**Supplementary Appendix**

# Germline Cas9 Expression Yields Highly Efficient Genome Engineering in a Major Worldwide Disease Vector, *Aedes aegypti*

**Short Running Title**

Germline-Specific Cas9 expression in *Aedes aegypti*

**Authors Affiliation**

Ming Li[1,3], Michelle Bui[1,3],Ting Yang[1,3], Christian S. Bowman[1], Bradley J. White[1,2] and Omar S. Akbari[1,3*]

[1]Department of Entomology and Center for Disease Vector Research, Institute for Integrative Genome Biology, University of California, Riverside, Riverside, CA 92521, USA

[2]Current address: Verily Life Sciences, 259 East Grand Avenue, South San Francisco, CA

[3]Current address: Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, California, United States of America

## Supplementary Appendix Contents

## SI Materials and methods

### Insect rearing

Mosquitoes used in all experiments were derived from of the *Ae. aegypti* Liverpool strain, which was the source strain for the reference genome sequence(1). Mosquitoes were raised in incubators at $28^0$C with 70–80% humidity and a 12 hour light/dark cycle. Larvae were fed ground fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany) and adults were fed with 0.3M aqueous sucrose. Adult females were blood fed three to five days after eclosion using anesthetized mice. All animals were handled in accordance with the guide for the care and use of laboratory animals as recommended by the National Institutes of Health and supervised by the local Institutional Animal Care and Use Committee.

### *piggyBac* mediated Cas9 plasmid construction

To generate the Cas9 constructs, the *piggyBac* plasmid pBac-3xP3-dsRed (a kind gift from R. Harrell) was digested and linearized with Fse1/Asc1 and this was used as a backbone for construct assembly using the Gibson method(2). The Opie2 promoter region(3) was amplified from vector pIZ/V5-His/CAT (Invitrogen) using primers AE001 and AE002, to drive the expression of dsRed. The promoters that we selected in the current study were based on three parameters: 1) an optimal location for primer design for ideal PCR conditions, 2) include as much regulatory information as possible without venturing into other genes or transcribed features as identified through (www.vector.caltech.edu), and 3) limit the size of our transgene to ensure integration events would occur at high frequency. A total of 2133 bp putative promoter region for gene AAEL010097, a 2500 bp putative promoter region for gene AAEL007097, a 3041 bp putative promoter region for gene AAEL007584, a 3034 bp putative promoter region for gene AAEL005635, a 1386 bp putative promoter region for gene AAEL003877, a 421 bp putative promoter region for gene AAEL006511 was PCR amplified from *Ae. aegypti* genomic DNA with primers AE003 and AE004, AE005 and AE006, AE007 and AE008, AE009 and AE010, AE011 and AE012, AE013 and AE014, respectively(4, 5). Downstream of each promoter element, a NLS-Cas9-NLS-T2A-eGFP-p10 UTR which consisted of elements i) 120 bp N-terminal nuclear localization signal (NLS) sequence; ii) a 4101 bp human codon optimized

Cas9 (hspCas9) in which the sequence was derived from plasmid p3xP3-EGFP/*vasa*-3xFLAG-NLS-Cas9-NLS(6); iii) a 48 bp C-NLS, a 54 bp self cleaving peptide 2A (T2A); iv) a 720 bp green fluorescent protein (eGFP). A template DNA containing elements i-vi was generated using gene synthesis (Genescript), and subcloned into plasmids to be driven by promoters mentioned above. A 677 bp p10 3' untranslated region (UTR)(7) was amplified with primers AE015 and AE016 from vector pJFRC81-10XUAS-IVS-Syn21-GFP-p10 and included as the 3'UTR for NLS-Cas9-NLS-T2A-eGFP (Addgene plasmid 36432). All plasmids were grown in strain JM109 chemically competent cells (Zymo Research #T3005), and isolated using Zyppy Plasmid Miniprep (Zymo Research #D4037) and Maxiprep (Zymo Research #D4028) kits; the full plasmid sequence was verified using Source Bioscience Sanger sequencing services. A list of primer sequences used in the above construct assembly can be found in Supplementary Table 1. We have also made available all Cas9 plasmids and sequence maps for order and download at www.addgene.com with addgene ID's #100580 (AAEL006511-Cas9), #100581 (AAEL003877-Cas9), #100608 (AAEL005635-Cas9), #100705 (AAEL007097-Cas9), #100706 (AAEL007584-Cas9) and #100707 (AAEL010097-Cas9).

**Generation of *Ae. aegypti* Cas9 transgenic lines**

Transgenic *Ae. aegypti* Cas9 mosquitoes were created by injecting 0-1 hr old pre-blastoderm stage embryos with a mixture of *piggybac* vector containing the Cas9 expressing plasmid designed above (200ng/ul) and a source of *piggyBac* transposase (phsp-Pbac, (200ng/ul))(8–10). Embryonic collection and microinjections were largely performed following previously established procedures(11). The injected embryos were hatched in deoxygenated $H_20$, and the surviving adults were put into cages, and blood feed 4 days later after eclosion. The larvae with positive fluorescent signals were selected under the fluorescent stereo microscope (Leica M165FC) for at least 8 generations to establish stable lines (mostly homozygous). To ensure that these lines represented single chromosomal insertions, we also backcrossed single individuals from each of the lines for four generations to our wildtype stock, and measured the Mendelian transmission ratios in each generation. In all cases, we observed a 50% transmission ratio in each generation, indicating that our strains represent insertions into single chromosomes. We have placed a pending request (under review with NAIAD) with BEI collection to deposit all the Cas9 expressing *Aedes aegypti* strains to make them easily accessible to the community.

**Characterization of AAEL010097-Cas9 insertion site**

To characterize the Cas9 insertion site for AAEL010097-Cas9, we utilized a previously described inverse polymerase chain reaction (iPCR) protocol(12). Briefly, genomic DNA (gDNA) was extracted from 10 *Aedes aegypti* fourth instar larvae using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The eluted DNA was diluted, and two separate restriction digests were performed on the gDNA to characterize both the 5' and 3' ends using Sau3AI (5' reaction) or HinP1I (3' reaction) restriction enzymes. A ligation step using NEB T4 DNA Ligase was then performed on the restriction digest products to promote circularization of the digested DNA. Two rounds of PCR were performed using primers AE049-AE056 (with their corresponding restriction digest reaction, AE049-AE056) and sequence confirmation (AE057, and AE058) are listed in supplementary table 2. PCR products from the second round of PCR were cleaned using the MinElute PCR Purification Kit (Qiagen) in accordance with the manufacturer's protocol, and subsequently sequenced via Sanger sequencing (Source BioScience). Both the location and orientation were confirmed by PCR using primers designed from the mapped genomic region in combination with both 3' *piggyBac* end forward primers. Sequencing data was then blasted to the AaegL5.0 reference genome available on NCBI. An alignment of the sequencing data was performed using EMBOSS Water, an online pairwise sequence alignment tool, for determination of the orientation of the Cas9 insertion site. Sequencing data mapped the genomic region flanking the 3' *piggyBac* end of the Cas9 insertion site, placing it on chromosome 3 at the locus 212,799,454 – 212,799,858.

