Suppressing mosquito populations with precision guided sterile males

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The mosquito *Aedes aegypti* is the principal vector for arboviruses including dengue/yellow fever, chikungunya, and Zika virus, infecting hundreds of millions of people annually. Unfortunately, traditional control methodologies are insufficient, so innovative control methods are needed. To complement existing measures, here we develop a molecular genetic control system termed precision-guided sterile insect technique (pgSIT) in *Aedes aegypti*. PgSIT uses a simple CRISPR-based approach to generate flightless females and sterile males that are deployable at any life stage. Supported by mathematical models, we empirically demonstrate that released pgSIT males can compete, suppress, and even eliminate mosquito populations. This platform technology could be used in the field, and adapted to many vectors, for controlling wild populations to curtail disease in a safe, confinable, and reversible manner.
mosquitoes are the world’s deadliest animals, killing more humans on earth than any other animal\textsuperscript{1}. They transmit the majority of vector-borne diseases, such as the notorious arboviruses dengue, Zika virus, yellow fever, and chikungunya transmitted by Aedes mosquitoes. The predominating strategy to control these devastating diseases is the use of insecticides, though mosquitoes are evolving and spreading insecticide resistance\textsuperscript{2}, hampering control efforts. Therefore, there is an urgent demand for innovative mosquito-control technologies that are effective, sustainable, and safe.

Alongside traditional control measures, several genetic-based techniques are being used to combat mosquitoes. These include multiple male (♂) release programs aimed at population suppression, such as the classical radiation-based sterile insect technique (SIT), relying on releasing irradiated sterile ♂’s (plural)\textsuperscript{3}. Alternative approaches include the Wolbachia-based incompatible insect technique (IIT), relying on the release of Wolbachia-infected ♀’s\textsuperscript{4,5}, or the antibiotic-based release of insects carrying a dominant lethal gene (RIDL)\textsuperscript{6}. Moreover, emerging CRISPR-based homing genes drives that spread target genes through a population faster than through traditional Mendelian inheritance are presently under development with the aim of safe implementation in the future\textsuperscript{8–10}.

As an alternative, a CRISPR-based technology termed precision guided SIT (pgSIT), was recently innovated\textsuperscript{11}. pgSIT uses a binary approach to simultaneously disrupt genes essential for female (♀) viability and ♀ fertility, resulting in the exclusive survival of sterile ♂’s that can be deployed at any life stage to suppress and eliminate populations. It requires two breeding strains, one expressing Cas9 and the other expressing guide RNAs (gRNAs). Mating between these strains results in RNA-guided mosaic target gene mutations throughout development, ensuring complete penetrance of desired phenotypes. Compared to alternatives, pgSIT does not require the use of radiation, Wolbachia, or antibiotics, and will not persist in the environment longterm. Unfortunately, this technology is presently only accessible in flies, and for population control techniques, its equivalent needs to be developed for mosquitoes.

To address this need, here we systematically engineer pgSIT in Ae. aegypti using a system that simultaneously disrupts genes essential for ♀ fertility and ♀ flight, which is essential for mating, blood feeding, reproduction, and predator avoidance—meaning survival in general\textsuperscript{12}. Using our technology, we demonstrate that generated sterile ♀ progeny can compete, suppress and even eliminate mosquito populations in multigenerational population cages when released as either eggs or adults. Mathematical models suggest that releases of Ae. aegypti pgSIT eggs could effectively eliminate a local Ae. aegypti population using achievable release schemes. Taken together, this study suggests that pgSIT may be an efficient technology for mosquito population control and introduces the first pgSIT system suited for real-world release.

Results

Validation of pgSIT target genes. To engineer pgSIT in Ae. aegypti, we first validated target genes by generating transgenic gRNA-expressing lines targeting two conserved genes: β-Tubulin 85D (βTub, AAELO18994), specifically expressed in mosquito testes\textsuperscript{13–15} and essential for spermatogenesis and ♀ fertility\textsuperscript{16}, and myosin heavy chain (myo-fem, AAELO05656), expressed nearly exclusively in ♀ pupae\textsuperscript{13,14} and essential for ♀ flight\textsuperscript{17} (Supplementary Fig. 1 and Supplementary Table 1). To ensure efficient disruption, each gRNA line encoded four U6–promoter-driven\textsuperscript{18} gRNAs targeting unique sites in the coding sequence of either βTub (U6-gRNA\textsuperscript{βTub}—marked with 3xP3-GFP) or myo-fem (U6-gRNA\textsuperscript{myo-fem}—marked with 3xP3-tTomato) (Supplementary Figs. 2–4). Multiple independent transgenic lines were generated given that position effects are expected to result in a variable expression\textsuperscript{19,20}. To assess the activity of each line independently, we conducted bidirectional crosses with Cas9 controlled by a homozygous nuclear pore complex protein (Cas9—marked with Opic2-CFP)\textsuperscript{21} (Supplementary Tables 2 and 3 and Supplementary Figs. 2–4). The resulting transheterozygous ♀ progeny (gRNA+/-, Cas9+/−) were assessed and crossed to wildtype (WT) for further evaluation. For the βTub crosses, the fecundity of the F₁ transheterozygous ♀’s ranged from 0 to 94.9%, with two lines out of ten demonstrating sterility resulting from immotile sperm\textsuperscript{16}, while ♀’s transheterozygous ♀’s maintained normal fertility (Fig. 1, Supplementary Fig. 2, Supplementary Table 3, and Supplementary Video 1). For myo-fem crosses, all ♀ transheterozygous ♀’s generated from three out of five lines were flightless, while ♀ transheterozygous ♀’s maintained normal flight (Fig. 1, Supplementary Table 3, Supplementary Fig. 3, and Supplementary Videos 2 and 3). As expected, ♀ flightlessness significantly reduced mating ability, and blood consumption, as many flightless ♀’s get trapped on the water surface following eclosion, resulting in reduced fertility, fecundity, and survival. Sanger sequencing of genomic DNA revealed expected mutations at the βTub- and myo-fem-targeted loci.

