalent construct by replacing the phiC31 integrase open reading frame (ORF) in the Vasa-phiC31int vector with the ORF of a human codon-optimized Cas9 gene.⁷ The resulting construct pVasa-Cas9 retains the original attB site and was integrated into the X chromosome bearing attP site (Zh-2A) via phiC31 integrase-mediated transgenesis (Fig. 1A). The eyes of transgenic Vasa-Cas9 flies are dual-marked by EGFP and mRFP proteins under 3xP3 promoter control (Fig. 1A). Vasa-Cas9 flies are healthy, fully fertile, and do not show any morphological abnormalities (data not shown), consistent with the inactive nuclease activity of Cas9 without loading

gRNAs. This transgenic line was maintained and expanded under standard laboratory conditions for several months without any difficulty. We expect that this strain can be easily adopted by any fly lab for routine use.

To test the efficiency and specificity of this germline CRISPR/Cas9 gene editing platform, we identified 1 protospacer site in EGFP and 1 in mRFP ORFs. The main criterion for the protospacer site selection is its proximity to the critical residues forming the corresponding fluorophores (Gln⁶⁶ for mRFP and Thr⁶⁴-Tyr⁶⁵-Gly⁶⁶ for EGFP). The predicted cutting sites are within the codon of Gln⁶⁶ in mRFP and Thr⁶⁴ in EGFP (Fig. 1A, cyan triangles). We expected that indel mutations, generated by Cas9-mediated DNA cleavage followed by non-homologous end joining (NHEJ) near the key chromogenic residues, would diminish corresponding fluorescent signals, which could be easily detected and scored using a fluorescence stereoscope on a large population of offspring.

We cloned the EGFP and mRFP gRNA sequences under the *Drosophila* U6 promoter via a nested PCR method (see methods section for details). Because plasmid DNA is more stable during injection and cost-effective to generate, we chose to inject DNA vectors encoding gRNAs rather than in vitro transcribed small gRNAs. Embryonic injection was performed following standard DNA injection protocol.

Vasa-Cas9 embryos that were injected with gRNA-expressing vectors retained high viability, typical of standard injection practices (~50% of injected embryos developed into adulthood). No detectable mosaicism of EGFP or mRFP expression in the adult eyes was observed when they developed (0/152 G0 flies scored). This is in contrast to the widespread somatic mutations within G0 generation adult eyes we observed when injecting highly potent TALEN mRNAs against the *white* gene (Lee et al., manuscript in preparation). Previous reports targeting several morphological markers using TALENS or the 2-component CRISPR/Cas9 system also observed somatic mutations^{10-12,19,20}. A possible explanation is that the Vasa-driven expression is anchored at the germ plasma in oocytes and restricted to the posterior tip of syncytial embryos,^{21,22} thus the anterior diffusion of gRNA-loaded Cas9 endonuclease is spatially limited. The lack of detectable somatic mutations and the excellent survival rate of injected embryos suggest that Vasa-Cas9 is indeed working in a strictly germline-specific manner.

Despite the lack of apparent somatic mutations in the G0 generation, a significant portion of the G0 adult flies are infertile or exhibit low fertility (68% for EGFP and 45% for mRFP). This could be the consequence of toxicity introduced by off-target Cas9 activity in the germline, mechanical damage caused by injection, or both. However, among the G1 offspring produced by fertile G0 parents, we observed a large portion of flies expressing only 1 of the 2 fluorescent proteins in their eyes. To be specific, 24.7% (115 out of 465) of G1 from EGFP-gRNA vector injected G0 parents failed to express EGFP, while all these flies retained robust mRFP expression (Fig. 1B, top panels). On the other hand, 7.7% (109 out of 1418) of G1 from mRFP-gRNA vector injected G0 parents failed to express mRFP, while all these flies expressed EGFP (Fig. 1B, bottom panels). These results are consistent with our design that EGFP and mRFP gRNAs only target their corresponding fluorescent proteins around the critical chromogenic residues.

We also observed a significantly higher ratio of EGFP-gRNA vector injected G0 adults harboring transmittable germline mutations among fertile individuals (15 out of 21, 71%) compared with mRFP-gRNA vector injected flies (17 out of 48, 35%). EGFP-gRNA vector injected G0 adults also showed an overall higher probability distribution of mutation ratio within the germline, compared with mRFP-gRNA vector injected flies (Fig. 1C). Despite the difference of germline transmission rates between the EGFP- and mRFP-gRNAs, we achieved a specific and consistent gene targeting performance using the 1-component germline CRISPR/Cas9 platform. The efficiency achieved with our trials is comparable to the best scenario of the 3 fly reports published so far. In several cases, we observed a 100% germline mutation rate (2 lines for EGFP and one for mRFP gRNAs). The prompt availability of this resource to the entire fly community will greatly facilitate site-specific genomic engineering using the CRISPR/Cas9 system.