**Production of sgRNAs**

Linear double-stranded DNA templates for all sgRNAs were generated by template-free PCR with NEB Q5 high-fidelity DNA polymerase (catalog # M0491S) by combining primer pairs (sgRNA F and sgRNA R) following(13, 14). PCR reactions were heated to 98°C for 30 seconds, followed by 35 cycles of 98°C for 10 seconds, 58°C for 10 seconds, and 72°C for 10 seconds, then 72°C for 2 minutes. PCR products were purified with Beckman Coulter Ampure XP beads (catalog #A63880). Following PCR, sgRNAs were synthesized using the Ambion Megascript T7 in vitro transcription kit (catalog # AM1334, Life Technologies) according to the manufacturer's

protocols using 300ng of purified DNA template overnight at 37°C. Following in vitro transcription, the sgRNAs were purified with MegaClear Kit (catalog #AM1908, Life Technologies) and diluted to 1000 ng/ul in nuclease-free water and stored in aliquots at -80°C. Recombinant Cas9 protein from *Streptococcus pyogenes* was obtained commercially (CP01, PNA Bio Inc) and diluted to 1000 ng/ul in nuclease-free water and stored in aliquots at -80°C. All primer sequences can be found in table S1.

**Donor plasmids construction**

We designed two donor plasmids, *white*-donor and *kh*-donor based on the sgRNAs (W1-sgRNA and Kh-sgRNA) that target the *white* and *kynurenine hydroxylase*c (*kh*) gene, respectively. Donor plasmids were constructed using standard molecular biology techniques based on Gibson assembly(2). The plasmids contained the following elements: (1) a 3xP3-DsRed fragment, which was amplified from vector pBac-3xP3-dsRed using primers AE039 and AE040 and expresses the dsRed dominant fluorescence marker visible in larvae photoreceptors as well as adult eyes, (2) DNA fragments ~ 1 kb in length each that are homologous to the *Ae. aegypti white* and *kh* locus immediately adjacent to the 5' and 3' ends of the W1-sgRNA and Kh-sgRNA target cutting site, amplified from *Ae. aegypti* genome DNA with primer pairs of AE041 and AE042, AE043 and AE044, AE045 and AE046, AE047 and AE048, respectively.

**CRISPR mediated microinjections**

Embryonic collection and CRISPR microinjections were performed following previously established procedures(11, 14). The concentration of components used in the study was as follows; Cas9 protein at 300 ng/ul, sgRNA at 100 ng/ul, and donor plasmid at 100 ng/ul. To identify mutants, injected $G_0$s and $G_1$s were visualized at the life stages of larva, pupae, and adult carefully under a dissecting microscope (Olympus, SZ51). The heritable mutation rates were calculated as the number of mutant $G_1$s out of the number of all $G_1$ progeny crossed with respective mutant stocks. To molecularly characterize the induced mutations, genomic DNA was extracted from an individual mosquito with the DNeasy blood & tissue kit (QIAGEN) following the manufacturer's protocol. Target loci were amplified by PCR, PCR products were gel purified and were either sent directly for Sanger sequencing or cloned (TOPO-TA, Invitrogen). In the latter case, single colonies were selected and plasmids were extracted (Zyppy$^{TM}$ plasmid

miniprep kit) and sequenced. Mutated alleles were identified by comparison with the wild-type sequence. Primers used for PCR and sequencing are listed in supplementary table 1. All photographs were obtained using fluorescent stereo microscope (Leica M165FC) and confocal microscope (Leica SP5).

## Supplementary Appendix Tables

**SI Table 1. Primer sequences used in this study**

| Primer | Primer sequence (5'-3') |
|---|---|
| **AE001** | TTCCCACAATGGTTAATTCGAGCTCGCCCGGGGTTTAAACCATACTCGGTGGCCTCCCCAC |
| **AE002** | AACTCCTTGATGACGTTCTTGGAGGAGCGCACCATCTCGAGCACCAGAGACAGGTTGCGGC |
| **AE003** | CACGGCGGGCATGTCGACGCGGCCGCCGTCGTCAGTTATGGCGATGATGATGGTTGATGA |
| **AE004** | AAGAGGTTATCTTTTGTCTACAGAGTGAAACTCGAGATGGACTATAAGGACCACGACGGAG |
| **AE005** | CGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCGGCCGCACGTTTTGTGCGCTCCGGG |
| **AE006** | CTCCGTCGTGGTCCTTATAGTCCATGTTTAAACTTGGATTTTGGACACTTATTAACACAC |
| **AE007** | CGACGGTCACGGCGGGCATGTCGACGCGGCCGCTCACCCGAAATTACGGTACTTATGATCA |
| **AE008** | GTCTCCGTCGTGGTCCTTATAGTCCATCTCGAGGCCTGTGATAATGCGAAAAAACATTTT |
| **AE009** | CGACGGTCACGGCGGGCATGTCGACGCGGCCGCATGTGAATCAACATTAGGTTGTCATGA |
| **AE010** | TCCTTGTAGTCTCCGTCGTGGTCCTTATAGTCCATCTCGAGTTTCCCCAGGGTGGAGCCTTC |
| **AE011** | CGACGGTCACGGCGGGCATGTCGACGCGGCCGCTATCTTTACATGTAG |

| | |
|---|---|
| | CTTGTGCATTGAA |
| **AE012** | GTAGTCTCCGTCGTGGTCCTTATAGTCCATCTCGAGATTCGTTGAAATCTCTGTTGAGCAG |
| **AE013** | GTTCTCGACGGTCACGGCGGGCATGTCGACGCGGCCGCAGCGTGAACCTTGCATGCGTGCA |
| **AE014** | AGTCTCCGTCGTGGTCCTTATAGTCCATCTCGAGCTTGGATCAGTCTGTGGAAAAGTCG |
| **AE015** | CGGCATGGACGAGCTGTACAAGTAATTAATTAACTAGAATGAATCGTTTTTAAAATAACAAATCA |
| **AE016** | AAAAGTTGGTGGTGGGGAGGCCACCGAGTATGGGCGCGCCCCGGCCGTTAACTCGAATCG |
| **sgRNA-R** | AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC |
| **sgRNA-*kh*-F** | GAAATTAATACGACTCACTATAGGGAATGAATGCCGGATTCGGTTTTAGAGCTAGAAATAGC |
| **AE017** | GGCTATCTGGAGCTGTGTATTC |
| **AE018** | CTCCCAACGCGTATCACTAAA |
| **AE019** | CATAGGCCACCTATTCGTCTTC |
| **AE020** | ACCGATACCGATCGAATTTCTC |
| **AE021** | CATTTGGCCGTCGGAATTAAC |
| **AE022** | GTACCACACAGAACACGATGA |
| **sgRNA-** | GAAATTAATACGACTCACTATAGGTCCTTCAGGTCGCCTCCGGGTTTTA |

