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CRISPR/Cas9-based split homing gene drive targeting *doublesex* for population suppression of the global fruit pest *Drosophila suzukii*

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Genetic-based methods offer environmentally friendly species-specific approaches for control of insect pests. One method, CRISPR homing gene drive that target genes essential for development, could provide very efficient and cost-effective control. While significant progress has been made in developing homing gene drives for mosquito disease vectors, little progress has been made with agricultural insect pests. Here, we report the development and evaluation of split homing drives that target the *doublesex* (*dsx*) gene in *Drosophila suzukii*, an invasive pest of soft-skinned fruits. The drive component, consisting of *dsx* single guide RNA and DsRed genes, was introduced into the female-specific exon of *dsx*, which is essential for function in females but not males. However, in most strains, hemizygous females were sterile and produced the male *dsx* transcript. With a modified homing drive that included an optimal splice acceptor site, hemizygous females from each of the four independent lines were fertile. High transmission rates of the DsRed gene (94 to 99%) were observed with a line that expressed Cas9 with two nuclear localization sequences from the *D. suzukii nanos* promoter. Mutant alleles of *dsx* with small in-frame deletions near the Cas9 cut site were not functional and thus would not provide resistance to drive. Finally, mathematical modeling showed that the strains could be used for suppression of lab cage populations of *D. suzukii* with repeated releases at relatively low release ratios (1:4). Our results indicate that the split CRISPR homing gene drive strains could potentially provide an effective means for control of *D. suzukii* populations.

gene drive | CRISPR | *Drosophila suzukii* | spotted wing *Drosophila* | *doublesex*

Significance

Spotted wing drosophila (*Drosophila suzukii*) is a vinegar fly with a worldwide distribution that does significant economic damage to soft-skinned fruits. We made and evaluated split CRISPR homing gene drives that target the conserved female-specific exon of the *doublesex* gene, which is essential for sexual development in *Drosophila*. Our results suggest that homing gene drives could provide a cost-effective approach for suppression of *D. suzukii* populations.

Several genetic strategies for suppression of insect pest populations such as female-specific release of insects carrying a dominant lethal (fsRIDL) (1, 2), X-shredder sex ratio distortion (3, 4), precision-guided sterile insect technique (pgSIT) (5, 6), and homing gene drives (7, 8) have been developed in the past few years. The gene drive concept emerged from naturally occurring selfish genetic elements that show biased or super-Mendelian inheritance (9, 10). In 2003, Austin Burt proposed the use of homing endonuclease genes (HEGs) for control of pest populations (11). The central idea was to adapt an HEG to recognize an essential gene in the species of interest, for example, a gene required for female development. Following the introduction of a double-strand break (DSB) by the homing endonuclease, repair through homology-directed repair (HDR) led to copying or "homing" of the HEG into the targeted gene. A homing event in the germline would convert a heterozygous allele to homozygous resulting in super-Mendelian inheritance of any given trait or genetic elements. HEG (I-SceI)-based homing gene drives have been successfully performed in *Anopheles gambiae* and *Drosophila melanogaster* (12–15). Since it proved difficult in practice to adapt HEGs to target essential genes, the advent of easily programmable "RNA-guided endonucleases" such as Cas9 has expedited gene drive research tremendously, showing the potential of this approach to genetically alter or suppress wild populations of insect pests (16–18).

CRISPR/Cas, which refers to Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein, was originally derived from the immune systems of bacteria and archaea (19, 20). In cells, Cas9 DNA endonuclease bound with a small single guide RNA (sgRNA) is guided to a specific target sequence through base pairing between the sgRNA and DNA. Once the target is recognized, Cas9 binds to its sequence and promotes DSBs. CRISPR/Cas9-based homing gene drive systems have been demonstrated in a wide range of organisms including yeast (21), *D. melanogaster* (18, 22), mice (23), and the mosquito disease vectors *A. gambiae* (24), *A. stephensi* (25), and *Aedes aegypti* (26). The systems typically contain three elements, a Cas9 gene driven by a promoter active in the germline, one or more sgRNA genes controlled by a U6 gene promoter, and a

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fluorescent protein marker gene to monitor biased inheritance (24, 25). The gene drive cassette is flanked by homologous sequences or "arms" that match the gene sequences to either side of the Cas9 cleavage site (27–31). Given their potential to spread, recommendations have been made for improved biocontainment of gene drive strains (32). One suggested approach is to test and evaluate gene drives as a "split" homing gene drive in which the Cas9 and sgRNA components are present at widely separated locations in the genome (17, 33).