Development of pgSIT and fitness assessments. To generate a pgSIT strain capable of targeting both βTub and myo-fem simultaneously, we combined two gRNA lines that exclusively produced sterile ♀’s (gRNA\textsuperscript{βTub γTub}7) or flightless ♀’s (gRNA\textsuperscript{myo-fem γfem}8) (Fig. 1, Supplementary Figs. 2 and 3, and Supplementary Table 3) by repeated backcrossing, generating a double-homozygous stock (termed gRNA\textsuperscript{βTub γTub γmyo-fem γfem}) (Supplementary Fig. 4). To assess its activity, we bidirectionally crossed gRNA\textsuperscript{βTub γTub γmyo-fem γfem} to Cas9. Importantly, these crosses yielded all flightless ♀’s (termed pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}) with normal flight and mating capacity (Fig. 2, Supplementary Fig. 5, Supplementary Tables 4–8, and Supplementary Videos 4–6). We next determined transgene integration sites, single copy number per transgene, and confirmed target gene disruptions by both ampiclon sequencing (Supplementary Fig. 6) and Nanopore genome sequencing using transheterozygous pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s (Supplementary Figs. 7–9 and Supplementary Tables 9 and 10). We also performed transcriptome sequencing of pupae comparing pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s and pgSIT\textsuperscript{myo-fem γfem}’s to WT to quantify target gene reduction, expression from transgenes, and to assess global expression patterns (Supplementary Figs. 8–10 and Supplementary Tables 11–15). As expected, we observed significant target gene disruption in pgSIT individuals, robust expression from our transgenes, and non-target gene misexpression, which would be expected given the significant phenotypes observed (i.e., flightless ♀’s and spermless ♂’s).

To explore potential fitness effects, we assayed several fitness parameters including ♀ fecundity, fertility, flight activity, ♂ mating capacity, ♂ sound attraction, larva–pupa development time, pupa–adult development time, and longevity (Fig. 2, Supplementary Fig. 5, Supplementary Tables 5–8, and Supplementary Videos 5 and 6). The pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s were flightless with significantly reduced fecundity, fertility, and survival, indicating they would be very unlikely to survive in the wild, let alone transmit pathogens. For pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s, other than slightly delayed larva–pupa development time, we did not detect significant differences in fitness parameters. Previous studies demonstrated that Ae. aegypti ♀’s typically mate only once in their lifetime, a behavior known as monandry\textsuperscript{22}. To explore whether prior matings with pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s could suppress ♀ fertility, we initiated experiments in which WT ♀’s were first mated with pgSIT\textsuperscript{βTub γTub γmyo-fem γfem}’s for a
Theoretical performance of pgSIT in a wild population. To explore the potential for pgSIT to suppress Ae. aegypti populations in the wild, we simulated releases of pgSIT eggs on the motu of Onetahi, Teti’aroa, French Polynesia (Fig. 4), a field site for releases of Wolbachia-infected ♂ mosquitoes, using the MGDrivE simulation framework. Weekly releases of up to 400 pgSIT eggs (♂ and ♀) per WT adult (♂ and ♀) were simulated in each human structure over 10–24 weeks. The scale of these releases was chosen considering adult release ratios of 10:1 are common for sterile ♂ mosquito interventions and ♀ Ae. aegypti produce >30 eggs per day in temperate climates. We also assumed 25% reductions in ♀ mating competitiveness and adult lifespan for pgSIT ♂’s by default because, although pgSIT fitness effects were not apparent from laboratory experiments, they may become apparent in the field. Results from these simulations suggest that significant population suppression (>96%) is observed for a wide range of achievable release schemes, including 13 weekly releases of 120 or more pgSIT eggs per WT adult (Fig. 4 and Supplementary Video 7). Population elimination was common for larger yet achievable release schemes, including 18 weekly releases of 200 or more pgSIT eggs per wild adult, and 24 weekly releases of 100 or more pgSIT eggs per wild adult. Results also suggest a wider range of pgSIT fitness profiles (e.g., a 50% reduction in ♀ mating competitiveness and 25% adult lifespan reduction) could lead to population elimination for these release schemes (Fig. 4).