| | |
|---|---|
| ***White* 1-F** | GAGCTAGAAATAGC |
| **sgRNA-*White* 2-F** | GAAATTAATACGACTCACTATAGGTCAGGGTTTCCGATGCAAGTTTTAGAGCTAGAAATAGC |
| **AE023** | GCACGAACGACTCACATACA |
| **AE024** | TAAGAGGCAAGACACCACAAG |
| **AE025** | GGAGATCGATGTGTTTGGAGAG |
| **AE026** | TGATTTGTCCGTGCGGTATG |
| **AE027** | CTCAAAGTACGCAGTCAGGAA |
| **AE028** | GGGTTCATCACAAAGCAACAG |
| **sgRNA-*yellow*-F** | GAAATTAATACGACTCACTATAGGCCAGTCACCGTGAATTCAGTTTTAGAGCTAGAAATAGC |
| **AE029** | CGGTTCGAGCATAGCTTCTT |
| **AE030** | GTTCTCTTCGGCATCGATCTT |
| **sgRNA-*Ebony*-F** | GAAATTAATACGACTCACTATAGGCCGTGTTCGGCGCAACGCGTTTTAGAGCTAGAAATAGC |
| **AE031** | CCTCACCAAGACCAACTACAA |
| **AE032** | CTACACTGGTAGGGACGTAAAG |
| **sgRNA-*Deformed*-F** | GAAATTAATACGACTCACTATAGGATAGGTCACCTGATGAAAGTTTTAGAGCTAGAAATAGC |
| **AE033** | AAAGATCTGTGGAGGCAGAATAG |

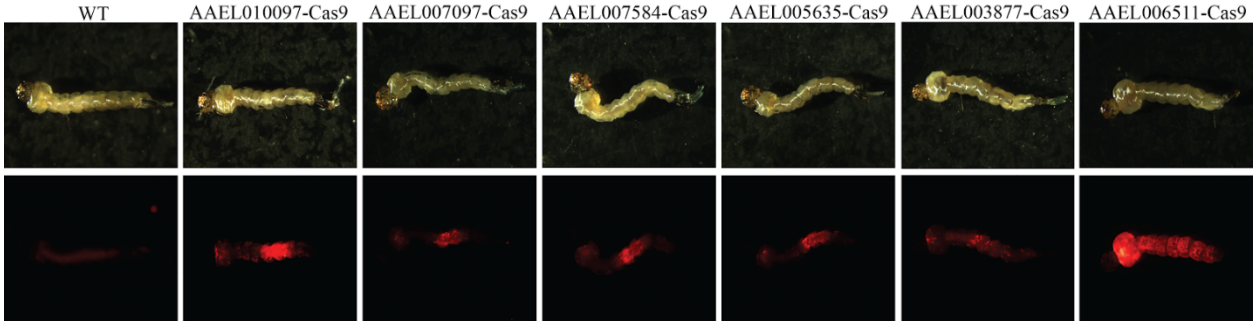
| | |
|---|---|
| **AE034** | CCTTGGTTGCAGAGAGTGATAA |
| **sgRNA-*Sine oculis*-F** | GAAATTAATACGACTCACTATAGGCGGTGGAAGTGGCACAGGAGGGTTTTAGAGCTAGAAATAGC |
| **AE035** | GACGAATCTAGAGCGTGTCATC |
| **AE036** | GGACTTAAGCCGAAGGAACAA |
| **sgRNA-*Vestigial*-F** | GAAATTAATACGACTCACTATAGGACGTGTGCCCGTACTGCTGGTTTTAGAGCTAGAAATAGC |
| **AE037** | TTCATCGGCGGAGAAGATTAC |
| **AE038** | TGCATCACTCTGAAGGCAATA |
| **AE039** | AAAGTGGCACCGAGTCGGTGCTTTTGCTAGCAATTCGAGCTCGCCCGGGGATCT |
| **AE040** | AACATTGTCAGATCCGAGATCGGCCGGCCTAGAAGCTTTAAGATACATTGATGAGTTTGGAC |
| **AE041** | TCCCACAATGGTTAATTCGAGCTCGCCCGGGGTCCTAGGCCTCACGCCACTCGTAGAATGA |
| **AE042** | ACAGTGGCCCAATTTCATATTCGTACCATTTAAATAAAGCGTTCAGCAAGGTTGTTTTA |
| **AE043** | GGCTTGGATAGCGATTCGAGTTAACGGCCGGGTTTAAACTCAAGATCGCACCGACTTCAG |
| **AE044** | TGGTGGGGAGGCCACCGAGTATGGGCGCGCCCCTAATGATTGAAGTCGTTTTTGGATG |
| **AE045** | CCCACAATGGTTAATTCGAGCTCGCCCGGGGTCCTAGGACGCCCTCCT |

| | |
|---|---|
| | CCAGCATGG |
| **AE046** | GGCTTCGAGACCGTGACCTACATCGTCGACTGCCCGTAGAAGGGAACCATGGCAT |
| **AE047** | TAGCGATTCGAGTTAACGGCCGGGTTTAAACATTGTACTGTGTTGACCGAGTTGTTC |
| **AE048** | TTGGTGGTGGGGAGGCCACCGAGTATGGGCGCGCCGTTTGTTGAATTTGCGACACGATAC |

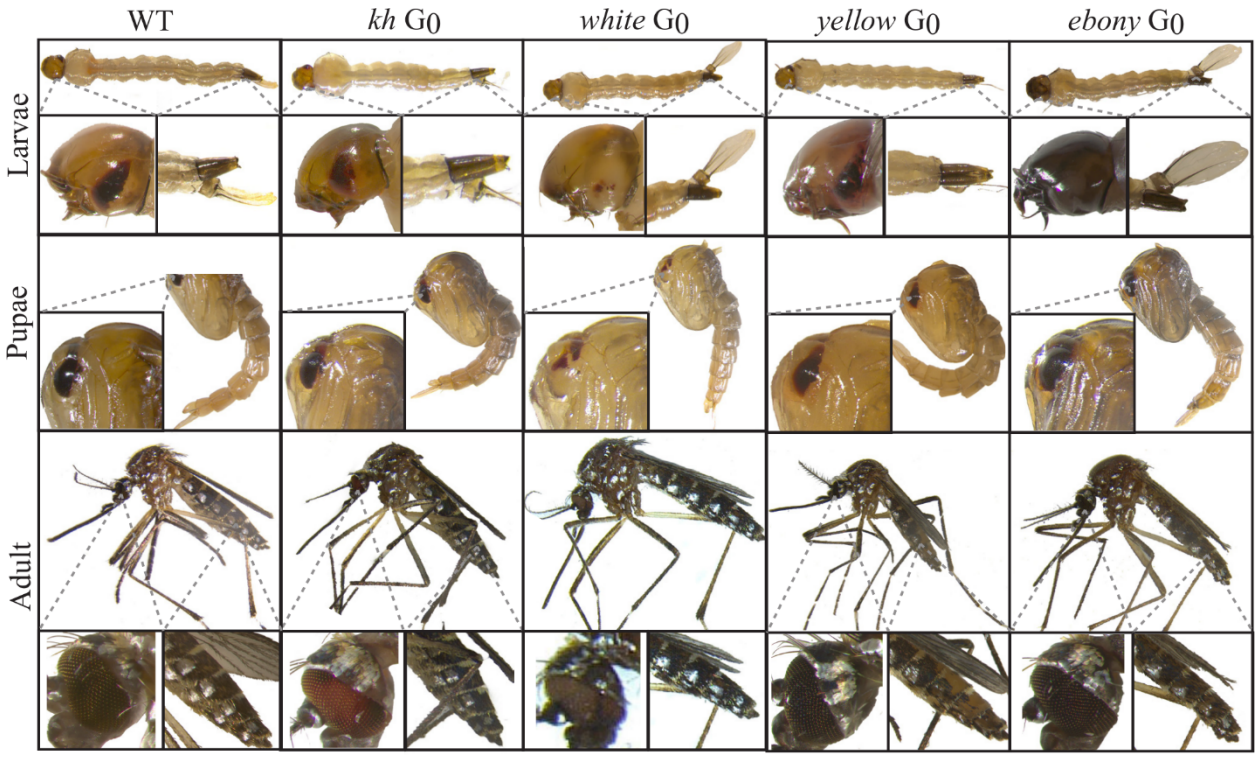
**SI Table 2. Inverse PCR Primer sequences used in this study**