Resistance development is one of the major challenges to develop an efficient gene drive system. This could simply occur due to naturally occurring allelic variants present in a population that are resistant to Cas9 cleavage (34, 35). Resistance can also arise as a consequence of DNA repair. In addition to HDR, the DSB at the homology site can also be repaired through nonhomologous end joining (NHEJ) and microhomology-mediated end joining pathways (36). Mutations within the sgRNA target site can prevent further Cas9-mediated cleavage, resulting in resistant-allele accumulation and loss of drive (24, 25). For example, a gene drive in *A. gambiae* was initially very successful, but the accumulation of short deletion mutations in the sgRNA target sequence that were resistant to Cas9 cleavage and restored functionality of the encoded protein led to drive resistance (37). More complex gene drive designs have been employed to minimize drive failure due to resistance. For example, using multiplexed sgRNAs along with a recoded rescue element targeting a haplolethal *RpL35A* gene, Champer et al have observed up to 91% of drive inheritance in *D. melanogaster* (29).

As population growth will largely be determined by female productivity, gene drives targeting a gene required for female development or reproduction could be particularly effective for population suppression (11, 24, 38). Drive males would be viable and fertile and thus pass on the disrupted female-essential gene to their offspring. Among insects, the sex-determining genes involved in sexual development and differentiation are functionally conserved (39) and have been exploited to develop genetic control methods of pest management (7). The bottom of the sex determination pathway in dipteran species such as *D. melanogaster, Ceratitis capitata,* and *Musca domestica* is quite conserved (40). The female-specific protein transformer (TRA) combines with non-sex-specific proteins (TRA2 and RBP1 in *D. melanogaster*) to regulate the sex-specific alternative splicing of *doublesex* (*dsx*) and *fruitless* (*fru*) gene transcripts (41, 42). In *D. melanogaster*, the TRA/TRA2/RBP1 complex binds to multiple sites in the 3′UTR of the female *dsx* transcript and enhance the use of a weak splice acceptor (SA) that immediately precedes the female exon (43). In the absence of the TRA/TRA2/RBP1 complex in males, this weak SA site is ignored, and instead, the common exon is spliced to a male-specific exon that is downstream of the female exon. The sex-specific DSX^F and DSX^M isoforms control the proper sexual and somatic tissue differentiation in a developing fly (44–47). As the female exon is highly conserved and essential for DSX^F function, it was a suitable target for a homing drive. At the laboratory scale, Kyrou et al. have shown a complete population suppression with a homing gene drive targeting the female-specific exon of the *dsx* gene in *A. gambiae* (7). The sequence matching the sgRNA spanned the intron–female exon boundary and was invariant among several *Anopheles* species. Drive-resistant alleles did not accumulate suggesting strong functional constraint on the targeted sequence.

