SUPPORTING INFORMATION

MATERIALS AND METHODS

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MATERIALS AND METHODS

***Anopheles gambiae cardinal* gene target site selection.** The *Anopheles gambiae heme peroxidase 6* gene (*AgHPX6*, AGAP003502) is the ortholog of the *cardinal* (*cd*) locus in *Drosophila melanogaster*. Genomic sequence of *Agcd* from the *An. gambiae* PEST (AgamP4) strain was accessed from VectorBase[1] (https://www.vectorbase.org/) and analyzed for potential targets and their associated off-target sites for Cas9 guide RNA- (gRNA) mediated endonuclease activity using the online tools Synthego (https://design.synthego.com/#/validate), ChopChop (http://chopchop.cbu.uib.no/) and SnapGene (https://www.snapgene.com/). Oligonucleotide primers and plasmids were designed using SnapGene (Table S1). The nucleotide sequence, 5'-GGTTAGCGACGATGCCAAGGCGG-3', within exon 3 of *Agcd* was chosen as the target of the gRNA. Regions flanking both ends of the target cut site were amplified from genomic DNA from the laboratory *An. gambiae* G3 strain (hereafter referred to as wild-type, WT) using oligonucleotide primers card-F1 and card-R1. The amplicon was sequenced to obtain the wild-type sequence for this genomic region.

Plasmid design and construction.

Target site. Complementary single-stranded oligonucleotides, Agcard-target_F and Agcard-target_R, containing the *Agcd* target sequence were hybridized at 55°C for 30 minutes and cloned into a *Bbs*I-linearized plasmid, pCO-13 (Table S2), using the In-Fusion HD Cloning Kit (Takara Bio) to generate pCO-33.

Homology arms. Homology arm 2 (cHM2) was amplified from a cardF1/R1 product using oligonucleotides AflII-cardhm2_F and AatII-cardhm2_R. The cHM2 fragment was digested with *Afl*II/*Aat*II, while pCO-33 was digested with *Asc*I/*Aat*II to excise the U6-cardinal gRNA fragment. Both the cHM2 and U6-*Agcd* gRNA fragments were cloned into *Asc*I/*Aat*II-linearized pCO-16 using T4 DNA Ligase (Promega) to generate pCO-35. Homology arm 1 (cHM1) was amplified from the cardF1/R1 PCR product using the oligonucleotides SbfI-cardhm1_F and PacI-cardhm1_R and ligated into pCO-35 that had been linearized with *Sbf*I/*Pac*I to generate pCO-36. Finally, oligonucleotides Inf_Agnos-cas9_F and Inf_Agnos-cas9_R were used to amplify the Agnos-Cas9-T2A-DsRed cassette from pCO-18 and this was cloned into *Asc*I-linearized pCO-36 using the In-Fusion

HD Cloning Kit to generate the gene-drive construct designated pCO-37. Gene amplifications was performed using the CloneAmp HiFi PCR Premix (Takara Bio). Restriction enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized by Sigma Aldrich. The integrity of all the plasmids was verified by sequencing. The final plasmid, pCO37, contains the *An. stephensi* codon-optimized SpCas9 endonuclease-encoding DNA[1] driven by the regulatory elements of the *An. gambiae* PEST strain *nanos* gene (AGAP006098) (Meredith et al. 2013); an *An. gambiae* U6 gene (AGAP013557) promoter expressing the gRNA complementary to the target site, three linked copies of the *D. melanogaster* P3 enhancer/promoter driving the expression of the cyan fluorescent protein marker gene (3XP3-CFP) for transgenic progeny identification, a *φC31 attP* site and genomic DNA fragments ~1 kilobase pairs (kb) in length homologous to *Agcd* immediately adjacent to the 5'- and 3-ends of the target cut site (Figure 1).

Microinjection of embryos and screening procedures. Mosquitoes were maintained at 27 °C with 77% humidity and a 12-h day/night, 30-min dusk/dawn lighting cycle. Larvae were fed powdered fish food (Tetra-Min) mixed with yeast. Adults were provided *ad libitum* with water and a 10% (wt/vol) sucrose solution. Blood meals, defibrinated calf blood (Colorado Veterinary Products) were provided by an artificial feeding apparatus (Hemotek). Mosquito containment followed accepted procedures[2]. Microinjections were performed as described in Methods in Anopheles Research[3]. Embryos were injected with a solution containing 300 ng/μL of pCO-37, and 100 ng/μL of SpCas9 protein (PNA BIO Inc). Resulting adult G_0 males and females were outcrossed to WT mosquitoes of the opposite sex in pools of ~5 G_0 males or 15–30 G_0 females and progeny screened for CFP fluorescence using UV-fluorescence microscopy.

Molecular validation of gene drive cassette integration. Validation of precise integration was confirmed by amplification analysis with primers card-seqF1, eCFPseqR, AgU6 seq F, and card-seqR1, and sequencing amplicons comprising both the left- and right-hand junctions of the homology arms and cargo. Integration at the target gene locus was determined by amplification (primers upHM1cdn-F, eCFPseqR, AgU6 seq F, and dwnHM2cdn-R) of the left and right junctions of regions outside the genomic sequence included in the homology arms and cassette cargo. Hemizygous mosquitoes (one copy of the dominant CFP fluorescence gene)

cannot be readily distinguished from homozygotes (two copies), therefore, left and right portions of the homology arm sequences were amplified (primers card F2 and card-seqR1) to ascertain the presence of a WT *Agcd* allele. Genomic DNA extracts from single mosquitoes were obtained from homozygotes (AgNosCd-1-IX [IX denotes an intercross of transgenic mosquitoes]), hemizygotes (AgNosCd-1-OX [OX denotes an outcross of transgenic mosquitoes to WT]), and control WT using the Extract-N-Amp Tissue PCR kit (Sigma-Aldrich), modified for mosquito extraction[4]. The left- and right-hand junctions of the cassette cargo and homology arms were amplified by forward and reverse primer pairs (left junction, card-seqF1 and eCFPseqR; right junction, AgU6 seqF and card-seqR1). Left and right junctions of the cassette cargo and genomic sequence outside the homology arms were amplified by forward and reverse primer pairs (left junction, upHM1cdn-F and eCFPseqR; right junction, AgU6 seqF and dwnHM2cdn-R). The WT allele was amplified by forward and reverse primer pairs (card F2 and card-seqR1). The oligonucleotide primer pairs were used in 25 µL reactions that included Phusion HF polymerase (New England Biolabs) with a DNA template from single AgNosCd-1-IX, AgNosCd-1-OX or WT mosquitoes. Cycling conditions to amplify the left junctions were 98°C initial denaturation for 1 minute; 30 cycles of 98°C denaturation for 10 seconds, 63°C annealing for 20 seconds and 72°C extension for 1 minute for amplicons < 2 kb in length or 2 minutes for amplicons > 2kb; and a 72°C final extension for 5 minutes. Cycling conditions for right junctions and the WT allele were identical except the annealing phases were carried out at 69°C. Ten µL of the amplification reactions were resolved and visualized in 1% agarose gels stained with Midori Green (BullDog-Bio, USA) with the 1 kb Plus ladder (ThermoFisher Scientific) size markers.

Approximately 40 µL of each amplification reaction was purified using DNA clean and concentrator (Zymo Research), purified product then was diluted to 4 ng/µL. Exactly 10 µL of product along with 5 µL of the corresponding 5 µM forward primer (left junction, card-seqF1; right junction, AgU6-gRNA-F) were premixed and submitted to GeneWiz (La Jolla, CA, USA) for Sanger sequencing. Sequences from the junction amplification fragments were aligned to a reference AgNosCd-1 cassette sequence using SnapGene to determine proper integration.

Male and female lineage gene-drive dynamics. The role of maternal effects and/or targeted SpCas9 activity at the *Agcd* gene in somatic cells[1] was assessed by crossing males and females hemizygous (originating from an outcross) for the AgNosCd-1 cassette with WT mosquitoes of the opposite sex and the progeny scored for eye phenotype and presence/absence of the CFP marker gene. Progeny with WT color eyes (deep purple/black) and the CFP marker gene (CFP^+) were recovered as virgin males and females and crossed again to WT mosquitoes. Male and female founder lineages were maintained until the fourth generation of progeny had been screened or two successive generations of males had produced progeny.

Mass spectrometry. Metabolite quantification in the ommochrome biosynthetic pathway was carried out at the UCI Mass Spectrometry Facility on a Waters Aquity UPLC system and Waters Quattro Premier XE mass spectrometer using an Aquity UPLC CSP C18 1.7μM column and guard column. Metabolite extraction was done by submerging 10 adult mosquitoes (WT and AgNosCd-1 males and females) in 500 uL of methanol with 1% formic acid (v/v) at 25^{o}C for 24 hours. The solvent was centrifuged 5 minutes at 15,000 rpm, the recovered supernatants diluted 20-fold in 30% methanol and internal standards added prior to HPLC-MS/MS analysis. Analyses were performed in positive ion mode over a 5-minute run time with a flow rate of 0.3 mL/minute. Column and source temperatures were 50°C and 120°C, respectively. Gradient elution used solution A (H_20, 2% HCN, 0.2% acetic acid) and solution B (acetonitrile, 0.2% acetic acid) from 90% A/10% B over 0-3 minutes, 10% A/90% B over 3-4 minutes, and 90% A/10% B over 4-5 minutes. Triplicate quantification were performed with QuanLynx software, utilizing standard curves and internal standards. Tryptophan (TRP), kynurenine (KYN), 3-hydroxykynurenine (3-HK), kynurenic acid (KYNA), and xanthurenic acid (XA) standards were purchased from Sigma Aldrich (St. Louis, MO); tryptophan d3 (TRP-d3) and kynurenic acid d5 (KYNA-d5) from CDN Isotopes (Quebec, Canada); and 4-chlorokynurenine (4-ClKYN) from Toronto Research Chemicals (Ontario, Canada). All standards, solvents and reagents used were HPLC-quality.

Tear Intercrosses. The total mosaic progeny (tear) from G_3 (11 males, 31 females), G_4 (18 males, 29 females), and G_5 (11 males, 38 females) of the AgNosCd-1 cage trial in addition to 14 males and 16 females of the maternal-effect experiment, were intercrossed, with each separate generation representing an experimental

replicate. Mosaic phenotype mosquitoes were identified during pupation as part of the screening protocol for the cage trial and general screening for the maternal-effect experiment. All individuals from each replicate were mated in a single small cage (5000 cm^3)[4] for 3-5 days. Gravid females oviposited for 3 days before eggs were collected. Early pupae were screened for CFP fluorescence and eye color. Hemizygous (CFP^+/WT) and WT (CFP^-) phenotypes were analyzed using Sanger sequencing. A total of 50 homozygous cardinal eye (red), CFP^+ individuals (CFP^+/cd) were selected randomly from each of the four replicates for ddPCR analysis to screen for hidden insertion/deletion (indel) alleles, with Sanger sequencing performed on any indels found. All G_0 mosquitoes were individually assayed using ddPCR to assess indel frequency. DNA extractions of adults and pupae were performed using the Sigma Aldrich Extract-N-Amp Tissue PCR kit.

Digital Droplet PCR (ddPCR) drop-off Assay. Reactions were prepared as described in Carballar-Lejarazú et al.[4]. Briefly, reactions of 12.5 µL Bio-Rad ddPCR 2X Master Mix for probes (no UTP), 10 µL DNA (1 ng/µL), 1.25µL FAM/Forward (5 µM FAM probe, 18 µM forward primer), and 1.25 µL HEX/Reverse (5 µM HEX probe, 18 µM reverse primer) were prepared in a 96 well PCR plate. An estimated 30,000 haploid genome copies per 20 µL reaction were used assuming one *An. gambiae* haploid genome is 0.27 pg[5]. Droplets were generated using a Bio-Rad QX200 Droplet Generator (UCI Genomics High Throughput Facility) and transferred to a Bio-Rad 96 well PCR plate and heat-sealed with foil at 180°C for 5 seconds. Amplification was performed using a Bio-Rad C1000 Touch™ Thermal Cycler with 96-deep well reaction module with the following conditions: 95°C for 10 minutes; 40 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 60°C for 2 minutes; and a final extension at 98°C for 10 minutes. A 2°C/second ramp rate was used for all steps. Droplets were read using a Bio-Rad QX200 Droplet Digital PCR (ddPCR) System. Data analysis was performed using the Bio-Rad Quantasoft Analysis Pro (version 1.0.596) in "Drop Off" experiment type requiring manual cluster designation.

Life table parameters. Life table parameters were assessed in AgNosCd-1 [hemizygous (AgNosCd-1-OX) and homozygous (AgNosCd-1-IX) mosquitoes and compared with the WT strain. Each experiment was performed in triplicate unless specified otherwise.

Larva/pupa development: Larval and pupal developmental time periods were recorded as the number of days it took for second-instar (L2) larvae to pupate and then form adults. One hundred hemizygous, homozygous or WT L2 were placed in containers with 400 ml of water and fed once a day. The larvae in each container were counted daily and survivors recorded. Individual pupae were removed, the sex ratio determined for each replicate and the animals placed in 6-ounce (~177 cm^3) containers and monitored daily until adulthood.

Adult longevity: One hundred hemizygous, homozygous or WT newly-eclosed adult mosquitoes (fifty males and fifty females) were maintained in a colony cage and supplied with cotton balls moistened with a 1 M sucrose solution. Dead mosquitoes were collected and recorded daily until all mosquitoes had died.

Female fecundity/fertility: A total of 150 one to three-day old hemizygous, homozygous or WT virgin females were mated with 15 one to three-day old virgin WT males. Females were offered a single blood meal after two days of mating, and non-engorged females were removed from the cage and excluded from analysis. After three days, fed females were placed into individual containers (16-ounce, ~473 cm^3) and provided with an egg cup for oviposition. The numbers of eggs laid and hatched were counted for each individual. Individuals that did not lay eggs were excluded from analysis. Fecundity was calculated as the number of eggs laid per female and fertility was calculated as the percentage of eggs hatched per female.

Male contribution: Male contribution to the next generation in a competitive environment was assessed by providing 150 one to three-day old virgin WT females either 75 AgNosCd-1-IX (homozygous) or 75 AgNosCd-1-OX (hemizygous) virgin males and 75 WT virgin males. Males were age-matched to the day and allowed three days to mate with females. After the mating period, females were provided with two successive days of blood meals using the artificial feeder. After feeding, females were allowed two days to complete their gonotrophic cycle and then provided an egg cup for oviposition for three days. A minimum of 1,000 larvae from each mating event were screened for the presence/absence of CFP fluorescence and the total number of CFP positive and negative mosquitoes were recorded. Experiments were performed with a minimum of four replicates for each mating and data tested using X^2 with df = 1.

Statistics: Data analyses were performed using R statistical software (version 3.5.2, stats and pgirmess v1.6.9 packages). The Wilcoxon/Kruskal-Wallis nonparametric test was used to compare each strain for fecundity, fertility and larval/pupal development time period because the data did not follow a normal distribution. The Mann-Whitney nonparametric test was used to compare each strain for larval viability (independent variable) and pupae sex ratio (dependent variable). Adult survivorship was analyzed using the Wilcoxon test.

Cage trials. Triplicate (1-3) 5000 cm^3 cages were seeded with three single release ratios of homozygous AgNosCd-1-IX to WT adult males (A series: AgNosCd-1-IX:WT): 1:1 (75:75), B series: 1:3 (37:111), and C series: 1:9 (15:135) and WT adult females added to each cage to achieve an equal male:female ratio with a total population of ~300 individuals per cage[6]. Adults were blood-fed over two consecutive days, dead adults removed three days later, and an egg cup provided for two consecutive days. Larvae were hatched from the oviposition containers and 300 L2 selected randomly to establish the subsequent generation. Six hundred L2 were selected randomly to screen the larval and pupal eye phenotypes and the rest of the population reared to L4 and stored in 100% ethanol for population counts and molecular analysis. Cage trials were carried out for eleven non-overlapping generations.