Discussion
While many technologies for halting the spread of deadly mosquito-borne pathogens exist, none are without significant drawbacks such that additional measures are needed. By disrupting essential genes throughout development, we demonstrate efficient production of short-lived, flightless pgSIT♂’s and fit sterile pgSIT♀’s. Importantly, when repeatedly released into caged populations, the pgSIT♂’s competed with WT ♂’s thereby
suppressing, and even eliminating, populations using release ratios that are achievable in the field. Mathematical models suggest that population elimination could be accomplished in the field through sustained releases of ~100–200 or more pgSIT eggs per wild Ae. aegypti adult, even if fitness costs significantly exceed those measured in laboratory experiments.

For pgSIT to be realized in the wild, the two engineered strains will first need to be separately and continuously mass-reared in a facility, without contamination, and crossed to produce sterile \( \delta \)s. While this can be viewed as rate-limiting, it offers stability, as the binary CRISPR system will remain inactive until crossed—thereby reducing the evolution of suppressors or mutations that could disrupt the system. In addition, each sorted \( \delta \) can produce up to 450 eggs in her lifetime (~90 eggs per gonotrophic cycle), which improves scalability. Moreover, once crossed, the resulting progeny are essentially dead-ends (i.e., sterile \( \delta \)s/flightless \( \gamma \)s), and flightless \( \gamma \)s hatched among high numbers of sterile pgSIT\( ^{\circ} \)s, should not contribute to the gene pool. We demonstrate here that the technology is fully penetrant by screening >100 K individuals. Notwithstanding these results, it’s possible that when this is scaled further to releasing millions, or even billions, there may be some error rate. What error rate is acceptable? What error rate will still enable population suppression?

We do not have these answers, however, we can compare to Oxitec’s OX513A RIDL system with the lethality trait penetrance. We do not have these answers, however, we can compare to Oxitec’s OX513A RIDL system with the lethality trait penetrance. We do not have these answers, however, we can compare to Oxitec’s OX513A RIDL system with the lethality trait penetrance. We do not have these answers, however, we can compare to Oxitec’s OX513A RIDL system with the lethality trait penetrance.
Fig. 3 Multigenerational cage trials demonstrating efficient population suppression. A To generate sufficient mosquito numbers, three lines were raised separately, including homozygous Cas9, double-homozygous gRNA/Tub + myo-fem, and WT. To generate pgSIT progeny, virgin Cas9 ♀’s were genetically crossed to gRNA/Tub + myo-fem ♂’s, and eggs were collected. B To perform multigenerational population cage trials of pgSIT, two strategies were employed: release of eggs (B, top panel); release of mature adults (B, bottom panel). For both strategies, multiple pgSIT:WT release ratios were tested, including: 1:1, 5:1, 10:1, 20:1, and 40:1. Each generation, total eggs were counted, and 100 eggs were selected randomly to seed the subsequent generation. The remaining eggs were hatched to measure hatching rates and score transgene markers. This procedure was repeated after each generation until each population was eliminated (Supplementary Table 16). C Multigenerational population cage data for each release threshold plotting the proportion of eggs hatched each generation. Source data are provided as a Source Data file.

outstanding success at suppressing populations in the wild. Taken together, this indicates that, while perfection is the ultimate goal, it should not be necessary for obtaining approvals nor for achieving success in the field.

The pgSIT system developed here resulted in sterile ♀’s and flightless ♂’s. Importantly, if these flightless ♀’s were to mate to WT ♂’s, blood feed, and lay eggs, the transgene could be introduced into the local population. However, the likelihood is very low. First, *Ae. aegypti* ♀’s detect ♂’s via the sounds produced by ♀’s beating wings, and following a fast-paced, mid-air pursuit, they mate. If ♀’s move slowly and in a lethargic manner (Supplementary Video 2), with significantly reduced survival (Supplementary Fig. 5A, B and Supplementary Table 5). Taken together, the chance to find a blood source, blood feed, mate, and then find oviposition sites is extremely low. Third, the release process will continue for several rounds, even if the transgene is introduced into the local population, the population should be completely eliminated after several rounds of releases.

Finally, an egg-release device could be developed that would require the mosquitoes to fly out of the rearing container to enter the environment. This would basically eliminate the chances of a flightless ♀’s from entering the environment. pgSIT offers an alternative approach to scalability that should help decrease costs and increase efficiency. For instance, the required genetic cross at scale can be initiated using existing robotic sex sorting devices (www.senecio-robotics.com) or an automated robotic system developed by Verily. Upon sex sorting and crossing, the resulting pgSIT progeny can be distributed and released at any life stage, mitigating requirements for sex separation at field sites. This strategy will be especially effective for mosquitoes that diapause during the egg stage (e.g., *Aedes* species) because it will enable long-term egg accumulation. Eggs could be distributed to logistically spaced remote field sites where they can hatch, develop into adults, and fly out to compete with wild mosquitoes (Supplementary Fig. 11). These hatching containers could be engineered in such a way to require the adults to fly out thereby preventing the release of flightless ♀’s. This attractive feature should reduce the costs of developing multiple production
facilities requiring on-site sex separation for the manual release of fragile adults. That said, to achieve adequate control in major urban settings, repeated releases will likely be required on a continuous basis and costs will need to be considered.