We have been interested in developing genetic strategies for control of the invasive fruit pest *Drosophila suzukii*. A native of Asia, *D. suzukii* is now present in North America, Europe, and parts of South America (48–50). Unlike most *Drosophila* species that are not economic pests, *D. suzukii* females have a serrated ovipositor that facilitates egg laying in ripe fruit before harvest (51). While farmers mostly rely on insecticides for control, use is weather dependent, resistance has been reported to spinosad and pyrethroids in California (52, 53), and a wide range of noncrop host plants can provide a refuge (54, 55). Area-wide genetic control strategies offer an attractive species-specific alternative for control of this pest. The sterile insect technique (SIT) has been used successfully for area-wide suppression of some pests for over 70 y (56). In an SIT program, a large excess of sterile males (at least 10:1) is repeatedly released over the targeted area. For implementation of SIT for control of *D. suzukii*, methods for mass rearing and for sterilization have been developed (57–59). A recent study found that repeated releases of sterile males led to significant suppression of a field population of *D. suzukii* (60). Kandul and colleagues used the pgSIT approach to generate sterile *D. suzukii* males (6). This required crossing Cas9 and sgRNA strains that produced offspring with Cas9-mediated loss-of-function mutations in genes essential for female survival and male fertility. Repeated releases of sterile males (3 to 5:1 release ratio) into lab cage populations led to effective population suppression. We recently developed fsRIDL *D. suzukii* strains (61, 62) that produce only males when reared on diet without tetracycline. We showed that repeated releases of an excess of fsRIDL males (3 to 10:1 release ratio) led to suppression of small cage populations of *D. suzukii*. While effective, implementing SIT, pgSIT, and fsRIDL control programs across a large area would likely be expensive because of the need to repeatedly release an excess of sterile or modified males. As homing gene drive offers a potentially much more efficient population suppression approach for control of *D. suzukii* (63), here we have developed and evaluated a CRISPR/Cas9-based split homing gene drive that targets the female-specific exon of *dsx*.

Results

***dsx* Female-Exon Disruption Causes Dominant Female Sterility in Most but Not All Lines.** The *dsx* female-specific exon (Fig. 1*A*) is highly conserved in the genomes of wild-type *D. suzukii* collected across the United States and several other locations in Europe and South America (64) (*SI Appendix*, Table S2). Six sgRNAs were evaluated using an in vitro DNA cleavage assay with recombinant Cas9 (*SI Appendix*, Fig. S1). One of the most effective, dsx-sgRNA3, was selected for the homing gene drive construct. The selected target is well conserved across *D. suzukii* genomes (*SI Appendix*, Table S2). The *dsx* homing cassette (6.1 kb) consists of *dsx* homology arms, an U6:3 promoter–dsx-sgRNA3 gene, and a constitutively expressed DsRed selection marker gene (Fig. 1*B*). The U6:3 promoter was from *D. melanogaster* (65) and was used previously to express a white sgRNA in *D. suzukii* (66). The sgRNA targets Cas9 to the *dsx* female exon to induce a DSB 52 bp downstream from the start of the exon. Following microinjection of the *dsx* homing construct and Cas9 protein precomplexed with the *dsx*-gRNA into precellular embryos (67, 68), four independent homing strains were established at a transformation rate of about 9% (Dataset S1). All of the transgenic G_1 females that had a single copy of the *dsx* homing transgene were sterile with the exception of two out of the six females obtained from the 1C G_0 parent (Dataset S1). Consequently, the homing lines have been maintained by crossing transgenic males with wild-type virgin females each generation. To further investigate sterility, hemizygous females were crossed with wild-type males, and the proportion that produced offspring was recorded. Hemizygous 1C2 female fertility was comparable to wild type (Fig. 1*D* and