Eye phenotype screening. The 600 randomly-selected individuals were screened as L4 for CFP fluorescence (CFP^+ or CFP^-) and separated in two containers; pupae emerging from each container were screened for eye color: CFP^+/WT^+ (CFP-positive drive mosquitoes with deep purple/black eyes [WT]); CFP^+/cd^- (CFP-positive drive mosquitoes with red eyes); CFP^+/cd^{mos} (CFP-positive drive mosquitoes with mosaic [tear] eyes).

Population count: Larvae in excess of the 300 and 600 used to re-establish the colony or reared to screen for eye color phenotypes were counted in bulk to determine population size at each generation.

Variability of *Agcd* target site. Whole genome sequencing data of individual mosquito were generated for 120 *An. gambiae sensu stricto* samples from natural populations collected in Mali (N=46), Cameroon (N=5), Tanzania (N=6), Zambia (N=7) and the Union of Comoros (N=56). Additionally, 100 samples from the closely-related species, *An. coluzzii*, from natural populations collected in Mali (N=66), Benin (N=11), Equatorial

Guinea (N=3), Cameroon (N=1) and São Tomé and Príncipe (N=19) were screened. Specimens were taken from the UC Davis Vector Genetics Laboratory's (VGL) archive and their genomic DNA sequenced on an Illumina HiSeq 4000 instrument. Quality-filtering of raw sequences for adapters was performed with Trimmomatic v0.36 (Bolger et al. 2014). Mapping of the short sequence reads used BWA MEM (Burrows-Wheeler Alignment MEM tool) v0.7.17-r1188[7] against the most current reference genome assembly 'AgamP4'[8]. Single nucleotide polymorphisms (SNPs) were called using Freebayes v1.2.0[9] applying default parameters but with theta = 0.01 and max-complex-gap = 3. No subsequent quality filtering of detected SNPs was conducted to ensure having the broadest possible set of potential variants available while being as conservative as possible.

The publicly available polymorphism data from the *Anopheles gambiae* 1000 Genomes Consortium (AG1000G,2 AR1 data release)[10] comprising calls from whole-genome sequence data from 590 *An. gambiae* specimens (N=81 from Burkina Faso, N=275 from Cameroon, N=56 from Gabon, N=31 from Guinea, N=44 from Kenya, N=103 from Uganda) was used[10]. An additional 129 *An. coluzzii* specimen were included (N=60 from Angola, N=69 from Burkina Faso). No filtering of polymorphism data was applied. VCF files of polymorphism data from both sources were used to screen the target site by genomic coordinates. Gene amplification reactions verifying SNPs utilized the primers HP6E4-F1 and HP6E4-R1 (Table S1).

SpCas9 cleavage assay *in vitro* of WT and SNP variant target sites. An assay *in vitro* tested the ability of the gRNA/SpCas9 complex to cleave the target site containing SNPs found in wild populations of African *An. gambiae*. Single-stranded oligonucleotides having the desired target sequences were obtained from Sigma Aldrich (card-target series primers: wt F and R, F1 through F5 and R1 through R5; Table S2). Forward and reverse oligonucleotide pairs for each DNA target sequence version were annealed and used in amplification reactions utilizing DreamTaq Polymerase (Thermo Fisher Scientific) (95°C for 30 seconds; 30 cycles of 95°C

30 seconds, 65°C for 15 seconds, 72°C for 5 seconds; and a final extension for 72°C for 10 seconds) to amplify double-stranded DNA fragments that then were cloned into the pCR4-TOPO vector (Invitrogen) via the TA cloning strategy. The target plasmids were sequenced to verify their integrity. A total of 200-500 ng of each test target plasmid (pCO-58 to pCO-62, pCO-66 to pCO-68, and pCO72 to pCO74) and the control plasmid (pCO-57) were linearized with *Nco*I at 37°C for 45 minutes, mixed with 300 ng of SpCas9/cardinal-gRNA mix that had previously been kept at 25°C for 10 minutes and finally incubated at 37°C for 26 minutes. Each reaction was treated with 1µl of Proteinase K and allowed to incubate at room temperature for 10 minutes before proceeding with agarose gel analysis.

Off-target site analyses. The CRISPR software packages Synthego (https://design.synthego.com/#/validate), ChopChop (http://chopchop.cbu.uib.no/), CRISPR RGEN (http://www.rgenome.net/cas-offinder/) and CRISPOR (http://crispor.tefor.net/) were used to obtain a comprehensive prediction of the potential off-targets to the *Agcd* target sequence in the *An. gambiae* genome. The different predictions were compared and combined to yield a consensus final prediction. *Anopheles gambiae*-specific genomic DNA sequences were obtained from Vectorbase[1] (www.vectorbase.org). *A*nalysis *in silico* of DNA sequences and design of primers were performed using the Snapgene (www.snapgene.com) bioinformatics software. All primers designed (off-1-EZ-F and off-1-EZ-R, Table S1) were synthesized by Sigma-Aldrich (www.sigmaaldrich.com). Extraction of genomic DNA from mosquitoes was performed using the Wizard Genomic DNA Extraction Kit (Promega). Genomic DNA was extracted from randomly selected homozygous AgNosCd-1. A total of 253 individuals were used: 25 generation 8 (G_8) individuals (5 males; 20 females), 52 G_9 individuals (20 males; 32 females) and 176 G_{10} individuals (84 males; 92 females). AgNosCd-1 mosquitoes were verified by CFP epifluorescence as well as by molecular validation prior to utilization. All plasmids were constructed using pCR4-TOPO (Invitrogen) as the backbone (Table S2). For plasmids pCO-66, 67 and 68, single-stranded forward and reverse DNA primers; card-offtarget-F1 and card-offtarget-R1; card-offtarget-F3 and card-offtarget-R3; card-target-wt-noPAM-F and

card-target-wt-noPAM-R, containing the target sequences were used in amplification reactions utilizing the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), and the amplicons purified and cloned into the pCR4-TOPO vector backbone by TA cloning. Plasmids pCO-72, 73 and 74 were generated using primers off-2_F1 and off-2_R1 in an amplification reaction with genomic DNA template. Plasmids were sequenced to confirm their integrity. The plasmids were used for the SpCas9 cleavage assay *in vitro* as described previously. SpCas9 activity *in vivo* at the predicted off-target sites was assessed by amplification of the genomic regions surrounding the predicted sites, sequencing the products and analyzing the sequences obtained for the presence of indels. Sanger sequencing was performed to assess the predicted off-target sites for each of 25 G_8 homozygous AgNosCd-1individuals and the WT controls. Amplicon EZ deep sequencing was used for off-target 1 analysis of G_9 and G_{10} individuals. For these latter two generations, the individual male samples from each respective generation were pooled and sequenced as one group, while the individual female samples also were pooled and sequenced as one group. Primers off-1-EZ-F and off-1-EZ-R were used to amplify the regions around the off-target 1 site from samples for deep sequencing.

Sequencing mosquito samples.

DNA extractions: A total of 125µL of 4:1 extract:tissue preparation mix (Sigma Aldrich Extract-N-Amp Tissue PCR kit) was used for pools of five mosquitoes and 62.5µL for single mosquitoes.

Sanger sequencing: Amplification reactions comprising 2 µL of Sigma DNA extract, 12.5µL NEB Phusion High Fidelity Polymerase, 1.25 µL of 10µM each primer Cd_AmpF/R (Table S1), and 8 µL PCR-grade water were incubated at 98°C for 3 minutes; 35 cycles of 98°C for 30 seconds, 66.3°C for 30 seconds and 72°C for 30 seconds; followed by 72°C for 10 minutes and a 4°C hold. Reactions were resolved in 2% agarose gels for 15-30 minutes at 139V, the ~400 bp band was excised and DNA extracted using the Zymo Gel Extraction kit. Purified products were sequenced by Genewiz (La Jolla, CA, USA).

Next Generation Sequencing (NGS): Dual 50 µL amplification reactions were prepared for each sample using 4µL of Sigma DNA extract or 1 µL Promega DNA extract, 25 µL NEB Phusion High Fidelity Polymerase,

2.5µL of 10µM each primer Cd_SeqF1/R1 (Table S1), and PCR-grade water. Samples were incubated at 98°C for 3 minutes; 35 cycles of 98°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds; followed by 72°C for 10 minutes and a 4°C hold. Sample were purified using the Zymo DNA Clean and Concentrator kit and the products sequenced by Genewiz (La Jolla, CA, USA).

Table S1. List of oligonucleotides and primers.

Name of primer	Sequence (5'– 3')
card-F1	CTGGAACAGCCGCTCGTCGT
card-R1	CGGTACCGGCACCGTAGGCACC
Agcard-target_F	TTCCCCTCGTCCTTGGTTAGCGACGATGCCAAGGGTTTTAGAGCTAGAA
Agcard-target_R	TTCTAGCTCTAAAACCCTTGGCATCGTCGCTAACCAAGGACGAGGGGAA
AflII-cardhm2_F	TGACTTAAGTGGCATCGTCGCTAACCGCCGT
AatII-cardhm2_R	GAGGACGTCCGGTACCGGCACCGTAGGCACC
SbfI-cardhm1_F	ATTCCTGCAGGCTGGAACAGCCGCTCGTCGT
PacI-cardhm1_R	GTATTAATTAAAGGCGGCCGCCATAGCGGATG
Inf_Agnos-Cas9_F	TACGCGTACGGCGCGCCGGCCGTGTGGTTTAACCCACCA
Inf_Agnos-Cas9_R	GTCTCTACGGGCGCGCCATAACTTCGTATAGCATACAT
card-target-wt-F	TAGTTGACTGAGTACGGCGGTTAGCGACGATGCCAAGGCGG[1]
card-target-wt-R	CCGCTATGGCGGCCGCCTTGGCATCGTCGCTAACC[1]
card-target-F1	TAGTTGACTGAGTACGGCGGTTAGCGACGATGCCAAGGCG**A**[1]
card-target-R1	CCGCTATGGCGG**T**CGCCTTGGCATCGTCGCTAACC[1]
card-target-F2	TAGTTGACTGAGTACGGCGGTTAGCGACGATG**A**CAAGGCGG[1]
card-target-R2	CCGCTATGGCGGCCGCCTTG**T**CATCGTCGCTAACC[1]
card-target-F3	TAGTTGACTGAGTACGGCGGTTAGCGACGA**A**GCCAAGGCGG*
card-target-R3	CCGCTATGGCGGCCGCCTTGGC**T**TCGTCGCTAACC[1]
card-target-F4	TAGTTGACTGAGTACGGCGGT**A**AGCGACGATGCCAAGGCGG[1]
card-target-R4	CCGCTATGGCGGCCGCCTTGGCATCGTCGCT**T**ACC[1]
card-target-F5	TAGTTGACTGAGTACGGC**A**GTTAGCGACGATGCCAAGGCGG[1]
card-target-R5	CCGCTATGGCGGCCGCCTTGGCATCGTCGCTAAC**T**[1]
card-offtarget-F1	tagttgactgaGTACGGCGGTTA**A**C**A**ACGAAACC**AA**GG**T**GG[1,2]
card-offtarget-R1	CCGCTATGGCGGCC**A**CC**TT**GGTTTCGT**T**G**T**TAACC[1]
card-offtarget-F3	tagttgactgaGTACGGCGGTTAGCGA**A**GATG**G**CA**TT**G**T**GG[1,2]

card-offtarget-R3	CCGCTATGGCGGCCACAATGCCATCTTCGCTAACC[1]
card-target-wt-noPAM-F	tagttgactgaGTACGGCGGTTAGCGACGATGCCAAGGCCC[1,2]
card-target-wt-noPAM-R	CCGCTATGGCGGGGGCCTTGGCATCGTCGCTAACC[1]
off-1_F1	GTACTGGTGCTAAAGCTCACGAGAG
off-1_R1	CTTTGCTTCCAGTGCTGCCGTAATG
off-2_F1	GTAGCGCGCACCCCGAGAACAC
off-2_R1	CCGGGCTCGGCGGCTTCGGTTG
off-3_F1	ACGGTGCCGTCGGACGGACCGG
off-3_R1	GTTCGTGAAGGTGATCGCGGT
off-1-EZ-F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACAAGTGGATGATATCCCTGAACA
off-1-EZ-R	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGTGCCTGTCTCGGCTGCTGTATG
card-seqF1	CGATATGCCATCGCCATAG
eCFPseqR	GCCTTGATGCCGTTCTTCT
AgU6 seqF	CCCGAGCACACACTTTCTTGC
card-seqR1	GTGCATTTGCGCTGGCGATCGT
upHM1cdn-F	CAAACTCGGCGTACGTGAT
dwnHM2cdn-R	GGACTGGTCGTCGACACATCT
card F2	GCAAGGCTGTTGTGGTGCCGG
AgU6-gRNA-F	CGTGCAATGTTCTTTCGCAACC
Cd_Amp_F	GTACTCGTACGGTCGCTCCTTA
Cd_Amp_R	ATTGTTGTTGCAGATGAGTCGT
ddPCR_F	AAGGAGGGCGAGTTAAG
ddPCR_R	TGTTGTTGCAGATGAGTC
HP6E4-F1	CGCAC CGGCAAATCCATC
HP6E4-R1	GCAGATGAGTCGTCACCCGAG

1. Wild-type, variant and potential off-target gRNA target sites underlined with the polymorphic nucleotides shown in bold red.
2. Lower case nucleotides in card-offtarget-F1, card-offtarget-F3 and card-target-wt-noPAM-F prevent expression of the lethal gene, *ccdB* in the plasmid vector pCR4-TOPO. This cloning plasmid uses expression of this gene to kill insert-negative clones, instead of the classical blue/white selection for positive clones. The *cardinal* target sequences cloned into the vector are short and the inserted nucleotides prevent read-through translation following transcription to ensure successful cloning.

Table S2. List of plasmids.

Name of plasmid	Description
pCO-13 (pBSK_U6-BbsI-gRNA scaffold)	
pCO-16 (pHM1_3xp3-CFP_U6-pugilist gRNA_pHM2)	
pCO-18(pB_Agnos-Cas9-T2A-DsRed_3XP3CFP)	
pCO-33 (U6-cardinal gRNA)	
pCO-35 (pHM1_3xp3-CFP_U6-cardinal gRNA_cHM2)	
pCO-36 (cHM1_3xp3-CFP_U6-cardinal gRNA_cHM2)	
pCO-37 (cHM1_3xp3-CFP_Agnos-Cas9-T2A-DsRed_U6-cardinal gRNA_cHM2)	AgNosCd1 gene-drive plasmid
pCO-57, pCR4-TOPO_cardinal (GGTTAGCGACGATGCCAAGGCGG)*	Wild-type, on-target positive control
pCO-58, pCR4-TOPO_cardinal SNP1 (GGTTAGCGACGATGCCAAGGCG**A**)*	Wild-type SNP variant, SNP in PAM
pCO-59, pCR4-TOPO_cardinal SNP2 (GGTTAGCGACGATG**A**CAAGGCGG)*	Wild-type SNP variant
pCO-60, pCR4-TOPO_cardinal SNP3 (GGTTAGCGACGA**A**GCCAAGGCGG)*	Wild-type SNP variant
pCO-61, pCR4-TOPO_cardinal SNP4 (GGT**A**AGCGACGATGCCAAGGCGG)*	Wild-type SNP variant
pCO-62, pCR4-TOPO_cardinal SNP5 (**A**GTTAGCGACGATGCCAAGGCGG)*	Wild-type SNP variant
pCO-66 (GGTTA**A**C**A**ACGA**AA**CCAAGG**T**GG)*	Off-target 1 variant
pCO-67 (GGTTAGCGA**A**GATG**G**CA**TT**G**T**GG)*	Off-target 3 variant
pCO-68 (GGTTAGCGACGATGCCAAGG**CCC**)*	No PAM, on-target negative control
pCO-72 (GGTTAGC**C**A**G**G**CG**GCCAAGG**T**GG)*	Off-target 2-1 variant
pCO-73 (GGTTAGC**C**A**G**G**CA**GCCAAGG**T**GG)*	Off-target 2-2 variant
pCO-74 (GGTTAGC**C**A**G**G**CG**GCCAAG**AT**GG)*	Off-target 2-3 variant

*(5'-3'target sequence + PAM, nucleotide variants from the wild-type target sequence are shown in bold red).

Table S3. Gene drive frequencies and eye phenotypes from male- and female-derived AgNosCd-1 lineages.