| Reaction | Restriction enzyme | Primer name | Primer sequence |
|---|---|---|---|
| 5’ | Sau3A I | AE049 | GACGCATGATTATCTTTTACGTGAC |
| | | AE050 | TGACACTTACCGCATTGACA |
| | | AE051 | GCGATGACGAGCTTGTTGGTG |
| | | AE052 | TCCAAGCGGCGACTGAGATG |
| 3’ | HinP1 I | AE053 | CAACATGACTGTTTTTAAAGTACAAA |
| | | AE054 | GTCAGAAACAACTTTGGCACATATC |
| | | AE055 | CCTCGATATACAGACCGATAAAAC |
| | | AE056 | TGCATTTGCCTTTCGCCTTAT |
| **Confirmation PCR Primer name** | | | Confirmation PCR Primer sequence |
| **AE057** | | | CCAGGTTGTCGCCCTCCTTGTAG |
| **AE058** | | | CTACAAGGAGGGCGACAACCTGG |

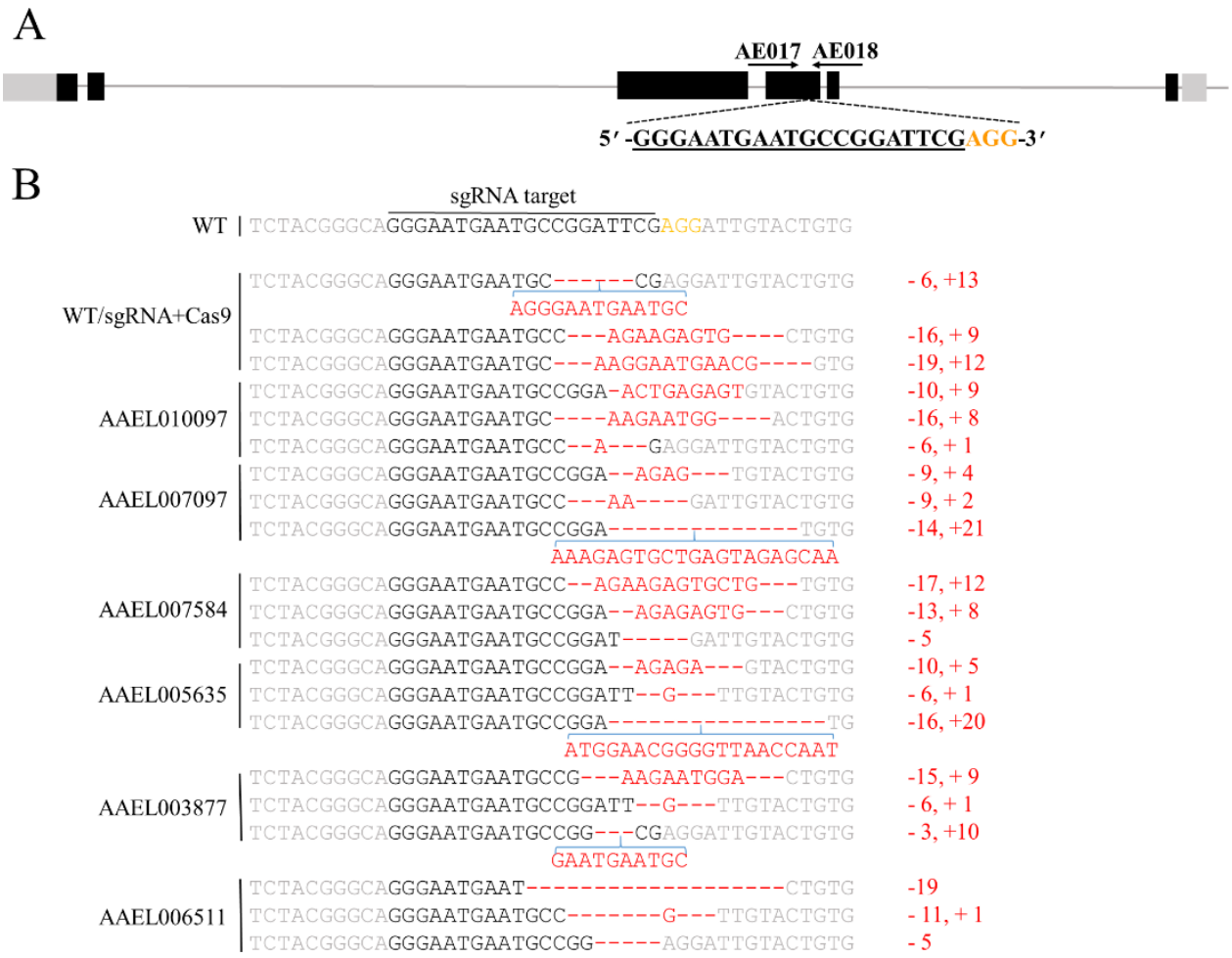