It should be noted that the releases of adult pgSIT♂'s unexpectedly resulted in faster population suppression as compared to egg releases in multigenerational population cage experiments. We believe this to result from the slightly reduced egg hatching rates of pgSIT♂'s and their delayed larva–pupa development time, which likely enabled the co-released WT ♀'s first access to WT ♂'s. While this could impact the discrete generation population cage experiments conducted here, it should not be problematic for suppressing continuous populations in the wild.

Finally, notwithstanding its inherently safe nature, pgSIT requires genetic modification, and regulatory use authorizations will need to be granted prior to implementation. While this could be viewed as a limitation35, we do not expect obtaining such authorizations to be insurmountable. In fact, we envision pgSIT to be regulated in a similar manner to Oxitec’s RIDL technology, which has been successfully deployed in many locations including the USA.

Overall, the inherent self-limiting nature of pgSIT, offers a controllable safe alternative to technologies that can persist and spread in the environment, such as gene drives9. Going forward, pgSIT may provide an efficient, safe, scalable, and environmentally friendly alternative next-generation technology for wild population control of mosquitoes resulting in wide-scale prevention of human disease transmission.

Methods

Mosquito rearing and maintenance. Ae. aegypti mosquitoes were derived from the Liverpool strain (wild-type (WT)) previously used to generate the reference genome37. Mosquitoes were reared in incubators at 27.0°C with 40–45% humidity and a 12-h light/dark cycle in cages (Bugdorm, 24.5 × 24.5 × 24.5 cm). Adults were provided 0.3 M aqueous sucrose ad libitum, and ♀'s were blood-fed on anesthetized mice for 2 consecutive days for ~15 min at a time. Oviposition substrates were provided ~3 days following the second blood meal. Eggs were collected and aged for ~4 days to allow for embryonic development, then hatched in deionized H2O in a vacuum chamber. Roughly ~400 larvae were reared in plastic containers (Sterilite, 34.6 × 21 × 12.4 cm, USA) with ~3 liters of deionized H2O, and fed an energy-rich diet of Tetramin Tropical Flakes (TetraWerke, Melle, Germany). For genetic crosses, to ensure ♀ virginity, pupae were separated and sexed under the microscope by sex-specific morphological differences in the genital lobe shape (at the end of the pupal abdominal segments just below the paddles) before being released to eclose in cages. These general rearing procedures were followed unless otherwise noted. Mosquitoes were examined, scored, and imaged using the Leica M165FC fluorescent stereomicroscope equipped with the Leica DMC2900 camera. For higher-resolution images, we used a Leica DM4B upright microscope equipped with a View4K camera enabling time-lapse videos. Time-lapse videos of caged adult mosquitoes were taken with a mounted Canon EOS 5D Mark IV using a 24–105-mm image stabilizer ultrasonic lens.

Guide RNA design and testing. Two target genes were selected for gRNA design: β-Tubulin 85D (βTub, AAE1019894) and myosin heavy chain (myo-fem, AAE103658). For each target gene, DNA sequences were first identified using reference genome assembly38, and genomic target sites were validated using PCR amplification and Sanger sequencing (Supplementary Table 18 for primer sequences). Gene structures, transcripts, and exon–intron junction boundaries were carefully evaluated using comprehensive developmental transcriptome data38 loaded into an internal genome browser. Target gRNA sequences were selected to be 20 bp (N20) in length, excluding the PAM (NGG)39. For in silico gRNA selection, we used either CHOPCHOP V3.0.0 or CRISPOR to minimize potential genomic off-target cleavage events. In total, we designed four gRNAs...
targeting gTub and four gRNAs targeting myo-fem (Supplementary Table 18). To confirm gRNA activity in vivo, each gRNA was in vitro synthesized prior to construct design (Synthego, CA, USA). Then 100 ng/µl of gRNA was individually injected into 50 preblastoderm stage embryos (0.5–1 h old) derived from Exu-Cas9 maternally depositing mothers, per previous embryo-injection protocols.[8,9] The surviving G0 progeny were pooled (2–5 individuals per pool), and genomic DNA was extracted using the DNeasy blood and tissue kit (Qagen, Cat. No./ID: 69506) following the manufacturer’s protocols. To confirm the targeted mutations, target loci were PCR amplified from extracted genomic DNA, and the PCR products were gel-purified (Zymo Research, Zymoclean Gel DNA Recovery Kit, Cat. No./ID: D4007). The purified products were either sent directly for sequencing to a third-party sequencing facility (TOPO-TA, Cat. No./ID: LS50641), wherein single colonies were selected and cultured in Lauret Broth (LB) with ampicillin before plasmid extraction (Zymo Research, Zypplid plasmid miniprep kit, Cat. No./ID: D4036) and Sanger sequencing. Mutated alleles were identified in silico by alignment with WT target sequences. All primers used for PCR and sequencing, including gRNA target sequences, are listed in Supplementary Table 18.