Generation	Male founder lineage drive efficiency[1,2]			Female founder lineage drive efficiency[1,2]				
F_1	♂ 97.8% (895/915)			♀ 96.2% (769/799)				
F_2	♂ 97.3% (918/943)	♀ 97.9% (559/571)		♂ 95.7% (381/398)		♀ 85.0% (164/193)		
F_3	--	♂ 98.3% (914/930)	♀ 95.3% (344/361)	♂ 99.3% (664/669)	♀ 95.9% (445/464)	♂ 98.2% (490/499)	♀ 96.2% (930/967)	
F_4	--	--	♂ 98.4% (362/368)	--	♂ 99.1% (112/113)	♀ 92.4% (73/79)	♂ 100% (222/222)	♀ 90.6% (125/138)
Generation	**Male founder lineage cardinal eye[3]**			**Female founder lineage cardinal eye[3]**				
F_1	♂ 0% (0/915)			♀ 0% (0/799)				
F_2	♂ 0% (0/943)	♀ 0.3% (2/571)		♂ 0% (0/398)		♀ 0% (0/193)		
F_3	--	♂ 0% (0/930)	♀ 0% (0/361)	♂ 0% (0/669)	♀ 0% (0/464)	♂ 0% (0/499)	♀ 0.1% (1/967)	
F_4	--	--	♂ 0% (0/368)	--	♂ 0% (0/113)	♀ 1.3% (1/79)	♂ 0% (0/222)	♀ 1.4% (2/138)
Generation	**Male lineage tear eye[4]**			**Female lineage tear eye[4]**				
F_1	♂ 0% (0/915)			♀ 7% (53/799)				
F_2	♂ 0% (0/943)	♀ 0.3% (2/571)		♂ 0% (0/398)		♀ 0% (0/193)		
F_3	--	♂ 0.1% (1/930)	♀ 3.9% (14/361)	♂ 0.9% (6/669)	♀ 8.2% (38/464)	♂ 1.0% (5/499)	♀ 6.0% (58/967)	
F_4	--	--	♂ 0.3% (1/368)	--	♂ 0.9% (1/113)	♀ 6.8% (5/79)	♂ 0.5% (1/222)	♀ 3.0% (4/138)

1. Symbols refer to the sex of the gene-drive parents: ♂ male gene-drive parents; ♀ female gene-drive parents.
2. Drive efficiency was determined by calculating the percentage of progeny expressing the CFP marker gene out of the total originating from the mating event.
3. Eye phenotype was determined by calculating the percentage of heteroallelic progeny containing one copy of the drive element (CFP^+) and one resistant mutant *cardinal* (cd^-) allele out of the total originating from the mating event.
4. Eye phenotype was determined by calculating the percentage of progeny showing the tear phenotype (Figure S3) out of the total originating from the mating event.

--, Not determined.

Table S4. Insertion and deletions discovered using next-generation sequencing of genome *cardinal* gRNA target sites in tear-eye phenotype mosquitoes.

Target Sequence[1]	Reads	Type	% Reads[2]	Indel Size
CCTTGGCATCGTCGCTAACC	62148	WT	56.55	0
----GGCATCGTCGCTAACC	8045	Deletion	7.32	-4
-----GCATCGTCGCTAACC	6642	Deletion	6.04	-5
CC------TCGTCGCTAACC	3695	Deletion	3.36	-6
CC---------TCGCTAACC	3374	Deletion	3.07	-9
CCT-------GTCGCTAACC	2949	Deletion	2.68	-7
------------CGCTAACC	1875	Deletion	1.71	-12
CCT--------TCGCTAACC	1860	Deletion	1.69	-8
CCT----ATCGTCGCTAACC	1704	Deletion	1.55	-4
CCT-----TCGTCGCTAACC	1274	Deletion	1.16	-5
CCT----------GCTAACC	950	Deletion	0.86	-10
CC-TGGCATCGTCGCTAACC	862	Deletion	0.78	-1
CCT-----------------	738	Deletion	0.67	-17
----------GTCGCTAACC	627	Deletion	0.57	-10
CCT---CATCGTCGCTAACC	594	Deletion	0.54	-3
----------------CC	592	Deletion	0.54	-18
CCT----------CTAACC	472	Deletion	0.43	-11
----AGCATCGTCGCTAACC	438	Deletion	0.39	-4
---TGGCATCGTCGCTAACC	438	Deletion	0.39	-3
---------CGTCGCTAACC	357	Deletion	0.32	-9
------------GCTAACC	311	Deletion	0.28	-13
----GTCATCGTCGCTAACC	299	Deletion	0.27	-4
-------ATCGTCGCTAACC	297	Deletion	0.27	-7
--------TCGTCGCTAACC	245	Deletion	0.22	-8
----------TCGCTAACC	228	Deletion	0.21	-11
CCT-------GCCACTAACC	227	Deletion	0.20	-7
CCTT----TCGTCGCTAACC	225	Deletion	0.20	-4
C----------CTAACC	220	Deletion	0.20	-13
------CATCGTCGCTAACC	216	Deletion	0.19	-6
CCT--GCATCGTCGCTAACC	215	Deletion	0.19	-2
----------------AACC	212	Deletion	0.19	-16
CC-TCGCATCGTCGCTAACC	194	Deletion	0.18	-1
---------------TACGC	177	Deletion	0.16	-15
CCT----------TAACC	177	Deletion	0.16	-12
C--------CGTCGCTAACC	162	Deletion	0.15	-8
----CGCATCGTCGCTAACC	134	Deletion	0.12	-4
--------TTGTCGCTAACC	116	Deletion	0.11	-8
C-----CATCGTCGCTAACC	115	Deletion	0.10	-5
----GGCCTCGTCGCTAACC	107	Deletion	0.097	-4

C-------------------	95	Deletion	0.086	-19
CCTAtGCCA------CTAACC	90	Insertion and Deletion	0.082	-5
CC--GGCATCGTCGCTAACC	87	Deletion	0.079	-2
CCTCagCGCATCGTCGCTAACC	87	Insertion	0.079	2
----------CCGCTAACC	85	Deletion	0.077	-11
----------------ACC	83	Deletion	0.075	-17
CC------------------	80	Deletion	0.073	-18
---TCGCATCGTCGCTAACC	79	Deletion	0.072	-3
----GGTTTTTTCGCTAACC	74	Deletion	0.067	-4
C---GGCATCGTCGCTAACC	73	Deletion	0.066	-3
C----------CGCTAACC	68	Deletion	0.062	-11
CCTCtGGCATCGTCGCTAACC	68	Insertion	0.062	1
TC------------------	67	Deletion	0.061	-18
CCT--------------ACC	64	Deletion	0.058	-14
CC---GCATCGTCGCTAACC	63	Deletion	0.057	-3
---ACGCATCGTCGCTAACC	61	Deletion	0.055	-3
CCTAtggcGGCATCGTCGCTAACC	61	Insertion	0.055	4
C--------CATAGCTAACC	60	Deletion	0.054	-8
------------GCTA---	57	Deletion	0.052	-16
-CT----ACtCGTCGCTAACC	57	Insertion and Deletion	0.052	-4
C-----AATCGTCGCTAACC	52	Deletion	0.047	-5
CtaaccgccacC-------CGTCGCTAACC	51	Insertion and Deletion	0.046	3
------CATCG-CGCTAACC	49	Deletion	0.044	-7
-CATGCCATCCggCCGCTAACC	47	Insertion and Deletion	0.043	1
CCTCgccGGCATCGTCGCTAACC	47	Insertion	0.043	3
-------------------C	46	Deletion	0.042	-19
--ATGGCATCGTCGCTAACC	46	Deletion	0.042	-2
--TT----------CTAACC	46	Deletion	0.042	-12
CCT--------------CC	45	Deletion	0.041	-15
CCTT--CATCGTCGCTAACC	45	Deletion	0.041	-2
--------------TAACC	44	Deletion	0.040	-15
CC-TGCCA-----GCTAACC	44	Deletion	0.040	-6
----------TAGCTAACC	43	Deletion	0.039	-11
-CTTACCTT--TCTatgCTAACC	43	Insertion and Deletion	0.039	0
CTatcctgcTAtGGCATCGTCGCTAACC	43	Insertion	0.039	8
CCTT-------TCGCTAACC	40	Deletion	0.036	-7
TCTTG--ATCGTCGCTAACC	40	Deletion	0.036	-2
---------GGTCGCTAACC	39	Deletion	0.035	-9
----GG----ATCGCTAACC	37	Deletion	0.034	-8
CC-TCGCGTCGTCGCTAACC	37	Deletion	0.034	-1
---------CATCGCCATCC	36	Deletion	0.033	-9
------TATCGTCGCTAACC	36	Deletion	0.033	-6
--TCGGCATCGTCGCTAACC	36	Deletion	0.033	-2
CGAT--AACCGTTccGCTAACC	36	Insertion and Deletion	0.033	0

CC---------------AGC	34	Deletion	0.031	-15
CCTAtGGCATCGTCGCTAACC	33	Insertion	0.030	1
CCTT---------GCTAACC	33	Deletion	0.030	-9
C--------GGTCGCTAACC	30	Deletion	0.027	-8
CCT----------------C	30	Deletion	0.027	-16
C---GGTACCGgTCGCTAACC	29	Insertion and Deletion	0.026	-2
CCTT---ATCGTCGCTAACC	26	Deletion	0.024	-3
CCTtTGGCATCGTCGCTAACC	26	Insertion	0.024	1
C-----TATCGTCGCTAACC	25	Deletion	0.023	-5
CCGT-------------ACC	25	Deletion	0.023	-13
CTatccgctaTCCGCATCGTCGCTAACC	25	Insertion	0.023	8
--TT----------------	24	Deletion	0.022	-18
--------TAACCGCTAACC	23	Deletion	0.021	-8
---------AGTCGCTAACC	22	Deletion	0.020	-9
--TTAGCATCGTCGCTAACC	22	Deletion	0.020	-2
C----GCATCGTCGCTAACC	22	Deletion	0.020	-4
CCTGgggcatcgtccggGGCATCGTCGCTAACC	22	Insertion	0.020	13
CCTT------GTCGCTAACC	22	Deletion	0.020	-6
------------CG----CC	21	Deletion	0.019	-16
-CAAGGCATCGTCGCTAACC	21	Deletion	0.019	-1
AtggccgcatcCGTCGCATCGTCGCTAACC	21	Insertion	0.019	10
C--TGGCATCGTCGCTAACC	21	Deletion	0.019	-2
---------CATCGCTAACC	20	Deletion	0.018	-9
C----TAATCGTCGCTAACC	20	Deletion	0.018	-4
CCTTctCGCATCGTCGCTAACC	20	Insertion	0.018	2
-----------------ACG	19	Deletion	0.017	-17
------------ACTAATC	19	Deletion	0.017	-13
--TTTGCA----CGCTAACC	19	Deletion	0.017	-6
-CT----ATCGTCGCTAACC	19	Deletion	0.017	-5
CCTtTGTCGTCGTCGCTAACC	19	Insertion	0.017	1
-------------CTAACC	17	Deletion	0.015	-14
-----GCCTCGTCGCTAACC	17	Deletion	0.015	-5
-----GCGTCGTCGCTAACC	17	Deletion	0.015	-5
----GGCATCGCCGCTAACC	17	Deletion	0.015	-4
----GGCATCGGCGCTAACC	17	Deletion	0.015	-4
CCAT---ATCGTCGCTAACC	16	Deletion	0.014	-3
CCTCagtGAagCATCGTCGCTAACC	16	Insertion	0.014	5
CTATGGC--GGTCGCTAACC	16	Deletion	0.014	-2
-----GCATCGCCGCTAACC	14	Deletion	0.013	-5
----GGCTTCGTCGCTAACC	14	Deletion	0.013	-4
---TAACCT-GTCGCTAACC	14	Deletion	0.013	-4
C----------GGATAACC	14	Deletion	0.013	-11
CC---GTACCGTccccgtaccgtttCGCTAACC	14	Insertion and Deletion	0.013	11
---------------AA--	13	Deletion	0.012	-18

---------CGTC-------	13	Deletion	0.012	-16
CC-------CGTCGCTAACC	13	Deletion	0.012	-7
CCT----ACCGTCGCTAACC	13	Deletion	0.012	-4
CCT---AATCGTCGCTAACC	13	Deletion	0.012	-3
CCTAaccgccgcctAGCATCGTCGCTAACC	13	Insertion	0.012	10
CCTCGTcggcggcgCATCGTCGCTAACC	13	Insertion	0.012	8
----GCCATCGTCGCTAACC	12	Deletion	0.011	-4
CCTCgctaaccgccgctaaccGGCATCGTCGCTAACC	12	Insertion	0.011	17
CCTT--------------C	12	Deletion	0.011	-15
------------CGCTA---	11	Deletion	0.011	-15
----------ACCGCTAACC	11	Deletion	0.011	-10
----------ATCGCTAACC	11	Deletion	0.011	-10
------CAT-----------	11	Deletion	0.011	-17
-----ACATCGTCGCTAACC	11	Deletion	0.011	-5
----GACATCGTCGCTAACC	11	Deletion	0.011	-4
---AGGCATCGTCGCTAACC	11	Deletion	0.011	-3
--TTTATTTTCTCGCTCACC	11	Deletion	0.011	-2
-CTAtGG------CGCTAACC	11	Insertion and Deletion	0.011	-6
AC----------CGCTAACC	11	Deletion	0.011	-10
C-----GATGGTCGCTAACC	11	Deletion	0.011	-5
CCATCcgctatggcgGCATCGTCGCTAACC	11	Insertion	0.011	10
CCT---CGTCGTCGCTAACC	11	Deletion	0.011	-3
CTATGGTcATCGTCGCTAACC	11	Insertion	0.011	1
-----GAATCGTCGCTAACC	10	Deletion	0.009	-5
-----GCATCTTCGCTAACC	10	Deletion	0.009	-5
-CTAACCAT-ATCGCTAACC	10	Deletion	0.009	-2
AtcC---GCATCGTCGCTAACC	10	Insertion and Deletion	0.009	-1
CATAtGGCATCGTCGCTAACC	10	Insertion	0.009	1
CC----------CGCTAACC	10	Deletion	0.009	-10
CCGtT----------------	10	Insertion and Deletion	0.009	-15
CCT-------GCCGCTAACC	10	Deletion	0.009	-7
CCTCGG--TCGTCGCTAACC	10	Deletion	0.009	-2
CCTTG-------CGCTAACC	10	Deletion	0.009	-7
CtaacC---GCACCGTCGCTAACC	10	Insertion and Deletion	0.009	1
------------CGCAAACC	9	Deletion	0.0081	-12
------------CTCTAACC	9	Deletion	0.0081	-12
---TGAaagaaacacgaatcgaaag-ATCGTCGCTAACC	9	Insertion and Deletion	0.0081	15
-CT----AT-GTCGCTAACC	9	Deletion	0.0081	-6
ACT-------GTCGCTAACC	9	Deletion	0.0081	-7
C-------TCGTCGCTAACC	9	Deletion	0.0081	-7
CC---------CCGCTAACC	9	Deletion	0.0081	-9
GCTCGGCTaTGgGTCGCTAACC	9	Insertion	0.0081	2
--------------CTA---	8	Deletion	0.0072	-17
------CACCAT-GCTAACC	8	Deletion	0.0072	-7