Discussion

To reduce the complexity of preparing 2-component CRISPR/Cas9 reagents for routine *Drosophila* genomic engineering applications, a germline-specific 1-component system (Vasa-Cas9) was developed. End users need only to inject embryos with DNA vectors expressing easy-to-make, short gRNAs to achieve highly specific and consistently efficient gene editing. Cloning such vectors with our reported methodology can be promptly accomplished. Introducing these vectors into Vasa-Cas9 embryos is done using standard injection protocol. The presence of endogenous Cas9 in germline cells might render the system more effective and robust; thus, we can achieve high editing efficiency with DNA injection previously only obtained by direct injection of highly concentrated guide RNA.¹⁰⁻¹² This simple CRISPR/Cas9 system can be an immediate routine tool to generate site-specific mutations in the fly genome.

This simplified scheme reduces technical variables that contribute to inconsistent outcomes of gene editing by different groups, or among different gRNA-mediated gene editing experiments performed by the same researcher. This advance can facilitate further experiments to systematically investigate the

biological variables (such as target site selection and chromatin accessibility) that contribute to differential efficiency of the same Cas9 endonuclease loaded with different gRNAs. The endogenously expressed Vasa-Cas9 transgene not only provides a uniform and consistent amount of Cas9 endonuclease specifically in the germline, making the nature of identified mutant phenotypes unambiguously recognizable as having a germline origin rather than somatic; it also limits any widespread toxicity compromising G0 animal survival and improves the germline transmission rate of acquired mutations.

Similar to Vasa, the Nanos regulatory sequence has been shown to drive efficient germline expression and has been adapted for stable germline site-specific gene integration.¹⁸ We predict a similarly constructed Nanos-Cas9 line should provide equivalent outcomes for generating germline mutations. Vasa-driven transgene expression is limited to the germline cells at posterior tips of embryos.²¹ In contrast, Nanos-driven maternal expression is distributed spatially throughout embryos during early developmental stages and then restricts to germline cells during later stages.²³ Thus, an efficient Nanos-driven CRISPR/Cas9 system should offer additional opportunities to generate somatic mutations in early syncytial embryos for developmental studies. This could become a uniquely powerful tool, since it is difficult to generate genetic mosaics at early stages using traditional mitotic recombination techniques.

Potential off-target effects and consequential cytotoxicity of the CRISPR/Cas9 system remains a challenging issue to be systematically addressed. Despite rare cases where off-target effects were examined through genome-wide studies and confirmed to be minimal,²⁴ a healthy skepticism is likely warranted based on accumulating evidence to the contrary.¹³⁻¹⁷ We suspect cytotoxicity is a contributing factor to the poor fertility rates we have observed. However, it is also unlikely that germ cells harboring chromosomal abnormalities are viable and therefore unlikely to contribute significantly to the genetic pools of the offspring. Furthermore, the transmitted off-target mutations are likely to be drastically diluted after several generations.

Utilizing a simple and standardized method to introduce gRNAs into fly embryos using the Vasa-Cas9 strain enabled us to get a glimpse of the biological causes of the variability in targeting efficiency. Although we cannot rule out all potential variables introduced during DNA preparation and injection, we speculate that the higher efficiency of EGFP gRNA compared with mRFP gRNA (Fig. 1C) reflects EGFP gRNA as a better guide for the Cas9 endonuclease. It is also possible that the fluorescence loss readout we used for scoring mutagenesis rates underestimates the real cutting events at the EGFP or mRFP genes. A more systematic effort is required to improve the targeting efficiency and stringency of the CRISPR/Cas9 system by improving gRNA designs and engineering a more specific Cas9 endonuclease.

A very appealing advantage of the CRISPR/Cas9 system is its ease in generating mutations in multiple genes simultaneously.^{6,7,9} Although our current trials focused on a single gRNA-expressing vector per injection, our Vasa-Cas9 platform offers the flexibility to mix multiple gRNA-expressing vectors at an optimal

concentration for a single injection. Injection with multiple in vitro transcribed gRNAs should be compatible with our 1-component germline CRISPR/Cas9 system as well. This multiplexing approach might be an immediate solution to improve the fidelity of the CRISPR/Cas9 system by using 2 gRNAs guiding a nicking nuclease (Cas9 with a point mutation in one nuclease domain so that it can only make a DNA lesion on one strand). With this approach, a double strand break can be generated only when 2 single-stranded cutting events are triggered adjacent to each other on opposite strands of the same DNA substrate.^{16,25} A Vasa-Cas9 D10A nickase^{4,7,16,25} transgene can be used to assist further testing of this application.