## Supplementary Appendix Figures



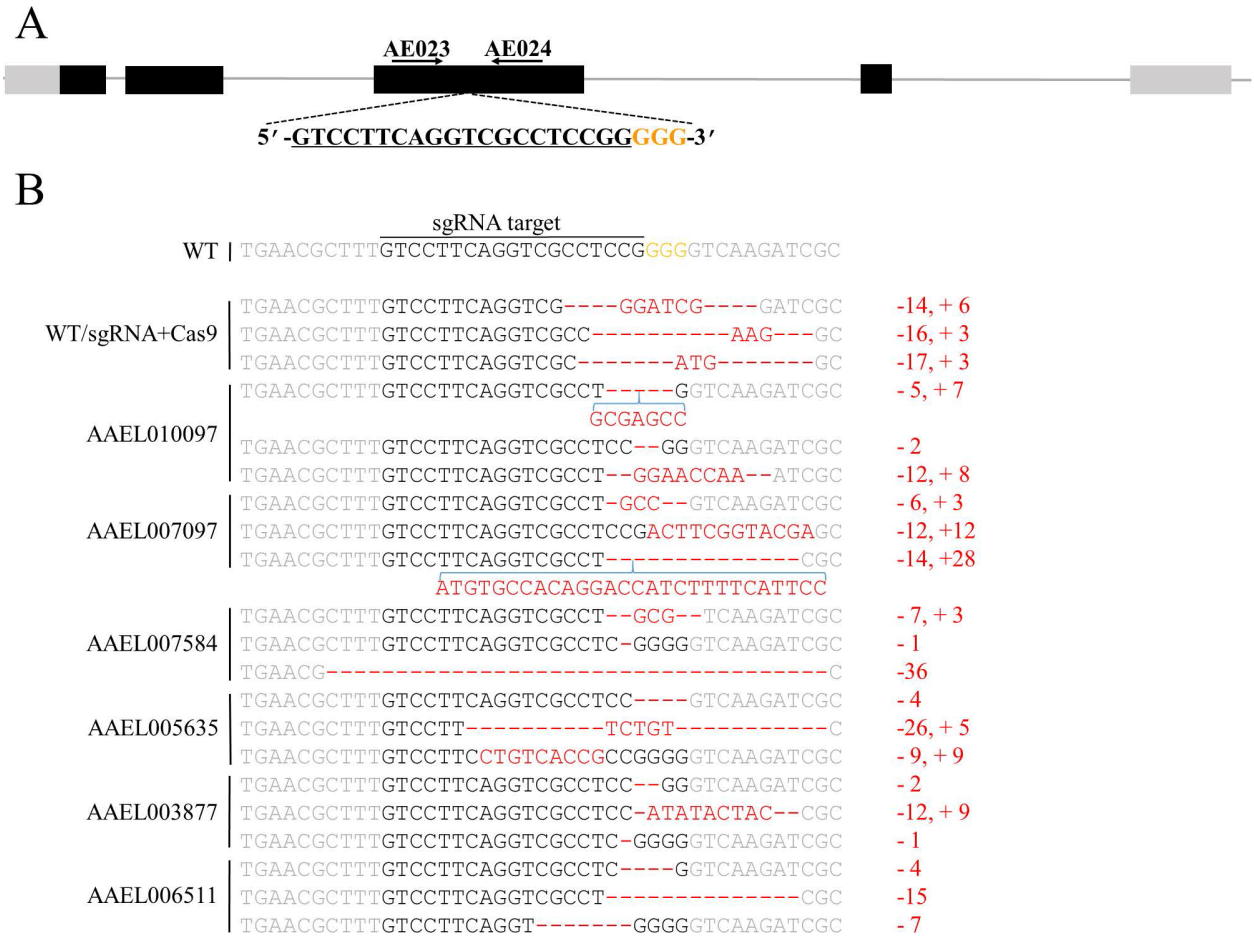

**SI Figure 1.** Cas9 expressing transgenic mosquitoes with dsRed selectable marker. Transgenic mosquitoes for each Cas9 strain showing the Opie2-dsRed fluorescent marker. Brightfield images (top) and corresponding dsRed fluorescent images showing robust expression in the larval gut and cuticle tissues.



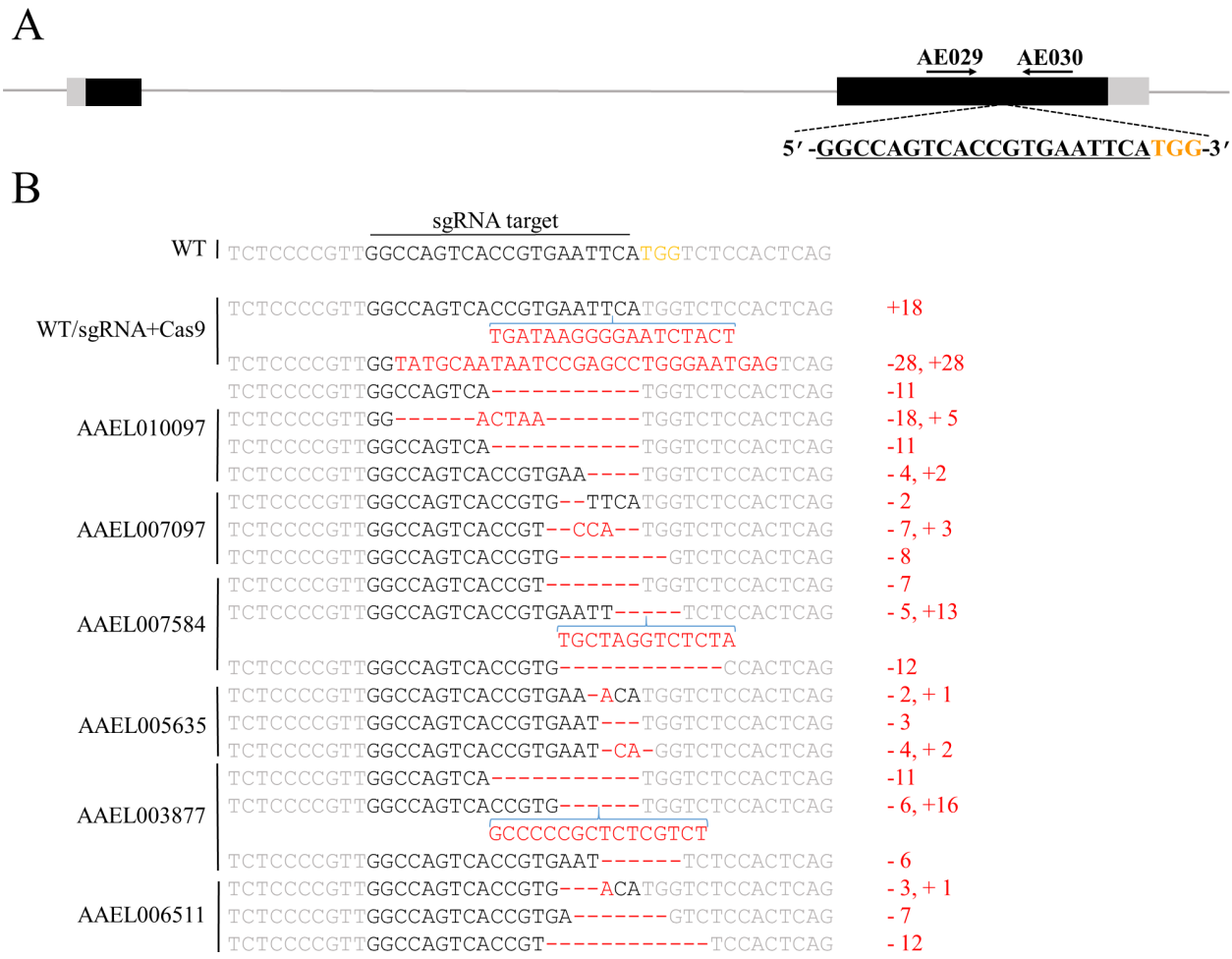

**SI Figure 2.** CRISPR/Cas9 induced G0 mosaic mutant phenotypes from single injections. Larval, pupa, and adult G0 mosaic phenotypes of wild type, *kh*, *white*, *yellow* and *ebony* mutants.