------CATC--CGCTAACC	8	Deletion	0.0072	-8
-----GCATCGGCGCTAACC	8	Deletion	0.01	-5
----GGAATCGTCGCTAACC	8	Deletion	0.01	-4
----GGCAGCGTCGCTAACC	8	Deletion	0.01	-4
----TGCATCGTCGCTAACC	8	Deletion	0.01	-4
-CT---AATCCggTCGCTAACC	8	Insertion and Deletion	0.01	-2
C-----CGTCGTCGCTAACC	8	Deletion	0.01	-5
CAgcacATCGCATCGTCGCTAACC	8	Insertion	0.01	4
CCT-------GATGCCAACC	8	Deletion	0.01	-7
CCT--CCA-----GCTAACC	8	Deletion	0.01	-7
CCTAccgt----TCGTCGCTAACC	8	Insertion and Deletion	0.0072	0
CCTT----------CTAACC	8	Deletion	0.0072	-10
CCTTAagG----GTCGCTAACC	8	Insertion and Deletion	0.0072	-2
CTaacctggggcTTTCCAGgCGTCGCTAACC	8	Insertion	0.0072	11
CTATGG--------------	8	Deletion	0.0072	-14
------------GCCAACC	7	Deletion	0.0063	-13
-----------TCGCCATCC	7	Deletion	0.0063	-11
---------CGTAtGCGGATC	7	Insertion and Deletion	0.0063	-8
--------ACGTCGCTAACC	7	Deletion	0.0063	-8
--------TAcGTCGCTAACC	7	Insertion and Deletion	0.0063	-7
-----ATATCGTCGCTAACC	7	Deletion	0.0063	-5
-----GCTTCGTCGCTAACC	7	Deletion	0.0063	-5
-----GTATCGTCGCTAACC	7	Deletion	0.0063	-5
-----TCATCGTCGCTAACC	7	Deletion	0.0063	-5
----GGCATCG---------	7	Deletion	0.0063	-13
----GGCGTCGTCGCTAACC	7	Deletion	0.0063	-4
----GGTATCGTCGCTAACC	7	Deletion	0.0063	-4
---TAGCGCCGTCGCTAACC	7	Deletion	0.0063	-3
--AAGGCTaTC-ACGCTATCC	7	Insertion and Deletion	0.0063	-2
--TT--CACCGT--------	7	Deletion	0.0063	-12
-CTAtG-------CGCTAACC	7	Insertion and Deletion	0.0063	-7
-CTTG-----GTCGCTAACC	7	Deletion	0.0063	-6
CC---------GCGCTAACC	7	Deletion	0.0063	-9
CC------TCGGCGCTAACC	7	Deletion	0.0063	-6
CCAtccgctaTGGCATCGTCGCTAACC	7	Insertion	0.0063	7
CCT-----------ATAACC	7	Deletion	0.0063	-11
CCT----ATAGTCGCTAACC	7	Deletion	0.0063	-4
CCT----CTCGTCGCTAACC	7	Deletion	0.0063	-4
CCTcgtCGGCATCGTCGCTAACC	7	Insertion	0.0063	3
CCTGcgGGCATCGTCGCTAACC	7	Insertion	0.0063	2
CTaac--GCCAACGgTCGCTAACC	7	Insertion and Deletion	0.0063	2
---------------TA--T	6	Deletion	0.0054	-17
-----------TCGCCAACC	6	Deletion	0.0054	-11
---------TATGGCTA---	6	Deletion	0.0054	-12

--------TGGTC-------	6	Deletion	0.0054	-15
-------ACCGTCGCTAACC	6	Deletion	0.0054	-7
------AAAAGTCGGGAACC	6	Deletion	0.0054	-6
------TATAac-TCGCTAACC	6	Insertion and Deletion	0.0054	-5
-----GCAGCGTCGCTAACC	6	Deletion	0.0054	-5
----GGCATCGTCACTAACC	6	Deletion	0.0054	-4
---CAGCATCGTCGCTAACC	6	Deletion	0.0054	-3
---TGGCGgTTGTCGCTAACC	6	Insertion and Deletion	0.0054	-2
-ATTCaGCATCGTCGCTAACC	6	Insertion and Deletion	0.0054	0
AC---ACATCGTCGCTAACC	6	Deletion	0.0054	-3
C----------CGC--ACC	6	Deletion	0.0054	-13
C--------CGCCACTAACC	6	Deletion	0.0054	-8
CAc---GCATCGTCGCTAACC	6	Insertion and Deletion	0.0054	-2
CC--------------AACC	6	Deletion	0.0054	-14
CC-------GGTCGCTAACC	6	Deletion	0.0054	-7
CC----CA-----GCCAACC	6	Deletion	0.0054	-9
CC-TAACATCGTCGCTAACC	6	Deletion	0.0054	-1
CCCTCGCtaacATCGTCGCTAACC	6	Insertion	0.0054	4
CCgcTATGCATCGTCGCTAACC	6	Insertion	0.0054	2
CCT----AAC--CGCTAACC	6	Deletion	0.0054	-6
CCT---CA-----GCTAACC	6	Deletion	0.0054	-8
CCT---CCaaTCGTCGCTAACC	6	Insertion and Deletion	0.0054	-1
CCTAccGCCATCGTCGCTAACC	6	Insertion	0.0054	2
CCTAccgccgt-ACATCGTCGCTAACC	6	Insertion and Deletion	0.0054	6
CCTcgtCGGTATCGTCGCTAACC	6	Insertion	0.0054	3
CCTT----------TAACC	6	Deletion	0.0054	-11
CCTTAgcGCATCGTCGCTAACC	6	Insertion	0.0054	2
--------------CT---C	5	Deletion	0.0045	-17
------------ACTAACC	5	Deletion	0.0045	-13
------------CGCCAACC	5	Deletion	0.0045	-12
------------CGCTAACccgccccC	5	Insertion and Deletion	0.0045	-5
------------CGCTACCC	5	Deletion	0.0045	-12
------------CGCTGACC	5	Deletion	0.0045	-12
-------CTCGTCGCTAACC	5	Deletion	0.0045	-7
------AAAGGTCGCTAACC	5	Deletion	0.0045	-6
------CAACGgTCGCTAACC	5	Insertion and Deletion	0.0045	-5
------CATAACCGCTAACC	5	Deletion	0.0045	-6
------CGTCGTCGCTAACC	5	Deletion	0.0045	-6
------TATGGT--------	5	Deletion	0.0045	-14
------TATGGTCGCTAACC	5	Deletion	0.0045	-6
-----CCATCGTCGCTAACC	5	Deletion	0.0045	-5
-----GCATCGTCGCCAACC	5	Deletion	0.0045	-5
-----GCATCGTCGCTACCC	5	Deletion	0.0045	-5
-----GCATCGTCTCTAACC	5	Deletion	0.0045	-5

----GGCACCGTCGCTAACC	5	Deletion	0.0045	-4
----GGCATCGACGCTAACC	5	Deletion	0.0045	-4
---ATGCATCGTCGCTAACC	5	Deletion	0.0045	-3
---TAGCATCGTCGCTAACC	5	Deletion	0.0045	-3
---TGCCATAG-CGCTAACC	5	Deletion	0.0045	-4
---TGG----GTCGCTAACC	5	Deletion	0.0045	-7
--ATGCCATTGTAttttgtatccGAT----	5	Insertion and Deletion	0.0045	4
--TTAGC----------	5	Deletion	0.0045	-15
-CTAtGGCATCGTCGCTAACC	5	Insertion and Deletion	0.0045	0
AC--GGCATCGTCGCTAACC	5	Deletion	0.0045	-2
C---------------AGaCC	5	Insertion and Deletion	0.0045	-14
C----------TCGCTATCC	5	Deletion	0.0045	-10
C--------AGACGAT----	5	Deletion	0.0045	-12
C--------CATCGCTAACC	5	Deletion	0.0045	-8
C--------CGTC-------	5	Deletion	0.0045	-15
C----GGATCGTCGCTAACC	5	Deletion	0.0045	-4
C---GGCAT-----------	5	Deletion	0.0045	-14
CC------CCGTCGCTAACC	5	Deletion	0.0045	-6
CC------GCGTCGCTAACC	5	Deletion	0.0045	-6
CC------TCGCCGCTAACC	5	Deletion	0.0045	-6
CC---GTACCGTCGCTAACC	5	Deletion	0.0045	-3
CCATCcgctatggcggGCATCGTCGCTAACC	5	Insertion	0.0045	11
CCT-------GGCGCTAACC	5	Deletion	0.0045	-7
CCT-----TCGCCGCTAACC	5	Deletion	0.0045	-5
CCT---AACCGTCGCTAACC	5	Deletion	0.0045	-3
CCTActG--ATCGTCGCTAACC	5	Insertion and Deletion	0.0045	0
CCTCatGGCATCGTCGCTAACC	5	Insertion	0.0045	2
CCTGgcatagtGGCATCGTCGCTAACC	5	Insertion	0.0045	7
CCTT--------------CA	5	Deletion	0.0045	-14
CCTT--------------CC	5	Deletion	0.0045	-14
CCTTG--ATCGTCGCTAACC	5	Deletion	0.0045	-2
CCTTGCCA------CTAACC	5	Deletion	0.0045	-6
CTaacgcT---AACCGTCGCTAACC	5	Insertion and Deletion	0.0045	2
CTAT------GTCGCTAACC	5	Deletion	0.0045	-6
CTAT---AT-GTCGCTAACC	5	Deletion	0.0045	-4
CTATGgcgGCATCGTCGCTAACC	5	Insertion	0.0045	3
GCTAtggcGGCATCGTCGCTAACC	5	Insertion	0.0045	4
TCGCGG-ATCGTCGCTAACC	5	Deletion	0.0045	-1

1. Sequences are listed as the anti-sense strand. Deletions are represented as dashes (-) and insertions are lower-case.
2. Total reads = 109,903.

Table S5. ddPCR analysis of cage trial G_2 tear phenotypes.	
Sample	**Indel %**
Wild Type (pool)	0.217
Wild Type (single) - 1	0.189
Wild Type (single) - 2	0.271
Tear (pool)	22.6
Tear (single) - 1	10.8
Tear (single) - 2	16.1
Tear (single) - 3	35.8
Tear (single) - 4	30.6
Tear (single) - 5	47.5
Tear (single) - 6	2.21
Tear (single) - 7	3.53
Tear (single) - 8	30.2
Tear (single) - 9	7.43
Tear (single) - 10	15.7
10 tear individuals and a single pool of 5 were analyzed by ddPCR for fractional abundance of indel alleles. Wild-type samples serve as controls, containing minimal indel alleles.	

Table S6. Tear intercrosses.

General phenotype screening (a)

Replicate	CFP$^+$/cd	CFP$^+$/WT	CFP$^-$/cd	CFP$^-$/WT	Drive %	G_0 indel average %
IX-1	328	4	0	0	100.0	16.02
IX-2	360	55	0	1	99.76	6.03
IX-3	326	11	0	0	100.0	9.47
IX-4	495	11	0	0	100.0	11.9
Total	1509	81	0	1	99.99	10.55

Hemizygous sequencing (b)

Replicate	CFP$^+$/WT sequenced	Indels/SNPs	Sequence change 5' – 3' WT: **GGTTAGCGACGATGCCAAGGCGG**
IX-1	4	0/0	all WT
IX-2	40	7/13	GGTTAGCGACGAT------GCGG Indel GGTTAGCGACGAT**AG**C**G**AGGCGG SNPs
IX-3	9	3/0	GGTTAGCGACGAT------GCGG (2) GGTTAGCGACG------AGGCGG (1)
IX-4	7	3/0	GGTTAGCGACGAT------GCGG

Homozygous (cardinal) sequencing (c)

Replicate	CFP$^+$/cd screened	Indels/SNPs	Sequence change 5' – 3' WT: **GGTTAGCGACGATGCCAAGGCGG**
IX-1	50	0/0	NA
IX-2	50	1/0	GGTTAGCGACGATGC **Δ37** CGgaGA
IX-3	50	0/0	NA
IX-4	50	0/0	NA

(a) General phenotype and genotype data.
(b) Sequencing results from CFP$^+$/WT individuals found. All non-indel alleles are either WT or possess SNPs (bold letters). The wild-type gRNA target and PAM sequence are shown with the latter underlined.
(c) 50 CFP$^+$/cd individuals were screened by the ddPCR method from each replicate, any indel individual identified was sequenced. Δ37 is a 37 bp deletion. Lower-case nucleotides are insertions.

Table S7. Comparisons of life parameters of wild-type and AgNosCd-1 mosquitoes.

Line[1]	Wild-type	AgNosCd-1 (OX)	AgNosCd-1 (IX)
Larval development in days[2]	2.71 ± 0.12 (N=561)	2.72 ± 0.09 (N=561)	2.70 ± 0.35 (N=539)
Pupal development time in days [2]	6.11 ± 1.07 (N=479)	5.76 ± 0.74 (N=512)	5.99 ± 1.51 (N=476)
Larval viability	0.82 ± 0.12 (N=600)	0.85 ± 0.12 (N=600)	0.81 ± 0.16 (N=600)
Percent female adults[3]	43.0 ± 5.93 (43.0%) (N=237)	42.8 ± 7.22 (39.6%) (N=234)	39.7 ± 8.47 (42.8%) (N=201)
Percent male adults[3]	49.2 ± 4.49 (49.1%) (N=261)	49.8 ± 5.03 (52.3%) (N=251)	52.3 ± 4.67 (49.8%) (N=223)
Adult female longevity in days ± SE	19.8 ± 0.97 (N=50)	23.0 ± 1.03 (N=45)	17.5 ± 1.04 (N=49)
Adult male longevity in days ± SE	23.9 ± 1.05 (N=49)	23.6 ± 1.12 (N=47)	23.0 ± 1.10 (N=49)
Fecundity[4]	71.8 ± 3.5	75.9 ± 3.8 *	65.3 ± 2.9
	(91; 4-171) (11)	(66; 1-128) (13)	(118; 1-171) (18)
Fertility (Egg hatchability)[5]	0.47 ± 0.03	0.71 ± 0.04 ***	0.52 ± 0.03
	(5,932; 0 - 1)	(4,883; 0 - 1)	(5,271; 0 - 0.99)
OX Male contribution[6] (N=4)	51.9% ± 2.8% (OX) (2901/5623)*	48.1% ± 2.8% (N=4) (2722/5623)*	---
IX Male contribution[6] (N=5)	57.9% ± 3.8% (IX) (4084/7023)***	---	42.1% ± 3.8% (N=5) (2939/7023)***

1. 'Line' refers to the control (wild-type G3) and transgenic (AgNosCd-1) mosquitoes that carry one (OX, outcross) or two (IX, intercross) copies of the transgenes.

2. Number of days to complete each instar stage (larval development), number of days for L2 larvae to reach pupal stage (pupal development). N = number of mosquitoes counted.

3. Sex was scored when adults emerged following seeding 100 larvae in each of 6 pans per line. Number of males and females do not add up to 100 due to larval and pupal mortality. N = number of mosquitoes counted.

4. Mean number of eggs per female ± standard error (SE) in the first gonotropic cycle. The numbers in the first parentheses are the total number of mosquitoes counted followed by the range in numbers of fecundity. Females that did not lay eggs were excluded from the calculation. The numbers in the second parentheses are the number of females that died during the experiment.

5. Average fraction of deposited eggs that hatched. The numbers in parentheses are the total number of eggs observed followed by the range of hatchability (0, no eggs hatching; 1, all eggs hatching).

6. Values represent percentage of offspring in a single generation with (AgNosCd-1) or without (wild-type) a fluorescent marker in the results of a mating event where 150 wild-type females were provided an equal ratio of 75 wild-type and 75 AgNosCd-1 males. N = number of repetitions, numbers in parentheses are total CFP[2]/wild-type first generation progeny. OX males are expected to have 98% drive and IX males are expected to be 100% homozygous for the drive construct.