**Construct molecular design and assembly.** The Gibson enzymatic assembly method was used to engineer all constructs in this study. To generate the Nap50-Cas9 construct marked with CFP, OA-874A (Addgene https://www.addgene.org/614846/) was used as the backbone template.[10] The fragments of T2A-eGFP–P10–3′UTR and OpIe2-disRed–SV40 were removed by cutting with restriction enzyme FseI. Then, the P10-3′UTR fragment was amplified from Addgene 100608 (https://www.addgene.org/100608/) with primers P740-P10 and 777B. Another fragment of Nap50-Cas9, was synthesized from gBlocks Gene Fragment (Integrated DNA Technologies, Coralville, Iowa). Both fragments were provided for the Gibson assembly into the cut backbone. We designed two constructs, OA-1067A1 (https://www.addgene.org/164487/) and OA-1067A2 (https://www.addgene.org/164488/), each carrying four different gRNAs targeting either β-Fibrinogen (gRNAβ-fib), myosin heavy chain (gRNamyo-fem), myostatin (gRNAmyost), and myo-fem (gRNAmyo-fem) genes.

To engineer these plasmids, four intermediate plasmids, OA-1055A (gRNAβ-fib), OA-1055B (gRNAmyo-fem), OA-1055W (gRNamyost), and OA-1055X (gRNAmyo-fem), each harboring two gRNAs, were generated by cutting a common plasmid (OA-984, [54]) with restriction enzyme FseI, which contains piggyBac elements and the 3′xP-ttdTomato transformation marker, with the restriction enzymes AvrII and Ascl. Two gBlocks Gene Fragments were then cloned in, each containing two gRNAs: one driven by U6b (AaegL017774) and one by U6c (AaegL017763) promoters.[11] To assemble the final plasmid OA-1067A1, an intermediate plasmid OA-1067A was generated by linearizing the plasmid OA-1055B with the restriction enzyme BgIII and inserting in the fragment of U6b-gRNAβ-fib–U6c-gRNAmyo-fem amplified with primers 1167.C1 and 1067.C2 from plasmid OA-1055A. Then, the fragment of 3′xP-ttdTomato was removed from plasmid OA-1067A using the restriction enzymes Ascl and NotI and replaced with the 3′xP-mCherry marker amplified from primers 1067A1.C2 from the plasmid OA-9618 (https://www.addgene.org/104967/). To assemble the final plasmid OA-1067K: OA-1055W was linearized with the restriction enzyme FseI, and the insertion of U6b-gRNAβ-fib–U6c-gRNAmyo-fem amplified with primers 1167.C5 and 1067.C6 from the plasmid OA-1055X. During each cloning step, single colonies were selected and cultured in LB medium with ampicillin, and then the plasmids were extracted (Zymo Research, Zypplid plasmid miniprep kit, Cat. No./ID: D4036) and Sanger sequenced. Final plasmids were maxi-prepped using (Zymo Research, Zypplid Plasmid Maxiprep kit, Cat. No./ID: D4020) and Sanger sequenced. All primers are listed in Supplementary Table 18. Complete annotated plasmid sequences and plasmid DNA are available at Addgene.

**Generation of transgenic lines.** Transgenic lines were generated by microinjecting preblastoderm stage embryos (0.5–1 h old) with a mixture of the piggyBac plasmid (OA-1067K) and transposase helper plasmid (OA-1067A). Embryonic collection and microinjection procedures were performed following established protocols.[21] After 4 days of development post-microinjection, G0 embryos were harvested in deionized H2O in a vacuum chamber. Surviving G0 pupae were separated and sexed and divided into separate ♀ and ♂ cages (~20 cages total). The pupae were allowed to eclosion (~4 days) and eclosion rate and sexed and divided into separate ♀ and ♂ cages (~20 cages total). The pupae were allowed to eclosion (~4 days) for 5 days, and the surviving G0 pupae were pooled (2–5 days). The surviving G0 pupae were then blood fed. Afterward, eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with tetracycline for 3 days, which corresponds to the time for development and mating, a blood meal was provided, and eggs were collected and cultured in LB medium with.
insertion in one of the remaining gaps in the genome. Finally, using nanopore data, we confirmed genomic deletions in both pgSIT target genes—see AEI019894 and AAE1005656 as expected (Supplementary Figs. 8 and 9). The nanopore sequencing data have been deposited to the NCBI sequence read archive (NCBI-SRA, PRJNA699282).