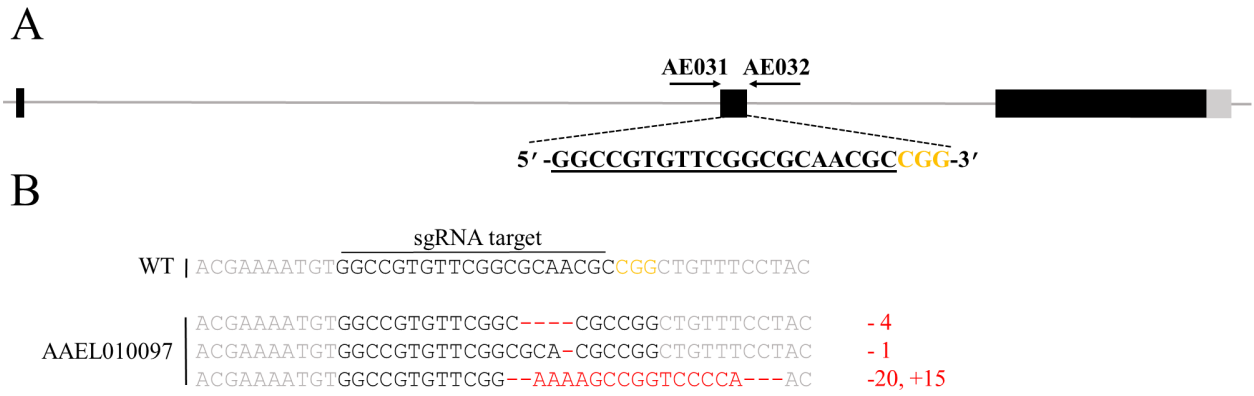

**SI Figure 3.** Confirmed mutagenesis of the *kh* locus. Schematic representation of the *kh* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing analysis of indels from individuals sequenced from the various Cas9 strain injections. Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *kh* gene disruptions resulting from insertions/deletions are indicated in red (B).



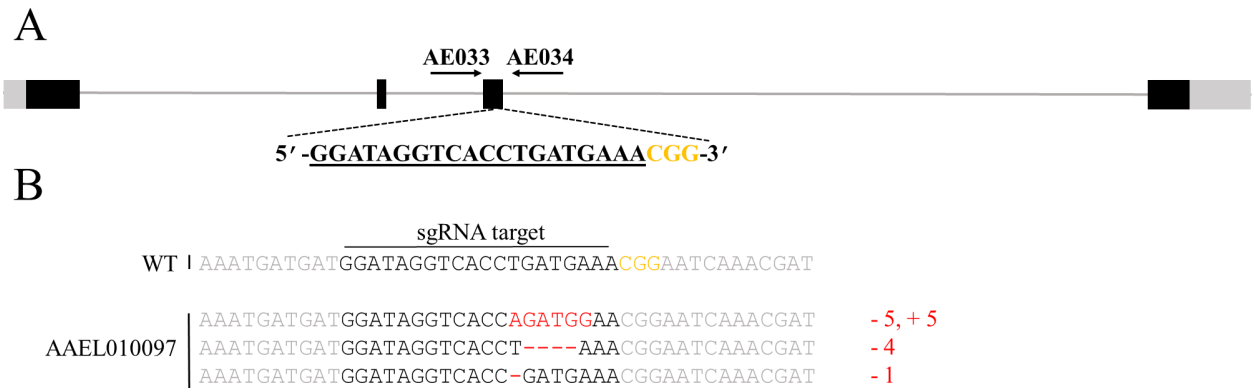

**SI Figure 4.** Confirmed mutagenesis of the *white* locus. Schematic representation of the *white* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing analysis of indels from individuals sequenced from the various Cas9 strain injections. Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *white* gene disruptions resulting from insertions/deletions are indicated in red (B).



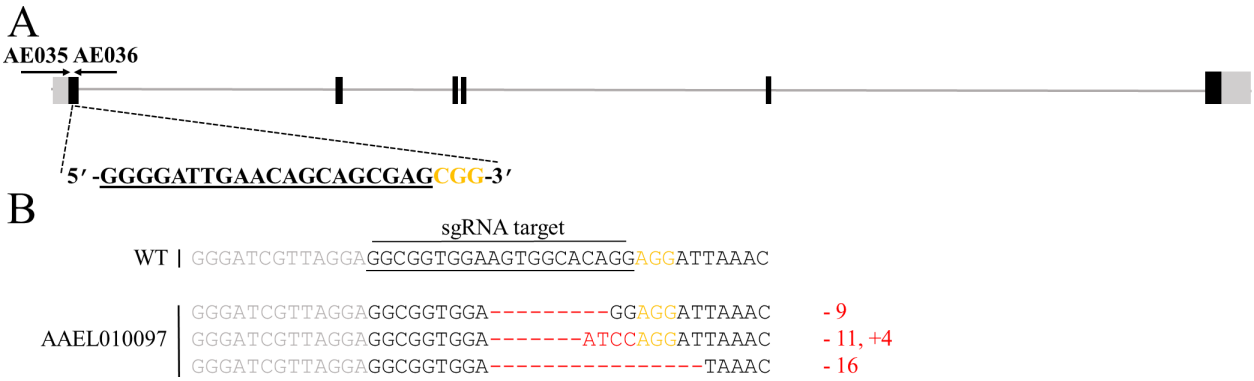

**SI Figure 5.** Confirmed mutagenesis of the *yellow* locus. Schematic representation of the *yellow* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing analysis of indels from individuals sequenced from the various Cas9 strain injections. Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *yellow* gene disruptions resulting from insertions/deletions are indicated in red (B).



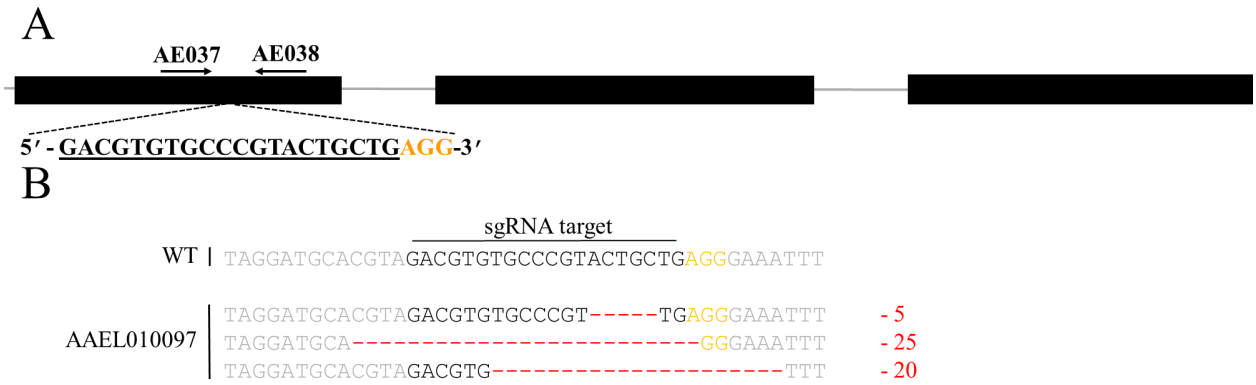

**SI Figure 6.** Confirmed mutagenesis of the *ebony* locus. Schematic representation of the *ebony* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing confirms the generation of small indels (B). Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *ebony* gene disruptions resulting from insertions/deletions are indicated in red.