*** P < 0.05; ** P < 0.01; *** P <0.001.**

Table S8. Small cage trial data 1:1 ratio cage A-1.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	75 25.0%	225 75.0%	75 25.0%						75 25.0%	150 50.0%			50		188		22	260
G1	3573	333 56.4%	257 43.6%	149 28.3%	137 26.0%					127 24.1%	113 21.5%			137		96		1	234
G2	2788	496 87.0%	74 13.0%	124 23.3%	127 23.8%	110 20.6%	100 18.8%	2 0.4%		32 6.0%	38 7.1%			201		29		22	252
G3	4760	484 95.8%	21 4.2%	63 12.5%	67 13.3%	177 35.0%	159 31.5%	9 1.8%	9 1.8%	7 1.4%	14 2.8%			187		16		24	227
G4	3863	596 100%		27 5.0%	24 4.4%	248 45.6%	245 45.0%							184				21	205
G5	4926	477 100%			1 0.3%	206 51.8%	191 48.0%							176				34	210
G6	2548	471 100%				231 50.7%	225 49.3%							243				16	259
G7	4636	549 100%				241 47.1%	271 52.9%							243				17	260
G8	4983	559 100%				260 46.5%	299 53.5%							168				24	192
G9	2569	546 100%				265 48.5%	281 51.5%							161				64	225
G10	3757	515 100%				261 50.7%	254 49.3%							147				15	162
G11	4072	461 100%				267 57.9%	194 42.1%							163				64	227
TOTAL	42775	5562	577											2060		329		324	2713

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S9. Small cage trial data 1:1 ratio cage A-2.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP $^+$		CFP $^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	75 25.0%	225 75.0%	75 25.0%						75 25.0%	150 50.0%			48		203		9	260
G1	3914	305 60.5%	199 39.5%	120 26.3%	146 31.9%					89 19.5%	102 22.3%			143		82		2	227
G2	3251	492 84.0%	94 16.0%	139 25.2%	130 23.6%	93 16.9%	92 16.7%	1 0.2%	9 1.6%	41 7.4%	46 8.3%			175		34		28	237
G3	4713	468 93.8%	31 6.2%	61 12.2%	64 12.8%	131 26.3%	202 40.5%	2 0.4%	8 1.6%	18 3.6%	13 2.6%			171		9		11	191
G4	3620	602 99.7%	2 0.3%	30 6.5%	31 6.7%	194 42.2%	200 43.5%	4 0.9%	1 0.2%					188				23	211
G5	3440	501 100%		7 1.8%	4 1.0%	181 45.3%	204 51.0%	2 0.5%	2 0.5%					193				19	212
G6	2590	452 100%				210 48.6%	222 51.4%							167				28	195
G7	3107	584 100%				262 47.1%	294 52.9%							162				17	179
G8	3092	540 100%				272 50.4%	268 49.6%							181				53	234
G9	3839	576 100%				285 49.5%	291 50.5%							221				17	238
G10	4175	502 100%				264 52.6%	238 47.4%							156				55	211
G11	2553	542 100%				267 49.3%	275 50.7%							201				6	207
TOTAL	38594	5639	551											2006		328		268	2602

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S10. Small cage trial data 1:1 ratio cage A-3.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	75 25.0%	225 75.0%	75 25.0%						75 25.0%	150 50.0%			42		240			282
G1	6444	175 31.5%	381 68.5%	74 14.7%	93 18.5%					155 30.9%	180 35.9%			73		168			241
G2	3291	311 52.7%	279 47.3%	90 17.1%	106 20.1%	33 6.3%	40 7.6%	1 0.2%	9 1.7%	122 23.1%	126 23.9%			108		107		26	241
G3	3641	347 71.3%	140 28.7%	117 23.1%	150 29.6%	26 5.1%	45 8.9%	13 2.6%	16 3.2%	95 18.7%	45 8.9%			189		33		13	235
G4	4929	597 98.7%	8 1.3%	59 12.2%	68 14.0%	160 33.0%	185 38.1%	3 0.6%	2 0.4%	3 0.6%	5 1.0%			160		5		17	182
G5	2675	456 98.7%	6 1.3%	7 1.9%	9 2.5%	154 42.2%	190 52.1%		1 0.3%	3 0.8%	1 0.3%			164				17	181
G6	3084	441 100%				199 46.4%	230 53.6%							212				43	255
G7	6198	521 100%				246 49.5%	251 50.5%							263				41	304
G8	6129	532 100%				246 46.2%	286 53.8%							186				42	228
G9	3891	593 100%				277 46.7%	316 53.3%							202				36	238
G10	1945	546 100%				295 54.0%	251 46.0%							217				9	226
G11	3303	533 100%				277 52.0%	256 48.0%							199				22	221
TOTAL	45830	5127	1039											2015		553		266	2834

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S11. Small cage trial data 1:3 ratio cage B-1.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	37 12.4%	261 87.6%	37 12.4%						111 37.2%	150 50.3%			21		223		4	248
G1	3507	31 6.5%	443 93.5%	15 2.8%	16 3.0%					225 42.6%	272 51.5%			26		220			246
G2	3366	100 19.7%	408 80.3%	47 9.7%	44 9.1%	4 0.8%	4 0.8%		2 0.4%	171 35.4%	211 43.7%			47		111		20	178
G3	3977	216 43.9%	276 56.1%	84 17.1%	78 15.9%	24 4.9%	28 5.7%	1 0.2%	1 0.2%	135 27.4%	141 28.7%			87		123		39	249
G4	3540	381 63.2%	222 36.8%	130 25.9%	121 24.2%	20 4.0%	30 6.0%	5 1.0%	21 4.2%	97 19.4%	77 15.4%			105		122		13	240
G5	4443	523 82.9%	108 17.1%	104 20.1%	103 19.9%	98 19.0%	106 20.5%	4 0.8%	13 2.5%	46 8.9%	43 8.3%			166		25		10	201
G6	2442	511 98.6%	7 1.4%	48 10.6%	48 10.6%	164 36.4%	177 39.2%	4 0.9%	3 0.7%	3 0.7%	4 0.9%			202				14	216
G7	4764	573 100%		3 0.6%	8 1.6%	233 46.0%	262 51.8%							174		4		29	207
G8	3789	496 100%		1 0.2%	1 0.2%	240 48.4%	254 51.2%							172				34	206
G9	1374	497 100%				265 53%	232 47%							113				64	177
G10	3302	516 100%				261 51%	255 49%							195				18	213
G11	1545	542 100%				269 50%	273 50%							204				11	215
Total	36349	4423	1725											1512		828		256	2596

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S12. Small cage trial data 1:3 ratio cage B-2

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	37 12.4%	261 87.6%	37 12.4%						111 37.2%	150 50.3%			27		246		6	279
G1	5156	55 9.5%	525 90.5%	27 4.7%	28 4.9%					264 46.3%	251 44.0%			34		217		1	252
G2	3931	152 23.4%	498 76.6%	50 8.9%	70 12.4%	5 0.9%		1 0.2%		233 41.4%	204 36.2%			52		177		32	261
G3	5416	232 38.5%	370 61.5%	81 15.3%	90 17.0%	10 1.9%	13 2.5%	1 0.2%	4 0.8%	168 31.7%	163 30.8%			82		148		18	248
G4	2392	302 59.6%	205 40.4%	78 20.7%	87 23.1%	23 6.1%	24 6.4%	5 1.3%	5 1.3%	80 21.3%	74 19.7%			78		51		2	131
G5	2905	462 82.2%	100 17.8%	86 17.2%	95 19.0%	88 17.6%	122 24.4%	7 1.4%	12 2.4%	43 8.6%	46 9.2%			120		38		54	212
G6	2994	572 92.7%	45 7.3%	76 13.1%	94 16.2%	165 28.4%	179 30.9%	12 2.1%	11 1.9%	22 3.8%	21 3.6%			197		10		62	269
G7	6395	548 99.6%	2 0.4%	9 1.8%	17 3.3%	235 46.3%	236 46.5%	7 1.4%	2 0.4%	2 0.4%				207		2		27	236
G8	4712	542 100%		6 1.1%	4 0.7%	274 50.6%	252 46.5%		6 1.1%					198				43	241
G9	2779	589 100%		1 0.2%	2 0.3%	275 46.7%	311 52.8%							224				21	245
G10	3921	466 100%		7 1.5%		222 47.6%	237 50.9%							183				14	197
G11	4254	542 99.8%	1 0.2%	8 1.5%	10 1.8%	255 47.0%	269 49.5%					1 0.18%		200		23			223
TOTAL	45155	4499	2007											1602		912		280	2794

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S13. Small cage trial data 1:3 ratio cage B-3.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	37 12.4%	261 87.6%	37 12.4%						111 37.2%	150 50.3%			27		186		9	222
G1	2901	24 4.7%	484 95.3%	16 3.1%	8 1.6%					228 44.9%	256 50.4%			12		212		4	228
G2	3968	50 8.5%	541 91.5%	16 2.9%	22 3.9%	3 0.5%	7 1.3%			262 47.0%	247 44.3%			17		153		58	228
G3	5477	80 18.1%	363 81.9%	37 8.4%	33 7.4%	4 0.9%	6 1.4%			177 40.0%	186 42.0%			23		166		31	220
G4	3029	126 21.7%	455 78.3%	18 5.0%	27 7.6%	2 0.6%	2 0.6%	1 0.3%		127 35.6%	180 50.4%			20		129		15	164
G5	3602	66 12.0%	482 88.0%	20 4.0%	31 6.2%	4 0.8%	3 0.6%		3 0.6%	216 42.9%	227 45.0%			75		139		85	299
G6	4809	1708 36.3%	3001 63.7%	61 13.9%	58 13.2%	23 5.2%	24 5.5%	5 1.1%	4 0.9%	150 34.2%	114 26.0%			57		126		70	253
G7	3520	2161 61.4%	1359 38.6%	109 20.2%	103 19.1%	39 7.2%	26 4.8%	17 3.1%	16 3.0%	109 20.2%	121 22.4%			141		35		26	202
G8	3477	532 93.5%	37 6.5%	87 15.3%	110 19.3%	165 29.0%	159 27.9%	4 0.7%	7 1.2%	18 3.2%	19 3.3%			154		16		48	218
G9	3027	610 99.2%	5 0.8%	49 8.0%	46 7.5%	229 37.2%	265 43.1%	13 2.1%	8 1.3%	1 0.2%	4 0.7%			225				15	240
G10	2663	637 100%		1 0.2%		339 53.2%	297 46.6%							204				15	219
G11	3399	561 100%				265 47.2%	296 52.8%							214				18	232
TOTAL	40172	6592	6988											1169		1162		394	2725

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S14. Small cage trial data 1:9 ratio cage C-1.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	15 5.0%	285 95.0%	15 5.0%						135 45.0%	150 50.0%			13		259		6	278
G1	5574	370 6.6%	5204 93.4%	32 6.1%	15 2.8%					266 50.4%	215 40.7%			22		235			257
G2	4999	769 15.4%	4230 84.6%	36 6.3%	50 8.7%				2 0.3%	248 43.1%	239 41.6%			39		153		75	267
G3	5426	1761 32.5%	3665 67.5%	61 13.1%	59 12.7%		11 2.4%	3 0.6%	5 1.1%	168 36.2%	157 33.8%			49		141		23	213
G4	4273	335 55.6%	267 44.4%	138 26.4%	109 20.9%	15 2.9%	21 4.0%	6 1.1%	3 0.6%	118 22.6%	112 21.5%			133		83		28	244
G5	4513	442 81.0%	104 19.0%	126 24.9%	127 25.1%	71 14.0%	80 15.8%	1 0.2%	6 1.2%	39 7.7%	56 11.1%			164		37		10	211
G6	3012	548 98.6%	8 1.4%	106 20.0%	88 16.6%	162 30.6%	161 30.4%	4 0.8%	3 0.6%	3 0.6%	3 0.6%			253		2		20	275
G7	6148	561 100%		6 1.1%	11 2.1%	254 47.8%	259 48.8%		1 0.2%					201		2		20	223
G8	5490	552 100%		2 0.4%	3 0.5%	274 49.6%	273 49.5%							148				52	200
G9	3956	568 100%				285 50.2%	283 49.8%							157				79	236
G10	2336	585 100%				301 51.5%	284 48.5%							202				21	223
G11	3710	502 100%				249 49.6%	253 50.4%							190		19			209
TOTAL	49737	7008	13763											1571		931		334	2836

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S15. Small cage trial data 1:9 ratio cage C-2.

Generation	Total population	Larvae CFP$^+$ (%)	Larvae CFP$^-$ (%)	Pupae and adults CFP$^+$ (%)						Pupae and adults (%)				Adults contributing to the next generation[1]					
				WT		cd		Tear eye		WT		cd		CFP$^+$		CFP$^-$		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	15 5.0%	285 95.0%	15 5.0%						135 45.0%	150 50.0%			15		258		2	275
G1	3992	462 11.6%	3530 88.4%	32 5.9%	33 6.1%					263 48.9%	210 39.0%			38		255		1	294
G2	4636	1303 28.1%	3333 71.9%	63 12.1%	66 12.7%	4 0.8%	3 0.6%			184 35.5%	199 38.3%			51		178		39	268
G3	4835	214 36.2%	377 63.8%	98 17.5%	94 16.8%	2 0.4%	5 0.9%	1 0.2%	3 0.5%	183 32.7%	173 30.9%			107		118		17	242
G4	5215	432 72.6%	163 27.4%	110 22.6%	138 28.4%	44 9.1%	49 10.1%	4 0.8%	8 1.6%	78 16.0%	55 11.3%			125		81		37	243
G5	4421	510 87.6%	72 12.4%	93 18.6%	98 19.6%	101 20.2%	128 25.5%	14 2.8%	11 2.2%	30 6.0%	26 5.2%			108		22		22	152
G6	3407	549 98.0%	11 2.0%	54 10.6%	54 10.6%	173 33.9%	196 38.4%	14 2.7%	8 1.6%	5 1.0%	6 1.2%			186		3		31	220
G7	5511	591 99.5%	3 0.5%	13 2.3%	9 1.6%	286 49.7%	251 43.6%	9 1.6%	5 0.9%	2 0.3%	1 0.2%			205				30	235
G8	4310	551 100%		3 0.5%	3 0.5%	260 47.2%	285 51.7%							153				51	204
G9	2533	563 100%		7 1.2%	13 2.3%	263 46.7%	279 49.6%		1 0.2%					161				87	248
G10	3048	543 99.1%	5 0.9%	4 0.7%	2 0.4%	261 47.6%	276 50.4%					2 0.36%	3 0.55%	196				15	211
G11	2742	552 100%		3 0.5%	2 0.4%	260 47.1%	287 52.0%							184				18	202
TOTAL	44950	6285	7779											1529		915		350	2794

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

Table S16. Small cage trial data 1:9 ratio cage C-3.

Generation	Total population	CFP+ (Larval) (%)	CFP- (Larval) (%)	CFP+ (Pupal and adult)						CFP- (pupal and adult)				Adults that contributed to the next generation*					
				WT		cd		Tear eye		WT		cd		CFP +		CFP -		Unknown	Total
				Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	WT	cd	WT	cd		
G0	300	15 5.0%	285 95.0%	15 5.0%						135 45.0%	150 50.0%			14		231		10	255
G1	3629	121 3.3%	3508 96.7%	3 0.6%	8 1.5%					246 45.6%	283 52.4%			14		255			269
G2	4235	633 14.9%	3602 85.1%	25 4.4%	28 5.0%	4 0.7%	6 1.1%	1 0.2%		266 47.2%	233 41.4%			26		191		45	262
G3	5429	1099 20.2%	4330 79.8%	61 11.7%	46 8.8%					211 40.5%	203 39.0%			40		147		45	232
G4	2517	178 31.2%	393 68.8%	62 12.9%	58 12.1%	5 1.0%	12 2.5%	1 0.2%	6 1.2%	155 32.2%	182 37.8%			57		123		39	219
G5	3255	335 54.2%	283 45.8%	94 19.6%	122 25.4%	23 4.8%	15 3.1%	4 0.8%	7 1.5%	110 22.9%	105 21.9%			104		89		22	215
G6	3259	441 76.8%	133 23.2%	112 20.6%	109 20.0%	99 18.2%	82 15.0%	6 1.1%	10 1.8%	72 13.2%	55 10.1%			169		59		57	285
G7	5836	483 94.0%	31 6.0%	64 14.2%	83 18.4%	121 26.8%	130 28.8%	12 2.7%	15 3.3%	16 3.5%	11 2.4%			182		15		17	214
G8	4348	507 96.2%	20 3.8%	33 6.3%	26 4.9%	207 39.3%	224 42.5%	8 1.5%	9 1.7%	7 1.3%	13 2.5%			165		7		39	211
G9	3855	643 100.0%		29 4.5%	25 3.9%	277 43.1%	286 44.5%	11 1.7%	15 2.3%					133				70	203
G10	1668	518 100.0%				276 53.3%	242 46.7%							197				7	204
G11	2979	550 100.0%				250 45.5%	300 54.5%							162				58	220
TOTAL	41310	5523	12585											1263		1117		409	2789

1. Adults contributing to the next generation: total number of emerged males and females that survived when oviposition egg cups provided. Abbreviations: cd, cardinal (pale red-eye, positive +, negative-); CFP, cyan fluorescent protein (positive +, negative -); WT, wild-type eye color.