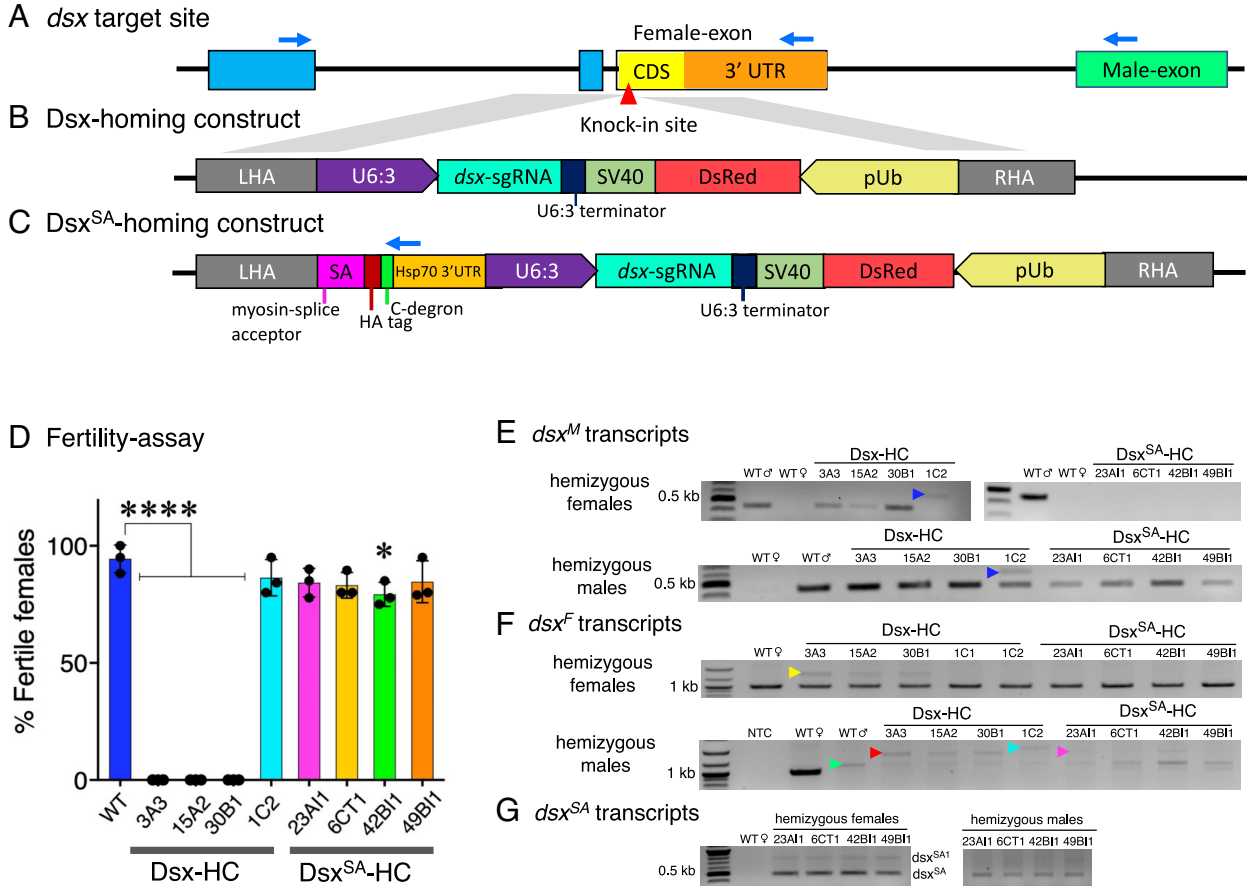

Fig. 1. Homing gene constructs targeting the *D. suzukii dsx* female-specific exon. (*A*) Schematic representation of the *dsx* gene showing the protein-coding exons and 3′UTR. There are two common exons (blue boxes) a female-specific exon (coding sequence/CDS: yellow box and 3′UTR: orange box) and a male-specific exon (green box). The target site into the CDS of female-exon is indicated by a red arrowhead. (*B*) The *dsx* homing construct (Dsx-HC) contains left and right homology sequences (LHA and RHA) flanking the *dsx* target site, the DmU6:3-dsx-sgRNA construct that provides constitutive expression of the sgRNA, and a DsRed selection marker. (*C*) *Doublesex* splice acceptor (SA) homing construct (Dsx^{SA}-HC) which is identical to the construct shown in panel *B* but also contains a myosin SA gene cassette inserted between the LHA and the U6:sgRNA gene. The myosin SA gene cassette contains the myosin SA site, an in-frame HA tag, a C-terminal degron sequence, a translation stop codon, and an hsp70 gene polyadenylation site and 3′UTR. (*D*) Fertility of hemizygous females from the homing strains. Wild type used as control (****$P < 0.0001$, *$P = 0.0260$). (*E–G*). RT-PCR with primers to detect the *dsx^{M}*, *dsx^{F}*, *and dsx^{SA}* transcripts in hemizygotes. (*E*) Transcripts that include the male exon. In wild-type controls, the *dsx^{M}* transcript is only detected in RNA from males. The dominantly sterile females of lines 3A3, 15A2, and 30B1 all showed *dsx* male transcript expression. Females from the 1C2, and the *dsx* SA homing strains (23AI1, 6CT1, 42BI1, and 49BI1) did not show any detectable *dsx^{M}* transcript. Males from all lines produce the *dsx^{M}* transcript. 1C2 females and males also produce the *dsx$^{M, C2}$* splice product (*E*, blue arrowhead). (*F*) Transcripts that include all or part of the female exon. Females from all lines produce the *dsx^{F}* transcript. Arrowheads indicate alternatively spliced transcripts that include the female exon detected in females (*dsx^{U1}*, yellow) and males (green, *dsx^{M2}*; red, *dsx^{U1}*; cyan, *dsx$^{U1, C2}$*; pink, *dsx^{U1SA}*). (*G*) In the *dsx^{SA}* homing strains, males and females produce the *dsx^{SA}* transcript, which uses the myosin splice acceptor site to join exon 2 with the HA tag exon. A minor unspliced product dsx^{SA1} was also detected. See *SI Appendix*, Tables S7 and S8, for details of alternatively spliced transcripts. Blue arrows indicate locations of RT-PCR primers.