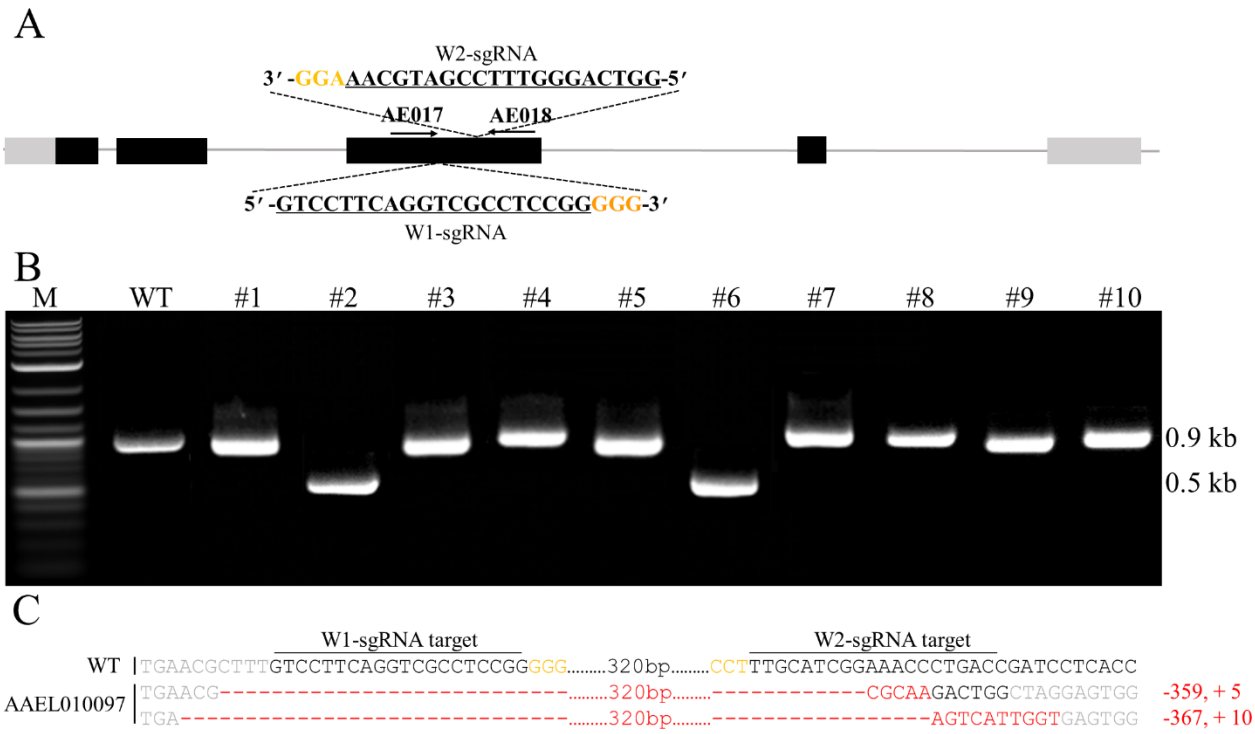

**SI Figure 7.** Confirmed mutagenesis of the *deformed* locus. Schematic representation of the *deformed* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing confirms the generation of small indels (B). Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *deformed* gene disruptions resulting from insertions/deletions are indicated in red.



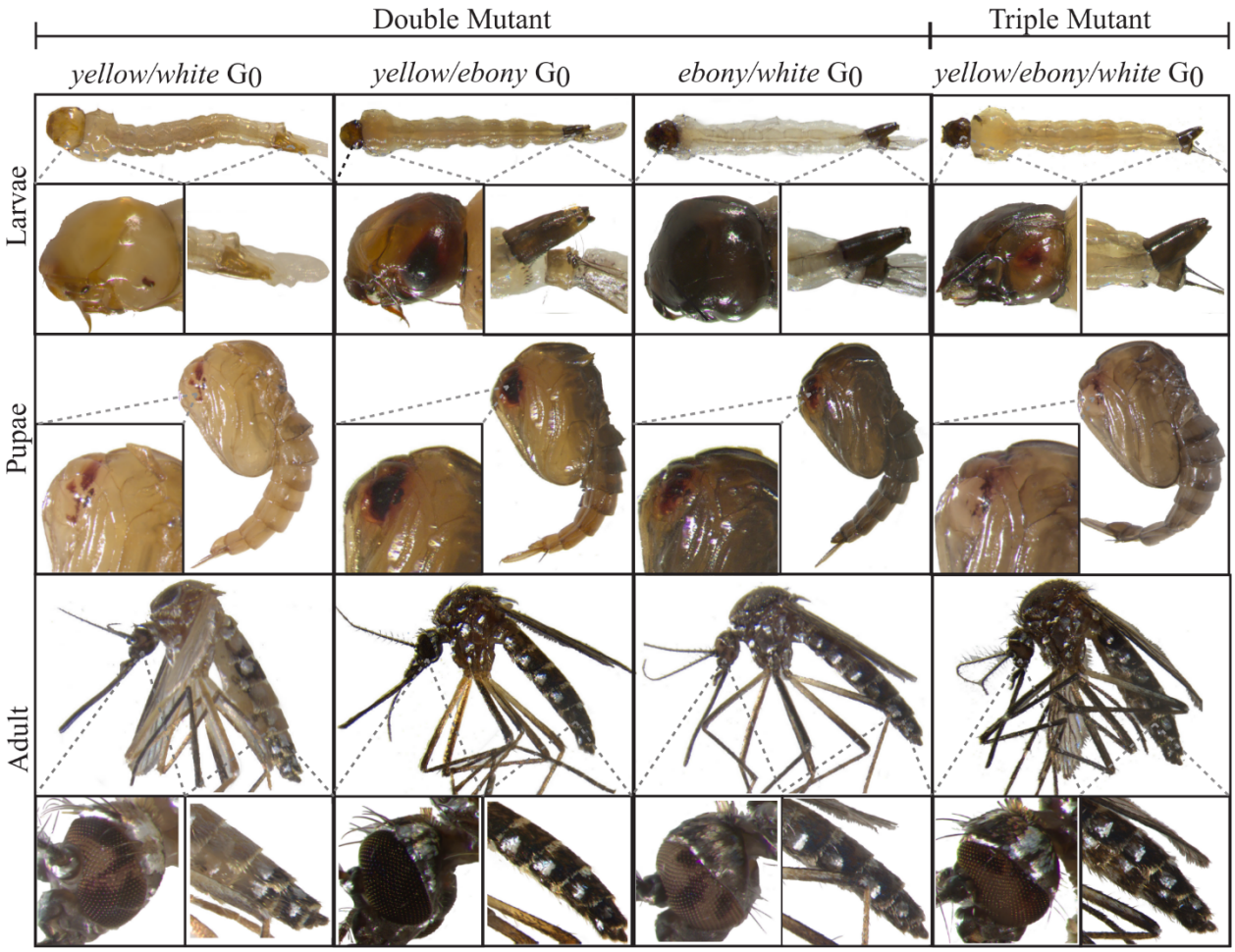

**SI Figure 8.** Confirmed mutagenesis of the *sine oculis* locus. Schematic representation of the *sine oculis* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing confirms the generation of small indels (B). Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *sine oculis* gene disruptions resulting from insertions/deletions are indicated in red.




**SI Figure 9.** Confirmed mutagenesis of the *vestigial* locus. Schematic representation of the *vestigial* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow (A). Genomic sequencing confirms the generation of small indels (B). Top line represents WT sequence; PAM sequences (NGG) are indicated in yellow, and *vestigial* gene disruptions resulting from insertions/deletions are indicated in red.