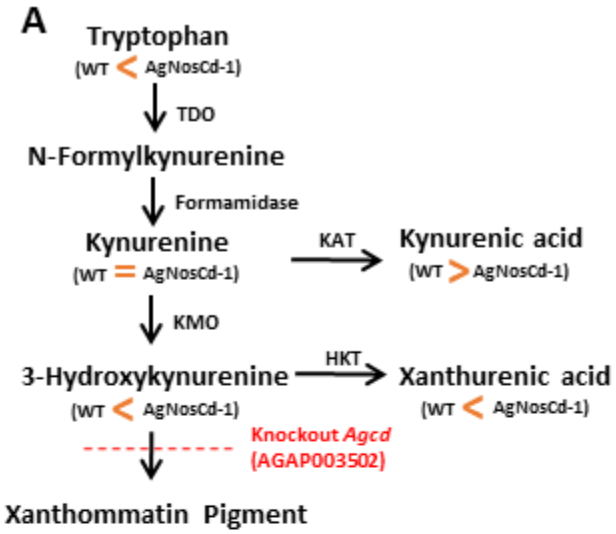
Table S17. Sequence analysis of exceptional phenotype individuals (CFP^-/cd^-) from cage trials.	
C/G/n/gen[1]	**Sequence 5'-3'[2]**
Wild-type	**ACCCGAGTGGAACGGTACGGCGGTTAGCGACGATGCCAAGGCGGCCGCCATAGCGGATGGCGAGAAGGCGCTGGGC**
B2/G11/1/he	**ACCCGAGTGGAACGGTACGGCGGTTAGCGACGAT----------GCCATAGCGGATGGCGAGAAGGCGCTGGGC**
B2/G11/1/he	**ACCCGAGTGGAACGGTACGGCGGTTAGCG----------GCCGCCATAGCGGATGGCGAGAAGGCGCTGGGC**
C2/G10/3/ho	**ACCCGAGTGGAACGGTACGGCGGTTAGCGACGAT----------GCCATAGCGGATGGCGAGAAGGCGCTGGGC**
C2/G10/1/ho	**ACCCGAGTGGAACGGTACGGCGGTTAGCGA----------CGGCCGCCATAGCGGATGGCGAGAAGGCGCTGGGC**
1. Cage/Generation/number of mosquitoes/heterozygous (he) or homozygous (ho). 2. Blue letters are the guide RNA target site and orange are the PAM site. Dashes are deletions.	

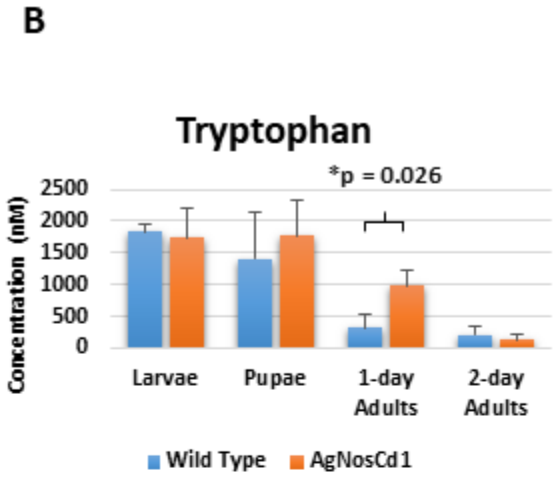
Table S18. Frequency of alternative alleles (single nucleotide polymorphisms [SNPs]) in the AgNosCd-1 guide RNA target site in colonized and wild populations of *Anopheles gambiae*.[1]

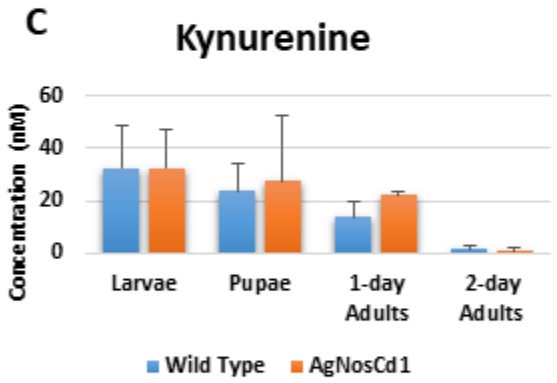
Region (impact)[2]	SNP Coordinate[3]	*Ref*[4]	*Alt*[4]	*MR4 An. gambiae* (N=68)	*MR4 coluzzii* (N=113)	*MR4 G3* (N=20)	*An. gambiae* VGL (N=238)	*An. gambiae* Ag1000G (N=664)	*An. coluzzii* VGL (N=54)	*An. coluzzii* Ag1000G (N=293)	*An. gambiae* overall (N=902)	*An. coluzzii* overall (N=347)
PAM (highest)	2R:38748879	C	T	-	-	-	0.0042	0.0030	-	-	0.0033	-
	2R:38748880	C	A/T	-	-	-	-	0.0008	-	-	0.0006	-
PAM (none)	2R:38748881	G	A	-	-	-	-	0.030	0.0185	0.0358	0.0022	0.0331
Seed region (high)	2R:38748882	C	T	-	-	-	-	0.0023	-	-	0.0017	-
	2R:38748883	C	A/T	-	-	0.025	0.0042	0.0008	-	-	0.0017	-
	2R:38748884	T	.	-	-	-	-	-	-	-	-	-
	2R:38748885	T	C	-	-	-	-	-	0.0093	-	-	0.0014
	2R:38748886	G	.	-	-	-	-	-	-	-	-	-
Non-seed protospacer (low)	2R:38748887	G	T	-	-	-	-	0.0166	-	0.0017	0.0122	0.0014
	2R:38748888	C	.	-	-	-	-	-	-	-	-	-
	2R:38748889	A	T	-	-	-	0.0189	0.0181	0.0093	-	0.0183	0.0014
	2R:38748890	T	.	-	-	-	-	-	-	-	-	-
	2R:38748891	C	.	-	-	-	-	-	-	-	-	-
	2R:38748892	G	.	-	-	-	-	-	-	-	-	-
	2R:38748893	T	C	-	-	-	-	0.0015	-	-	0.0011	-
	2R:38748894	C	.	-	-	-	-	-	-	-	-	-
	2R:38748895	G	T	0.0074	-	-	-	-	-	-	-	-
	2R:38748896	C	.	-	-	-	-	-	-	-	-	-
	2R:38748897	T	.	-	-	-	-	-	-	-	-	-
	2R:38748898	A	T	0.27721	0.0973	-	0.2815	0.4367	0.2685	0.3379	0.3958	0.3271
	2R:38748899	A	.	-	-	-	-	-	-	-	-	-
	2R:38748900	C	.	-	-	-	-	-	-	-	-	-
	2R:38748901	C	T	-	-	-	0.0588	0.0173	-	0.0051	0.0588	0.0283

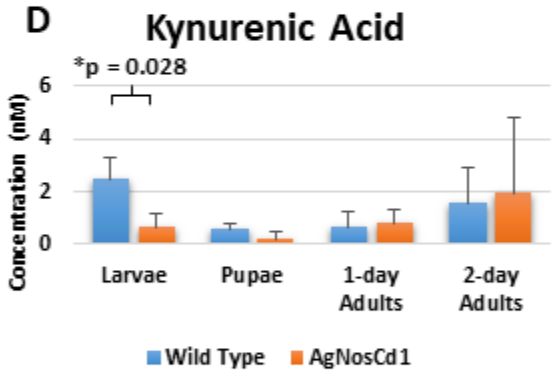
1. Frequencies are based on specimens from colonies supplied by the Malaria Research and Reference Reagent Resource Center (MR4; https://www.beiresources.org/MR4Home.aspx), the UC Davis Vector Genetics Lab (VGL) collection and publicly-available data from the *Anopheles gambiae* 1000 Genomes Consortium (Ag1000G). Overall consolidated frequencies from VGL and Ag1000G are shown in the sister species *An. coluzzii*. N= number of mosquitoes in each sample. Sites within the PAM are highlighted by orange shading.
2. Target site classification based on Drury et al.[11].
3. Guide RNA target site is listed in antisense (top to bottom) as it appears in the genome assembly (VectorBase; (https://www.vectorbase.org/)[1].
4. Nucleotides in the reference genome (https://www.vectorbase.org/[1]) and alternate (Alt) SNPs found in colonized and wild mosquito populations.

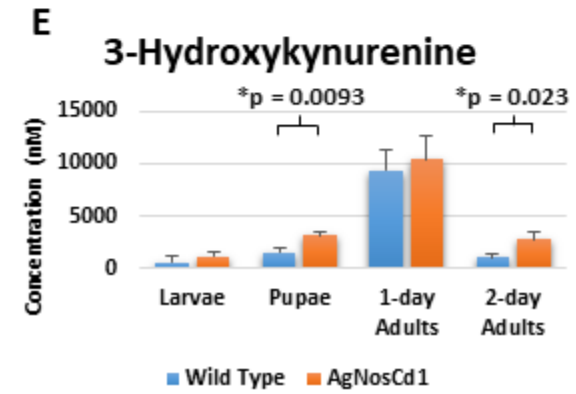
Figure S1. Mutations in *Agcd* result in modulations of ommochrome pathway metabolites. A) Ommochrome pathway[12,13]. Differences in metabolite accumulation in mosquito extracts are indicated by =, indicating no change, or < or >, indicating a significant change at some developmental stage (larva, pupa, or one or two day-old adults) in metabolite concentration. The gene drive knockout of the *Agcd* gene is indicated by the dashed red line. **B-F**) HPLC-MS/MS quantification of tryptophan metabolites in WT and gene drive (AgNosCd-1) mosquitoes. Quantification was performed on fourth instar larvae, pupae, 1-day adults and 2-day adults. Metabolites are quantified with standard curves and internal standards, TRP-d3 for TRP, 4-ClKYN for KYN and 3-HK, and KYNA-d5 for XA and KYNA. Mean concentrations in nanomoles (nM, Y-axes) of three replicates shown for tryptophan (**B**), kynurenine (**C**), kynurenic acid (**D**), 3-hydroxykynurenine (**E**), and xanthurenic acid (**F**). Students T-test performed between WT and AgNosCd-1 for each developmental stage. Differences considered significant when *P<.05. Error bars represent the standard deviation. Abbreviations: TDO, tryptophan-2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine 3-monooxygenase; HKT, 3-hydroxykynurenine transaminase.











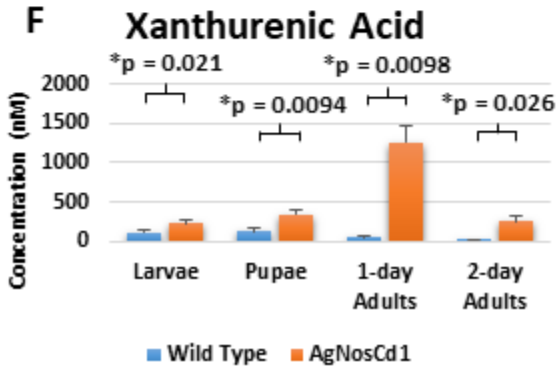
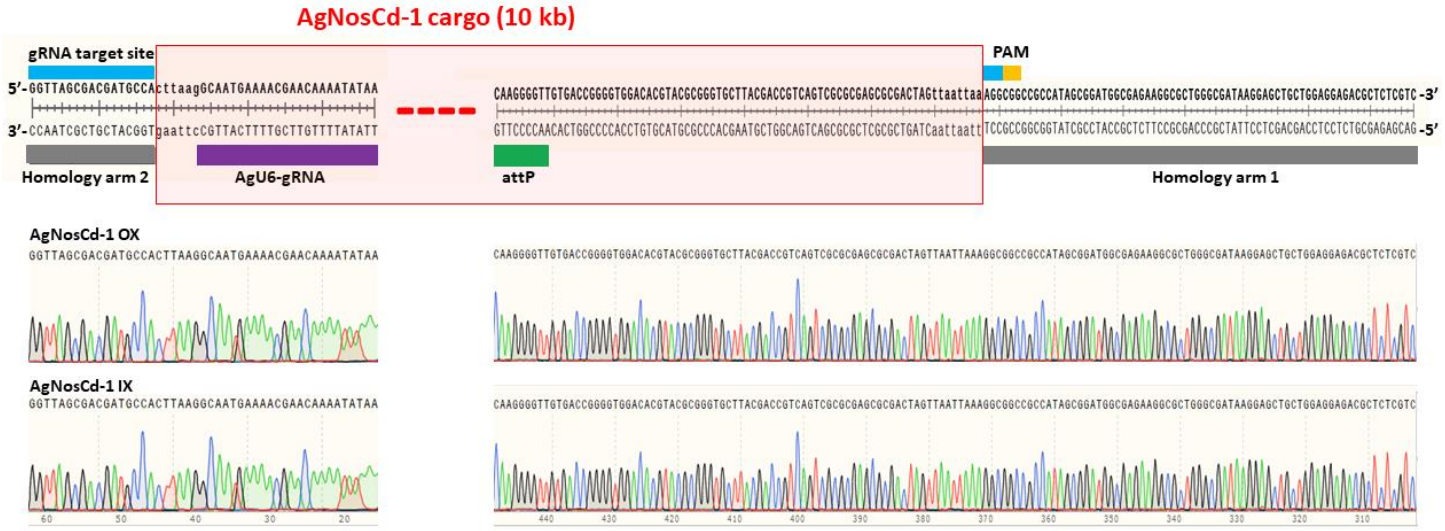


Figure S2. Molecular validation of precise insertion of the pCO-37 plasmid into the *Agcd* locus. A) Sequencing of the 5'- and 3'-end junction amplification fragments of the homology arms and construct amplified from a female heterozygous for the cassette (AgNosCd1-OX) and a female homozygous for the cassette (AgNosCd1-IX) showed a match to the reference Cas9-mediated cleavage sequence and precise integration at the *Agcd* locus. Positions of the Cd1 homology arms (grey boxes), guide RNA target sequence (blue box, PAM in yellow) and portions of the construct cargo (purple and green boxes) are shown. B) Single mosquito extractions containing individuals homozygous (IX) or heterozygous (OX) for AgNosCd-1, or wild type (WT) show amplicons with sizes diagnostic for the presence (1,682, 1,249, 2,409 and 2,238 base pairs in length) or absence (1,323 bp) of the gene drive construct using the oligonucleotide primers listed below each panel (Table S1). Individuals containing one copy of AgNosCd-1 had only the 1,323 bp product using primers (card F2/card-seq R1) that span the target sequence because the large size (~10 kilobases in length) of the integrated AgNosCd-1 prohibits amplification. The DNA ladder (M) used in each gel was 1 kb Plus (ThermoFisher Scientific) and the 1,500 bp standard is indicated.

A



B

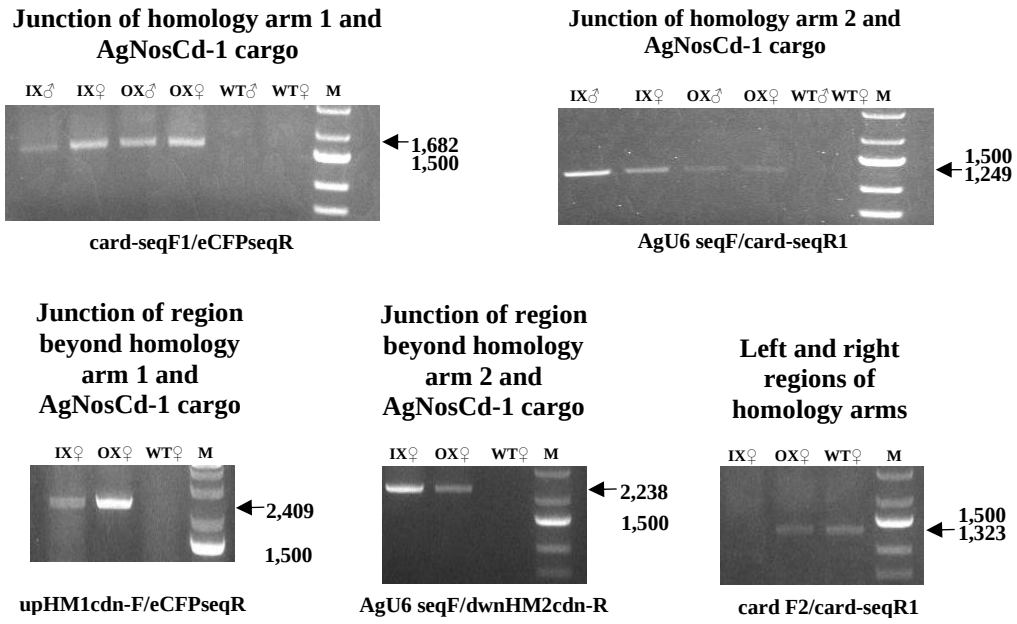


Figure S3. Mating scheme to test male and female drive properties. The scheme shows the mating events of male (left panel) and female (right panel) lineages. Forty males (left panel) and females (right panel) hemizygous for AgNosCd-1were outcrossed (OX) separately with 40 opposite sex WT *An. gambiae* G3 (male OX [outcross] indicated with blue boxes and female OX are indicated with red boxes). Male and female progeny of these parents that had expected phenotypes of WT eyes and the CFP^{+} marker were repeatedly outcrossed to WT individuals for four generations in order to determine role of maternal effects and/or targeted Cas9 in somatic cells when comparing successive male and/or female contributions of the drive system to offspring. Abbreviations: F1-4, first, second, third and fourth generations, respectively, following parental, P, matings.