Transcriptional profiling and expression analysis. To quantify target gene reduction and expression from transgenes as well as to assess global expression patterns, we performed Illumina RNA sequencing. We extracted total RNA using miRNeasy© kit (Qiagen, Cat. No. 277040) from ten sexed pupae. WT, WT♂, transheterozygous pgSIT♂, and pgSIT♀ harboring all three transgenes Cas9+; gRNA2Ub7+; gRNA4mio-fem+; with each genotype in biological triplicate (12 samples total), following the manufacturer’s protocol. DNase treatment was conducted using DNase I, RNase-free (ThermoFisher Scientific, Cat. No. EN0521), following total RNA extraction, RNA integrity was assessed using the RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513), and mRNA was isolated from –1 μg of the total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). RNA-seq libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770) following the manufacturer’s protocols. Briefly, mRNA was fragmented to an average size of 200 nt by incubating at 94 °C for 15 min in the first strand buffer. cDNA was then synthesized using random primers and ProtoScript® II Reverse Transcriptase followed by second-strand synthesis using NEB Second Strand Synthesis Enzyme Mix. The resulting DNA fragments were end-fragmented, DA-tailed, and ligated to NEBNext® Ultra II (NEB #E7355). Following ligation, adapters were con- verted to the “Y” shape by treating with USER enzyme, and DNA fragments were size-selected using Agencourt AMPure XP beads (Beckman Coulter #A6880) to generate fragment sizes between 250 and 350 bp. Adapter-ligated DNA was PCR amplified followed by AMPure XP bead clean up. Libraries were quantified using a Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific #DS10845), and the size range was confirmed using a High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies #5067-4626). Libraries were sequenced on an Illumina HiSeq2500 in single-read mode with the read length of 50 nt and sequencing depth of 20 million reads per sample. RNA-seq reads from the same sex and genotype (Supplementary Table 13 and 14), and a two-factor design consistently showed what changed in response to the genotype in both sexes (Supplementary Table 15). In a comparison between pgSIT♀ and WT♀, 660 genes were upregulated in pgSIT♀ and 392 were downregulated at an adjusted P value <0.05. The target gene, AAE1005656, was significantly upregulated in pgSIT♀ (Supplementary Table 10). A comparison between pgSIT♂ and WT♂ (Supplementary Table 13), 2067 genes were upregulated in pgSIT♂ and 2722 were downregulated at an adjusted P value <0.05. The target gene, AEI019894, was strongly downregulated in pgSIT♂ (Supplementary Table 10D). It is important to note here that the CRISPR/Cas9 pgSIT system disrupts the DNA (not the RNA) so transcription is expected to occur; however, the transcripts produced will encode mutations and should be degraded by nonsense-mediated mRNA decay (NMD) mechanisms. Indeed, these mutant RNA’s can be observed in the IGV (Supplementary Figs. 8 and 9). In the two-factor comparison, 1447 genes were upregulated in pgSIT♂ and 2563 were downregulated at an adjusted P value <0.05 (Supplementary Table 10E). For both DESeq2 comparison, gene ontology enrichment were performed on significantly differentially expressed genes, and these are provided as tabs in the corresponding tables (Supplementary Tables 13–15). All Illumina RNA sequencing data have been deposited to the NCBI-SRA, PRJNA699282.

Amplonc sequencing of target loci. To sample a variety of molecular changes at the gRNA target sites (myo-fem and Tubα), we used the Amplonc-EZ service by Geneviz© and followed the Geneviz® guidelines for sample preparation. Genomic DNA from 50 WT and 50 pgSIT sexed pupae (25♂:25♀) were extracted sepa- rately using DNeasy Blood and Tissue Kit (Qiagen, Cat. No./ID: 69506) following the manufacturer’s protocols. Primers with Illumina adapters (Supplementary Table 18) were used to PCR amplify the genomic DNA. PCR products were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat. No./ID: D4007). Roughly 50,000 one-directional reads were generated by Geneviz® and uploaded to Galaxy.org for analysis. Quality control for the reads was performed using FASTQC. Sequence data were then paired and aligned against the myo-fem or Tubα sequence using Map with BWA-MEM under “Simple Illumina mode”. Sequence data for both targets were detected using FreeBayes, with parameter selection level set to “simple diploid calling.” The amplonc sequencing data has been deposited as Supplementary File S1.

Prior mating with pgSIT’s suppress fertility. To determine whether prior matings with pgSIT♂ could reduce fertility, we initiated 15 cages each consisting of 20 newly emerged (2–3 days old) WT♂ and 20 newly emerged (2–3 days old) WT♀ virgin ♀. We allowed the pgSIT♂’s to mate with these ♀’s for a limited period of time (including 2, 6, 12, 24, and 48 h; all experiments begin at 9:00 am PST, three replicate cages each). Cages were shaken every 3 min for the first half hour to increase mating opportunities and left to mate for an additional 2 days. The ♀’s were then blood-fed, and each blood-fed ♀ was individually transferred to a single narrow Polystyrene vial (Genesee Scientific Cat. No. 1116), and eggs were counted and hatched for fertility determination. Following this, nonfertile ♀’s were then placed back into cages along with the original WT♂’s, plus an additional 50 mature WT♂’s, for another chance to produce progeny. This was repeated for up to five gonotrophic cycles. As controls, cages with 250 WT♂’s and 50 WT♀’s, or 50 unmated blood-fed WT♂’s with no ♀♂ added, or 50 unmated blood-fed WT♂’s with 250 WT♂ adults were also set up (Supplementary Table 8).