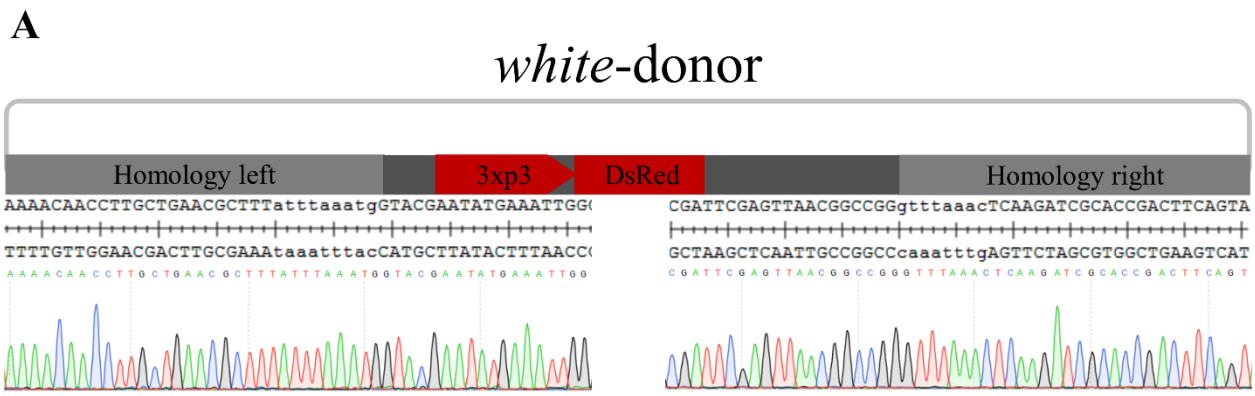



**SI Figure 10.** Generation of indels in the *white* locus by simultaneous injection of multiple sgRNAs. Schematic representation of the *white* locus with exons indicated as boxes, coding regions depicted in black, and the 5'and 3'UTR's in gray. Locations and sequences of the sgRNA targets are indicated with the PAM highlighted in yellow. PCR and gel analysis of the *white* locus following simultaneous injection of two sgRNAs into the AAEL010097-Cas9 strain. In total 10 independent injected G0's were tested along with wild type (WT) as a control. Generation of indels (0.4kb) for mosquitoes #2 and #6 can be straightforwardly distinguished on a DNA gel (B). Sequencing analysis of these indels confirms large deletions (C). Top line represents WT sequence; PAM sequences (NGG) are indicated in orange, and *white* gene disruptions resulting from insertions/deletions are indicated in red.

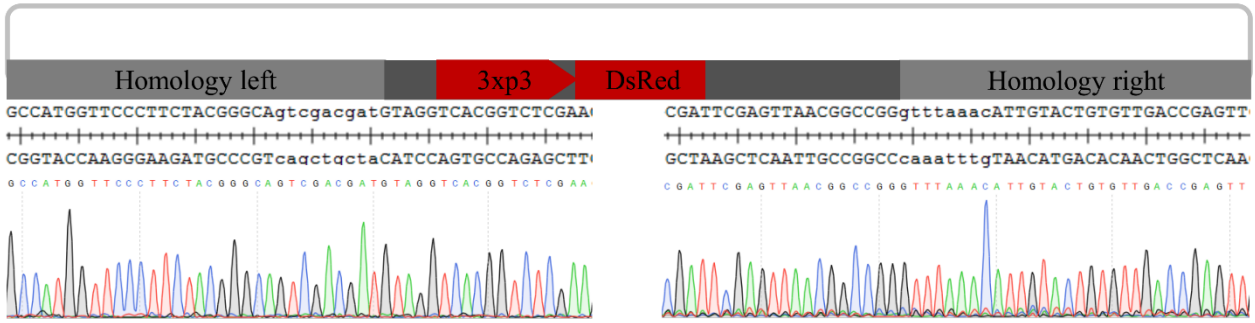



**SI Figure 11.** CRISPR/Cas9 induced double and triple G0 mosaic mutant phenotypes from single injections. Larval, pupa and adult G0 mosaic phenotypes of double mutants including yellow body and white eyes (*yellow/white)*, a mixture of yellow and dark body (*yellow/ebony)*, dark body and white eyes (*ebony/white)*, and one triple mutant which is a phenotypic mixture of yellow and dark body and white eyes (*yellow/ebony/white)*.

**A**




**B**




**SI Figure 12**. Confirmation of the precise donor insertions in the target loci via HDR. Genomic DNA sequencing was performed to confirm the donor construct insertions in *white* and *kh*. Sequencing of the 3'-end amplification fragments with primers of AE025 and AE026 (left), and 5'-end amplification fragments with primer AE027 and AE028 (right) from the *white* donor G1 founder (A). Sequencing of the 3'-end amplification fragments with primers of AE019 and AE020 (left), and 5'-end amplification fragments with primer AE021 and AE022 (right) from the G1 founder (B). Together, these results reveal precise integration of the *white*-donor and *kh*-donor construct into the target locus.

**SI Appendix References**


1. Nene V, et al. (2007) Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* 316(5832):1718–1723.

2. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343–345.

3. Ren L, et al. (2011) Comparative analysis of the activity of two promoters in insect cells. *Afr J Biotechnol* 10(44):8930–8941.

4. Akbari OS, Papathanos PA, Sandler JE, Kennedy K, Hay BA (2014) Identification of germline transcriptional regulatory elements in *Aedes aegypti*. *Sci Rep* 4:3954.

5. Anderson MAE, Gross TL, Myles KM, Adelman ZN (2010) Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*. *Insect Mol Biol* 19(4):441–449.

6. Gratz SJ, et al. (2014) Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196(4):961–971.

7. Pfeiffer BD, Truman JW, Rubin GM (2012) Using translational enhancers to increase transgene expression in *Drosophila*. *Proc Natl Acad Sci U S A* 109(17):6626–6631.

8. Handler AM, Harrell RA Ii (1999) Germline transformation of *Drosophila melanogaster* with the piggyBac transposon vector. *Insect Mol Biol* 8(4):449–457.

9. Kokoza V, Ahmed A, Wimmer EA, Raikhel AS (2001) Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. *Insect Biochem Mol Biol* 31(12):1137–1143.

10. Lobo NF, Hua-Van A, Li X, Nolen BM, Fraser MJ (2002) Germ line transformation of the yellow fever mosquito, *Aedes aegypti*, mediated by transpositional insertion of a piggyBac vector. *Insect Mol Biol* 11(2):133–139.

11. Aryan A, Myles KM, Adelman ZN (2014) Targeted genome editing in *Aedes aegypti* using TALENs. *Methods* 69(1):38–45.

12. Huang AM, Rehm EJ, Rubin GM (2009) Recovery of DNA sequences flanking P-element insertions in Drosophila: inverse PCR and plasmid rescue. *Cold Spring Harb Protoc* 2009(4):db.prot5199.

13. Li M, et al. (2017) Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Sci Rep* 7(1):901.

14. Kistler KE, Vosshall LB, Matthews BJ (2015) Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Rep* 11(1):51–60.