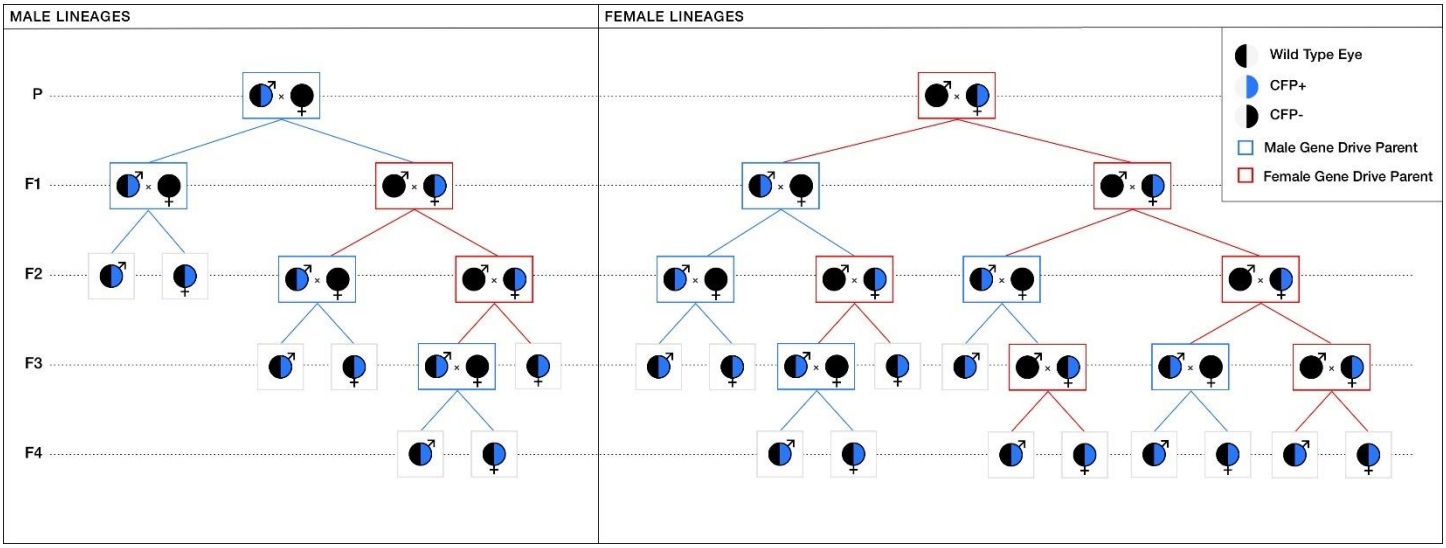
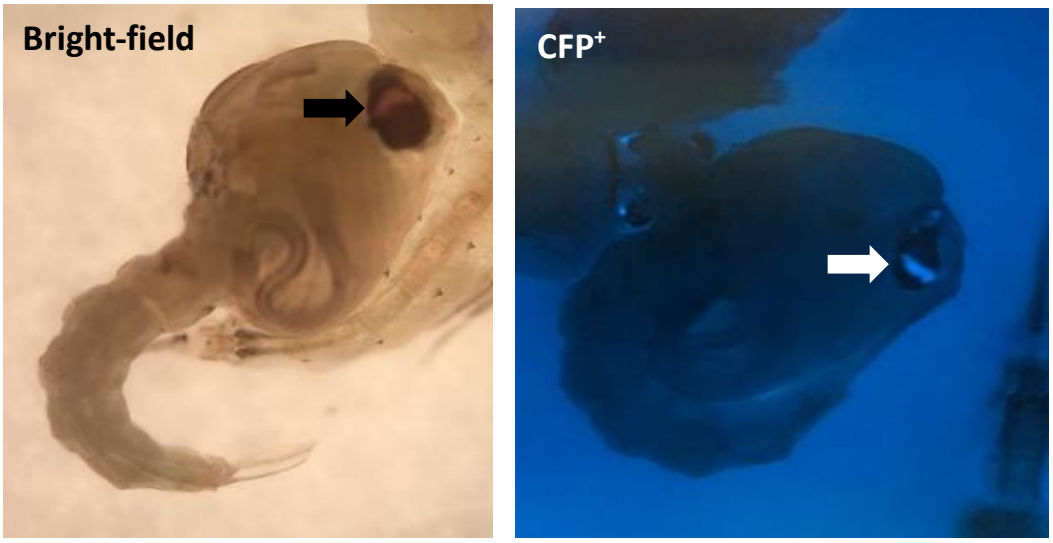


Figure S4. Phenotype and molecular analysis of mosaic individuals ('tear phenotype'). (A) Tear phenotype in AgNosCd1 pupae (CFP^+). Bright-field image (right) shows the tear phenotype in the background of a homozygous *cardinal* mutant (red eye) background. Fluorescent image (left) shows CFP^+ ommatidia in mosaic eye. Gene amplification and NGS analysis of *Agcd* insertion sites in pooled samples of wild-type (B) and tear (C) mosquitoes. The percentage of reads (Y-axis, note that the scale differs between B and C) with insertions (green line) and deletions (blue line) mapped across the sequences of the amplicons (X-axis). The target region blocked in orange designates the gRNA target sequence, with PAM sequence immediately adjacent to the left border. (D) Quantitative details of indels found in both sample cohorts.

A



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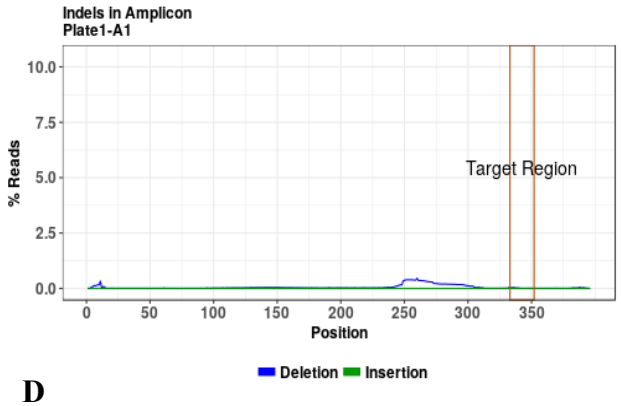
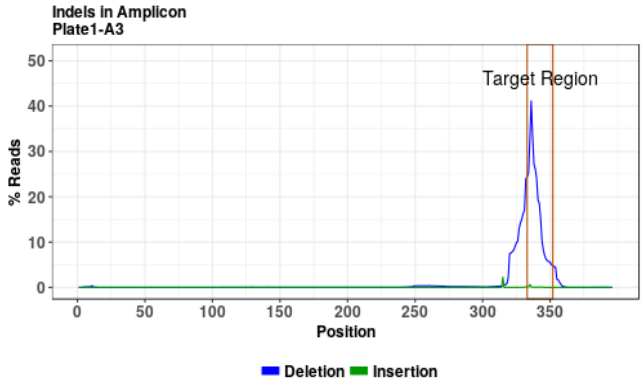


C



D

Sample (# individuals)	Total Reads	Indel reads near cut site (%)*	Indel Size Range
WT (15)	112,443	3 (0.0027)	-8, -5
AgNosCd1 "Tear" (20)	109,903	46,005 (41.86)	-19, +17
* Refers to any indel present within three base pairs of the Cas9 cleavage site, which is 3bp downstream from the PAM.			

Figure S5. Small cage trial schematic. Triplicate cages (A1-3, B1-3 and C1-3) were established with 150 wild-type females (black) and different ratios (1:1, 1:3 and 1:9) of AgNosCd-1 (blue) and wild-type (black) males, respectively, for a total population size of ~300 mosquitoes per cage. Adults were blood-fed, eggs collected and larvae hatched. Larvae were allotted as follows: 300 selected randomly to populate the next generation cages and 600 selected randomly and scored for eye phenotypes (CFP fluorescence and cardinal) and sex ratio. The remaining larvae were counted and the number added to the number removed to get a population size. Drive efficiency and generation of non-homologous end joining alleles were monitored by the accumulation of the eye phenotype.

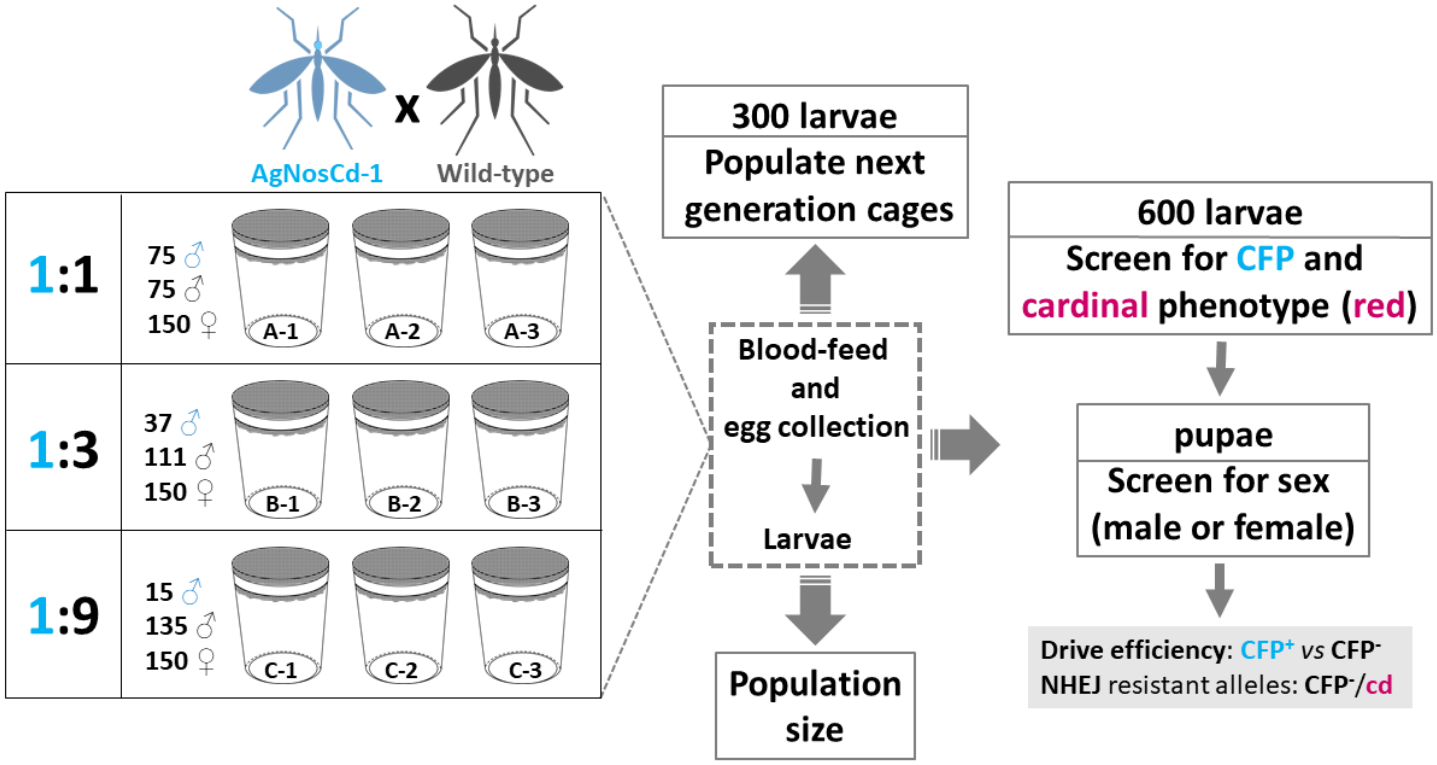


Figure S6. Genomic off-target nucleotide sequence predictions and cleavage assay *in vitro* for the gRNA targeting site in *Agcd*. A) The locus, chromosome location, predicted cut -site and sequence of the on-target *cardinal* target site and three predicted off site-targets are displayed. The PAM sequences for the targets are underlined, while nucleotide differences between the gRNA and the predicted off-target sequences are in red bold letters. B) Cas9 and gRNA-mediated cleavage *in vitro* of off-target and control target sites. Plasmids (Table S2) cleaved with *Nco*1 were incubated in cleavage reactions with Cas9 and the gRNA. Shown are a negative (–ve) control = pCO-68, positive (+ve) control = pCO-57, off-1 = pCO-66, off-3 = pCO-67. Versions of off-target 2 with known SNPs are off-2-1 = pCO-72, off-2-2 (pCO-73) and off-2-3 (pCO-74).

A

Name	Sequence[1]	Chromosome	Cut site[2]
Wild-type, on-target positive control	GGTTAGCGACGATGCCAAGGCGG	2R	38,748,885
No PAM, on-target negative control	GGTTAGCGACGATGCCAAGG**CCC**	synthetic	Not present in genome
Off-target 1 variant	GGTTA**A**C**A**ACGA**AA**CCAAGG**T**GG	2L	3,130,338
Off-target 2-1 variant	GGTTAGC**C**A**G**G**CG**GCCAAGG**T**GG	2R	21,710,305
Off-target 2-2 variant	GGTTAGC**C**A**G**G**CA**GCCAAGG**T**GG	2R	21,710,305
Off-target 2-3 variant	GGTTAGC**C**A**G**G**CG**GCCAAG**AT**GG	2R	21,710,305
Off-target 3 variant	GGTTAGCGA**A**GATG**G**CA**TT**G**T**GG	2R	40,155,032

1. 5'-3'target sequence, PAM (underlined), nucleotide variants from the wild-type target sequence are shown in bold red.
2. Coordinates based on PEST strain (VectorBase)[1].

B

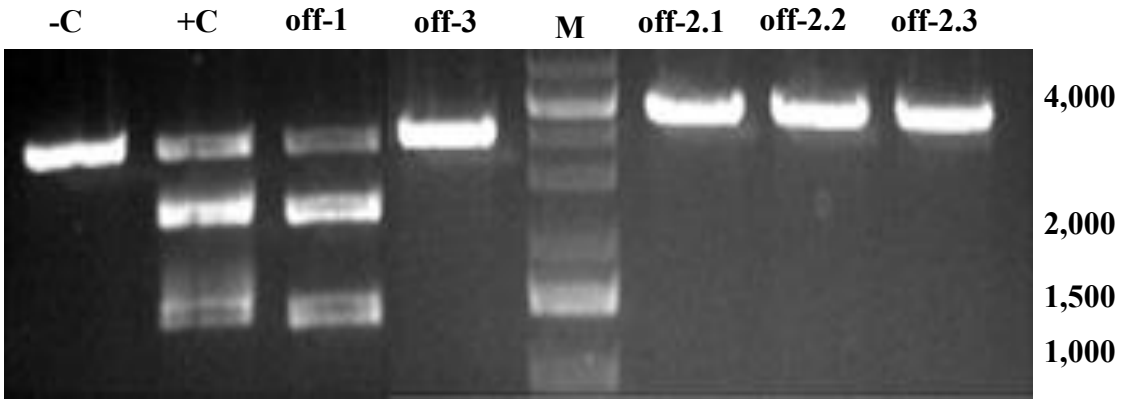


Figure S7. Sanger sequencing results from predicted off-target sites in G_8 individuals. Sequence analyses of predicted off-targets for the AgNosCd1 strain show no off-target activity from Cas9 and the gRNA. Sense (coding) and antisense strand sequences show the targets in bold letters, gRNA target highlighted in yellow and the PAM sequence underlined. The red letters are nucleotides in the off-targets that are different from those in the on-target sequence, while blue letters are SNPs.