Life table parameters. Life table parameters were assessed by comparing WT, homologous gRNA2Ub7+; myo-fem©, homozygous Cas9, and transheterozygous pgSIT♀ (gRNA1Ub7+; myo-fem+; Cas9+) generated with Cas9 inherited from either the mother (maternal Cas9) or father (paternal Cas9). Larva/pupa development times were recorded as the number of days from hatched larva to pupae and then to adults. One hundred larva from each line were placed in separate larval rearing containers (Sterile, 34.6 × 21 × 12.4 cm, USA), with each 3 liters of deionized water. Larvae were counted once daily until pupation, and then the date of pupation and emergence were recorded. Larval to pupal development time was calculated for each sex. Pupae were transferred to plastic cups (Karat, C- KC9) with 100 ml of water, and survivors were recorded until adulthood.

For measuring ♂ longevity, we tested the variation in ♂ longevity among different lines using two methods: (i) released along with WT of the opposite sex or (ii) without WT of the opposite sex. (i) One hundred WT, homologous gRNA2Ub7+; myo-fem©, homozygous Cas9 newly eclosed adult mosquitoes (fifty ♀♂’s and fifty ♀♂’s) were maintained in a cage; fifty newly eclosed pgSIT♂’s (maternal Cas9) and fifty newly eclosed pgSIT♂’s (paternal Cas9) were caged with fifty newly eclosed WT♂’s and, finally, fifty newly eclosed pgSIT♂’s (maternal Cas9) and fifty newly eclosed pgSIT♂’s (paternal Cas9) were caged with fifty newly eclosed WT♂’s. (ii) Fifty ♀♂’s or ♀♂’s from each line were released into a cage separately without the opposite sex. Adults were provided with 10% sucrose and monitored daily for survival until all mosquitoes had died (three replicates).

For measuring ♀ fecundity and fertility, ♀’s (n = 50) and ♀’s (n = 50) 3 days post emergence raised under the same standardized larval conditions were placed into a cage and allowed to mate for 2 days. ♀ mosquitoes were blood-fed until fully engorged and were individually transferred into plastic vials with oviposition substrate. Eggs were stored in the insectary for 4 days to allow full embryonic development and then were hatched in a vacuum chamber. Fecundity was calculated as the number of eggs laid per ♀♀ and fertility was calculated as the percentage of eggs hatched per ♀♀. ♀♂ mating capacity (how many ♀♀ can be mated by one ♀♂) was measured as follows. Fifteen mature WT♂’s were caged with 1 mature ♀♂ of each genotype for 24 h, the same as described for all cages. Two days after the single ♀♂ was removed, 75 WT♂’s were added to each cage that previously had a pgSIT♂ (5:5:19 ratio). Blood meals were provided, and each blood-fed ♀♂ was individually transferred to a single vial for egg collection. The fecundity and fertility of each ♀♀ was determined. The mating capacity was calculated as the total number of ♀♀’s-total number of fertile ♀♀’s. The mating capacity of WT, homologous gRNA2Ub7+; myo-fem©, and homozygous Cas9♀♂ was equal to the number of fertile ♀♀’s.

Flight activity quantification. Mosquitoes were reared at 28 °C, 80% relative humidity under a 12:12 h light:dark regime, and measurements of flight activity were performed using a Drosophila Activity Monitoring (DAM) System (TriKinetics, LAM25) and DAMSystem3 software (TriKinetics) using large tube designs for mosquitoes (TriKinetics, PGT 25 × 125 mm Pyrex Glass). Individual 4–7-day-old, non-blood-fed virgin ♀ and non-mated ♀♂ mosquitoes were intro- duced into the monitoring tubes, which contained 10% sucrose (Sigma, Cat. No. S0389) at both ends of the tube as the food source. The DAM System was positioned vertically during the assays. Flight activity was measured over a period of 24 h by automatically calculating the number of times that mosquitoes passed through the infrared beam in the center of the tubes. The walls of the monitoring tubes were painted with Signigril® (Cat. No. S0122), which allows mosquitoes from walking upward. For preparing the wingless mosquitoes, the animals were anes- thetized on ice, and the wings were removed using Vannas Scissor (World Preci- sion Instruments, Cat. No. 14003). The wingless mosquitoes were allowed to recover for 12 h before recording. Mosquitoes were manually checked after flight activity recording to ensure survival. Data acquisition was performed using the DAM System (TriKinetics) (Fig. 2D, Supplementary Video 5, and Supplementary Table 6).
Sound attraction assay. The sound attraction assay was performed in a chamber with a temperature of 28 °C and humidity of 80%. Seven-day-old female mosquitoes were sex separated after the pupal stage. The day before testing, 30–40 female mosquitoes were transferred by mouth aspiration to a 15-cm³ mesh cage with a 10% sucrose bottle. Female mosquitoes were allowed to recover in the cage under a 12 h:12 h light:dark regime for 24 h. For each trial, a 10–60 Hz sine tone was applied on one side of the cage as a mating behavior lure, mimicking a flight tone. The number of mosquitoes landing on the mesh area around the speaker box (10 cm² or 20 cm²) was counted at 10-s intervals throughout the stimulus. The average percent of mosquitoes landing around the speaker area out of the total cage post-sound presentation was calculated (Fig. 2E, Supplementary Video 6, and Supplementary Table 7). Heatmaps were generated using Noldus Ethovision XT 16.