Materials and Methods

Germline-specific Cas9 transgene

The p3xP3-EGFP.vas-int.NLS.attB (FBid: FBmc0002993) plasmid¹⁸ is a generous gift from Dr Johannes Bischof (University of Zurich). In addition to the phiC31 integrase gene under the control of the Vasa regulatory elements, this plasmid harbors an eye-expressing EGFP under the control of the 3xP3 promoter as a selection marker and an attB site for site-specific transgenesis. The phiC31 integrase ORF was excised by BsiWI digestion and replaced by the human codon-optimized Cas9 ORF, which was amplified from the hCas9 plasmid (Addgene plasmid 41815).⁷ The constructed plasmid pVasa-Cas9 was sequence-verified and injected into the ZH-2A (FBid: FBti0076477) embryos with a phiC31 integrase helper plasmid (Rainbow Transgenic Flies, Inc.). Site-specific integration of the Vasa-Cas9 transgene was verified by standard transposon mapping procedures.

gRNA design

The protospacer sequences were selected from EGFP and mRFP sequences with the predicted cutting sites at nucleotide 191 and 197, respectively. The predicted Cas9 cutting sites fall within the codons of Thr⁶⁴ for EGFP and Gln⁶⁶ for mRFP, both are critical for their respective fluorophore formations. The designs of the gRNAs start with the 20-nt target-specific spacer sequences (reverse-complement to the corresponding protospacer sequences) followed by a common 80-nt tracrRNA sequence. The full sequences of the guide RNAs are (bold letters indicate the spacer sequence):

EGFP gRNA

| | | |
|-------------------|------------------|------------|
| CUCGUGACCA | CCUGACCUA | GUUUUAGAGC |
| UAGAAUAGC | AAGUAAAAU | AAGGCUAGUC |
| CGUUAUCAAC | UGAAAAAGUG | GCACCGAGUC |
| GGUGCUUUUU | | |

mRFP gRNA

| | | |
|-------------------|-------------------|------------|
| UUGUCACCAC | AAUUUCAGUA | GUUUUAGAGC |
| UAGAAUAGC | AAGUAAAAU | AAGGCUAGUC |
| CGUUAUCAAC | UGAAAAAGUG | GCACCGAGUC |
| GGUGCUUUUU | | |

gRNA-expressing vectors

gRNA-expressing vectors were generated using a series of nested PCR reactions.²⁶ The first PCR amplified the *Drosophila* U6 promoter from genomic DNA. The second PCR extended

3' of the U6 promoter to include the target-specific 20-nt spacer sequences followed by the first 24-nt of the common tracrRNA sequence. The third PCR further extended 3' to include the remaining 56-nt tracrRNA sequence. Due to the long hairpin secondary structures of the gRNAs, the last PCR step was performed with GC rich buffer and a thermostable DNA polymerase. We achieved good amplification with GC rich buffer compatible with Phusion® High-Fidelity DNA polymerase (New England Biolabs). The final PCR products were cloned into pCR-blunt vector using the Zero Blunt® PCR cloning technique (Life Technologies). Cloned gRNA-expressing vectors were sequence-verified before injection into Vasa-Cas9 fly embryos. EGFP- and mRFP-gRNA expressing vectors were independently injected at 1µg/ml into 200 Vasa-Cas9 embryos following standard germline injection procedure (Rainbow Transgenic Flies, Inc.). Viability of injected embryos was scored.

Germline-transmitted mutation scoring

Individual G0 Vasa-Cas9 flies that developed from viable gRNA vector injected embryos were crossed to w¹¹¹⁸ flies (FBid: FBal0018186). The fertility of G0 flies was scored. In the G1 generation, flies that inherited the X chromosome from the injected G0 parents were scored for each individual cross (such that male G1s from injected male G0 were not counted). The loss of G1 adult eye EGFP or mRFP expression was used as the read-out for Cas9-induced mutagenesis in EGFP or mRFP genes. A Leica MZ10F fluorescence stereomicroscope equipped with ET filter set DsRed, filter set GFP plus, and a DFC345 FX cooled monochrome CCD camera was used for both visual scoring and digital imaging.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note added in revision

While this manuscript was under peer review, we learned of an independent study by Konda and Ueda.²⁷ By using a different germ-line Cas9 transgenic line, Nanos-Cas9, highly efficient gene editing is similarly achieved. Vasa-Cas9 and Nanos-Cas9 thus both serve as complementary and efficient genomic engineering tools. It is worthwhile to point out the important differences between these 2 similar lines as discussed in more detail in the discussion section, which explains advantages for each tool under different experimental situations.

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