Off-target 1:

Sense: 5′-**GGTTAACAACGAAACCAAGGTGG**-3′, Antisense: 5′-**CCACCTTGGTTTCGTTGTTAACC**-3′

WT genomic DNA extracted from 10 females

>wt1-292_F09.ab1
NNNNNNNNNNNNNNNNNGGCACTGTTCNTGTCAGTCATAAAAAATACACCATTAAACTGCTGTGGTGCAGCCGACTGTGAGACATGTTG
ATCGCGCTCCAGTGACTGAGCTCTAAGCAGACGCGATTCTTTATCGCGTGCGTCCATTATGACGTTGAGTATGGCTCGATTTGAATCCT
TCGTCGTAGGCTGCCGACTGCGTGAGTGATGTAGACCTTGTGCCGGCACAGTGCCTGTCTCGGCTGCTGTATGGATCACATCCGGCCCT
GCAATGACGCCCGGGTGCGATTCATGGGCAATTTCCAGTGGCAAGCTTGTTGCAGGTATATCGAAGTTTGTTTTCACAACTCCCGCGTC
CTCTTCATCTTCAAAAAAGT**CCACCTTGGTTTCGTTGTTAACC**ATCATCAAATCATAACCAGATCCTTCTCCATCAGATGGGTTCGAAA
TCAGATGTGCCAGCGATCTTGACTTTCGCGGCGGTTGCGGCAAAAGGTTTACAGGTACAATCTCGTGTTCGTTCGAAATTTCGAGACTG
TACAATGCCTGTTCAGGGATATCATCCACTTGTTGATAGAGAATCTCATTCTGGTCTGAAATTTCGCGCTCTGCATCGTTCACGATGTT
TTGTAGCTCCTCGATTACATCCTCGATCGTTGCGTTGGTATCGTCTTCGCCATTCCCATTCAGTTCGTGCATAGGAACGGTGCTCTCTT
GGGTAAGCGATATGCTGAACTTGTGCGAGCCTGAACTTGATCCTCCATTTTGAGCCGGATACAGACGTGGAGGTTCTGGAGTCGAGGAC
TGCCCTGTCTCCGAACTGCTAATACCTTCATCGTCGTTCTCTGAAGTGTTGTTTCGTTTTGGTTGAGGTGGTACCTGGCGCGGCAACGC
TACTGGTCTCTTTTCTAGACTCGAANAGTTGGAACTCTCGTGAGNTNNNNNNNNNNNNNNNNNNN

WT genomic DNA extracted from 4 males and 4 females

>wt2-292_H03.ab1
NNNNNNNNNNNNTTGCNGGCACTGTTCNTGTCAGTCATAAAAAATACACCATTAAACTGCTGTGGTGCAGCCGACTGTGAGACATGTTG
ATCGCGCTCCAGTGACTGAGCTCTAAGCAGACGCGATTCTTTATCGCGTGCGTCCATTATGACGTTGAGTATGGCTCGATTTGAATCCT
TCGTCGTAGGCTGCCGACTGCGTGAGTGATGTAGACCTTGTGCCGGCACAGTGCCTGTCTCGGCTGCTGTATGGATCACATCCGGCCCT
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Genomic DNA was extracted from a single female individual for each of samples 1-20 and from a single male individual for each of samples 21-25

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Off-target 2:

Sense: 5′-GGTTAGCCAGGCGGCCAAGGTGG-3′, Antisense: 5′-CCACCTTGGCCGCCTGGCTAACC-3′

WT genomic DNA extracted from 10 females

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Genomic DNA was extracted from a single female individual for each of samples 1-20 and from a single male individual for each of samples 21-25

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Off-target 3:

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WT genomic DNA extracted from 10 females

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WT genomic DNA extracted from 4 males and 4 females

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Genomic DNA extracted from a single female individual for each of samples 1-20 and from a single male individual for each of samples 21-25

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Figure S8. Indel length and number of off-target 1 NGS deep sequencing reads. A) WT, B) G_9 females, C) G_9 males, D) G_{10} females and E) G_{10} males. Blue bars represent deletions in the off-target 1 sequence found in the reads, while the orange bars represent reads that have the intact WT off-target 1 sequence. The numbers above the bars on the X axis are the total sequences recovered of each type. Small numbers cannot be seen because of the large scale of the Y axis.

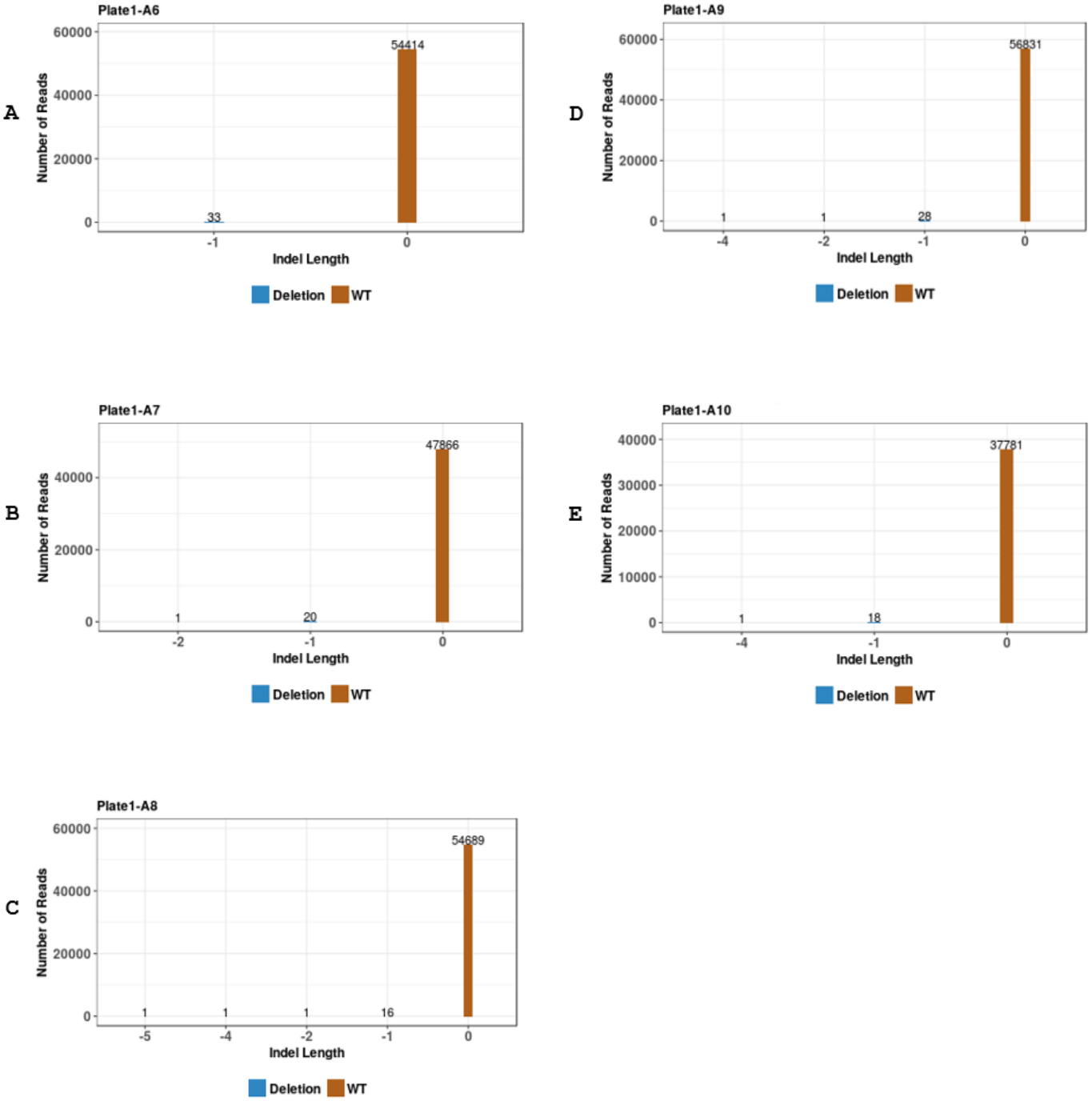
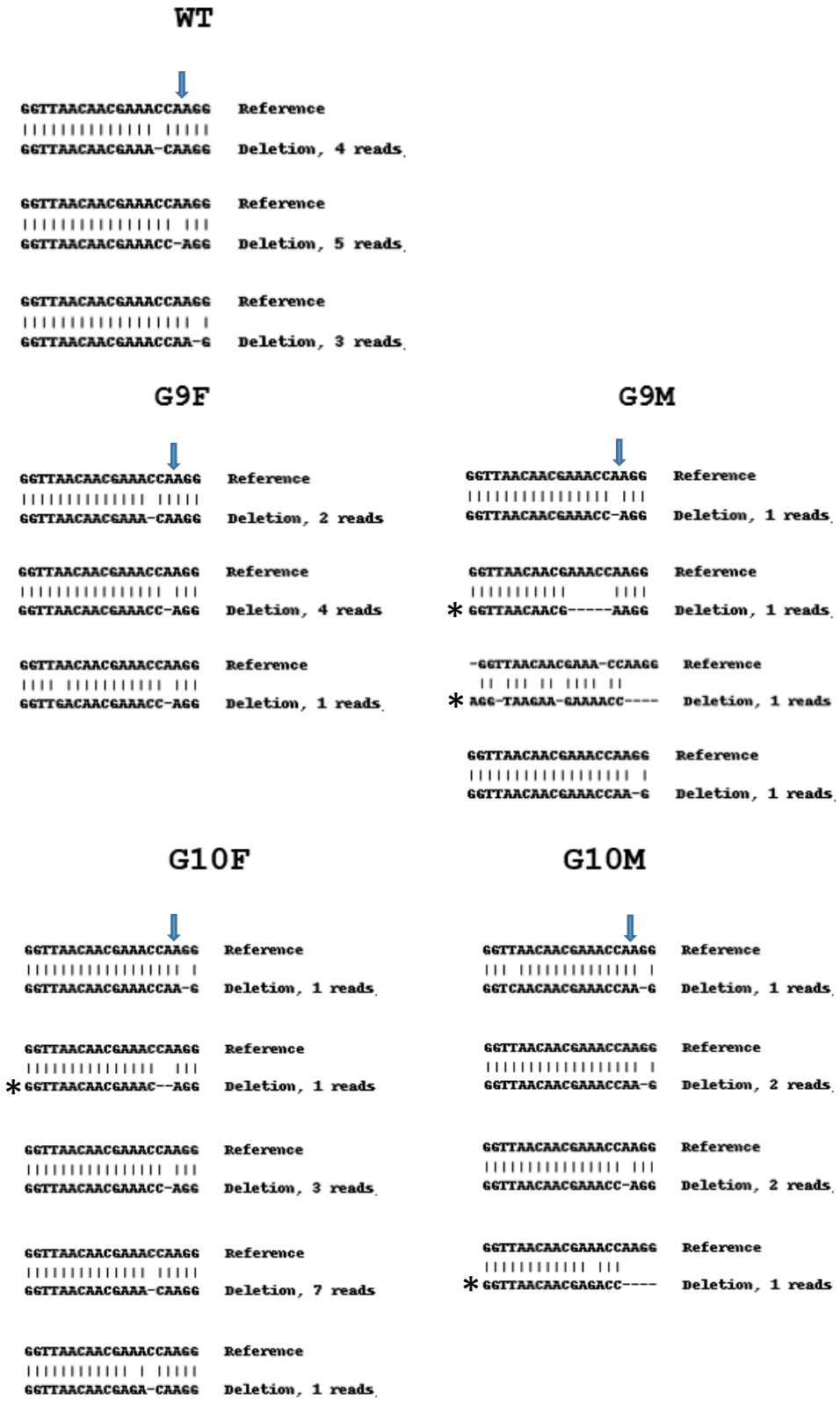


Figure S9. off-target 1 deletions detected by NGS near predicted AgNosCd-1 and WT cut-sites. Wild-type (WT), Generation 9 and 10 female and male samples were analyzed. Sequences are listed 5'-3' with the PAM (not shown) to the right. Dashes denote deletions compared to the wild-type reference target sequence. Vertical lines show identical nucleotides and an open space highlights polymorphisms. Asterisks are deletions found only in AgNosCd1 and not wild-type mosquitoes. The blue arrows are predicted cut sites.



SUPPORTING TEXT.

Analysis of tear phenotypes.

A pooled Next Generation Sequencing (NGS) analysis of *Agcd* amplicons from 20 tear mosquitoes supports the hypothesis that the distinctive phenotype results from mosaics of cells with indel and WT alleles. A total of 43.45% (47,755/109,903 reads) of the alleles show indels, 41.86% (46,005) of which are at and adjacent to the gRNA cut site (Table S4, Figure S4). The sizes of the lesions varied from deletions of 19 nucleotides to insertions of 17. A WT control sample comprising 15 mosquitoes had three deletions (0.0027%; 3/112,443) of 5-8 nucleotides near the cut site. Additional evidence in support of mosaicism comes from a ddPCR analysis of individual and pooled tear mosquitoes (Table S5). Individual mosquitoes revealed a wide range, 2.21% to 47.5%, of indels in the total loci examined with an average of 19.99%. All three alternate allele configurations, transgene-insertion, indel, and WT, can be present in mosaic individuals. Individual WT controls were on average ~80-fold lower. Five pooled tear mosquitoes had an average of 22.6% indels in all loci analyzed with pooled WT controls being ~104-fold lower.

To determine if indel alleles were present in the germline of tear mosquitoes, four intercrosses (IX) were performed with tear phenotype mosquitoes recovered from the cage experiments described below (IX-1, G_3 [11 males, 31 females]; IX-3, G_4 [18 males, 29 females] and IX-4, G_5 [11 males, 38 females]) in addition to IX-2 (14 males and 16 females) of the maternal-effect experiments (Table S6). Total pupae in all four intercrosses were >99% (1590/1591) CFP^+ and ~ 95% (1509/1591) of these had the mutant *cd* red-eye phenotype. Eighty-one (~5.1%) of the total progeny were hemizygous CFP^+ with WT eye-color. These could have a WT copy of the *cd* locus (no gene-drive insertion), an indel that creates a frameshift capable of encoding a functional enzyme, or a drive-resistant single nucleotide polymorphism (SNP) that also encodes a functional product. A single individual (1/1591, 0.06%) was CFP^- with WT eye-color.

A sample of 60 of the 81 CFP^+/WT presumed hemizygous mosquitoes were selected for sequencing around the target site, and of these, 56 produced DNA of sufficient quality to provide a result. Thirteen had functional indel alleles (capable of conferring a WT phenotype in a hemizygote) and the remaining were either WT (42 samples) or had a functional allele with three SNPs that was recovered 13 times (Table S6). Twelve of the functional indels had the same in-frame six nucleotide deletion sequence near the gRNA cut site and one had a six nucleotide deletion shifted two additional base pairs towards the 5'-end of the cut site.

Fifty CFP^+/cd individuals each from the four crosses were analyzed with ddPCR, and only one (1/200; 0.5%) non-functional indel allele was detected (Table S6). This allele has a mixed insertion/deletion sequence resulting most likely from NHEJ. We calculated the percentage of NHEJ indel alleles arising in the tear intercrosses as follows: 5.09% (81/1591) hemizygous progeny multiplied by 23.2% indels (13 /56 analyzed in hemizygous progeny) to give 1.18%. We add to this the percentage, 0.5%, found in the ddPCR experiments, for a total of 1.68%. This total is below the frequency of the indel alleles seen in the NGS and ddPCR analyses of tear phenotype mosquitoes (Figure S4 and Tables S4 and S5), indicating that the indels are predominantly restricted to the somatic cells.

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