Multigenational population cage trials. To perform multigenational population cage trials, two strategies were employed: (i) release of eggs; (ii) release of mature adults (Fig. 3A and Supplementary Table 16). Cage trials were carried out using discrete non-overlapping generations. For the first release of eggs strategy (i), pgSIT eggs and WT eggs were hatched together using the following ratios (pgSIT: WT) of: 1:1 (100:100), 5:1 (500:100), 10:1 (1000:100), 20:1 (2000:100), and 40:1 (4000:100), and three biological replicates for each ratio (15 cages total). All eggs were hatched simultaneously, then separated into multiple plastic containers (Sterile, 34.6 × 21.4 × 12.4 cm, USA). Roughly 400 larvae were reared in each container using standard conditions with 3 liters of deionized water and were allowed to develop into pupae. Pupae were placed in plastic cups (Karaf, C-C99) with ~100 ml of water (~150 pupae per cup) and transferred to large cages (BugDorm-2) to eclose. Each individual was allowed to mate for ~5–7 days. The females were blood-fed, and the eggs were collected. Eggs were counted and stored for ~4 days to allow full embryonic development, then 100 eggs were selected randomly and mixed with pgSIT eggs with ratios (pgSIT: WT) of: 1:1 (100:100), 5:1 (500:100), 10:1 (1000:100), 20:1 (2000:100), and 40:1 (4000:100) to seed for the following generations, and this procedure continued for all subsequent generations. The remaining eggs were hatched to measure hatching rates and to screen for the possible presence of transformation markers. The hatching rate was estimated by dividing the number of hatched eggs by the total number of eggs.

For the release of mature adults strategy (ii), 3–4-days-old mature WT adult females were released along with mature (3–4 days old) pgSIT adult females at release ratios (pgSIT: WT): 1:1 (50:50), 5:1 (250:50), 10:1 (500:50), 20:1 (1000:50), and 40:1 (4000:100), with three biological replicates for each release ratio (15 cages total). One hour later, 50 mature (3–4 days old) WT adult females were released into each cage. All adults were allowed to mate for 2 days. The females were then blood-fed and eggs were collected. Eggs were counted and stored for 4 days to allow full embryonic development. Then, 100 eggs were randomly selected, hatched, and reared to the pupal stage, and the pupae were separated into females and males and transferred to separate cages. Three days post eclosion, ratios (pgSIT: WT) of 50 (1:1), 250 (5:1), 500 (10:1), 1000 (20:1), and 2000 (40:1) age-matched pgSIT mature females adult mosquitoes were hatched from the total number of eggs. This procedure continued for all subsequent generations.

Ethical conduct of research
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 approved by the UCSD Institutional Animal Care and Use Committee (IACUC, Animal Use Protocol #S17187) and UCSD Biological Use Authorization (BUA #R2401).

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Acknowledgements
We thank Judy Ishikawa for helping with mosquito husbandry. This work was supported by funding from a DARPA Safe Genes Program Grant (HR0011-17-2-0047) and an NIH award (R01AI151004) awarded to O.S.A. to support CM from the “U.S. Army Research Office and accomplished under cooperative agreement W911NF-19-2-0026 for the Institute for Collaborative Biotechnologies”, support to J.M.M. from the Innovative Genomics Institute, and an NIH award (R56-AI153334). The views, opinions, and/or findings expressed are those of the authors and should not be interpreted as representing the official views or policies of the U.S. government.

Author contributions
O.S.A. and M.L. conceptualized and designed experiments; T.Y., J.L., L.A., and S.G. performed molecular analyses; M.L., J.R.E., T.W., H.L., and M.B. performed genetic experiments; Y.Z., Y.W., N.D., J.C., and C.M. performed behavioral experiments; J.B., H.M.S.C., and J.M.M. performed mathematical modeling; L.A. performed bioinformatics; O.S.A., M.L., N.P.K., and R.R. analyzed and compiled the data. All authors contributed to writing and approved the final manuscript.

Competing interests
O.S.A. is a founder of Agragen, Inc. and has an equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. All remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25421-w.

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Peer review information This article has been peer reviewed as part of Springer Nature’s Guided Open Access initiative. Nature Communications thanks Marcelo Jacobs-Lorena, Michael Smanski, Jeff Seckel and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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