SI Appendix, Table S3), but fecundity was reduced (*SI Appendix*, Table S5). However, hemizygous females from the other lines were fully sterile (i.e. dominant sterility) (Fig. 1*D*) and did not lay eggs (*SI Appendix*, Table S4). Morphologically, the dominant sterile females appeared to be very similar to wild type with a serrated ovipositor that allows the pest to lay eggs in ripe fruit (*SI Appendix*, Fig. S4). Further, upon dissection, the ovaries appeared to contain developing eggs, although the ovaries were often smaller than wild type (*SI Appendix*, Fig. S5). All homozygous 1C2 flies were intersexual but could be separated morphologically as male-like or female-like (*SI Appendix*, Figs. S2–S5). Molecular analyses with PCR primer pairs for X-linked (*yellow*) and Y-linked (*DsPp1-Y2*) genes showed that the male-like and most of the female-like intersex flies were genetically male (XY) and female (XX), respectively (*SI Appendix*, Fig. S10 *A* and *B*).

We hypothesized that the reason 1C2 hemizygous females were fertile but females from the other initial drive strains were sterile was because of nucleotide differences that somehow influence the splicing of *dsx* exon 2 to the male exon. To test this hypothesis, we initially amplified and sequenced a 6.7-kb region from the transgene to the male exon. However, very few nucleotide differences were found between the drive strains and none that were unique to 1C2 (*SI Appendix*, Fig. S8). Consequently, we next examined a 1.07-kb region upstream of the transgene that included the common exon 2 and part of the first intron. In this region, there were a few nucleotide differences between the 1C2 strain and the sterile strains, mostly within the first intron (*SI Appendix*, Fig. S9).

Hemizygous Females Express the *dsx*^{M} Transcript. We next examined whether any of the females from the homing strains produce the male-specific *dsx^{M}* transcript. Reverse transcriptase PCR and 3′RACE-PCR analyses showed that the hemizygous females from the dominant female sterile lines produce both the male and female transcripts, but 1C2 females only make the female transcript (Fig. 1 *E* and *F*). In addition, 3′ RACE analysis identified a shorter transcript in females from all lines that appears to use a cryptic polyadenylation signal in the U6:3 terminator (*SI Appendix*, Fig. S7 and Table S7).

All strains produce the female dsx^F transcript (Fig. 1*F*). However, this analysis also detected a minor splice variant that was present in all sterile strains but not 1C2 (Fig. 1*F*). This transcript was produced through joining of the exon 2 splice donor site with a cryptic SA site in the *D. melanogaster polyubiquitin* promoter (reverse strand), which is part of the PUb-DsRed marker gene (*SI Appendix*, Table S8). Male-like (XY) 1C2 homozygotes did not produce the dsx^M transcript (*SI Appendix*, Fig. S10*C*). However, a related transcript, $dsx^{M.C2}$, which is similar to dsx^M but has an extra copy of exon 2, was identified (*SI Appendix*, Table S8). The extra copy of exon 2 is in-frame and would encode a protein identical to DSX^M but with a duplication of the 46 amino acid domain encoded by exon 2 (*SI Appendix*, Table S12). These molecular analyses show that the female-exon knock-in disrupts splicing of *dsx* transcripts.

Addition of a Myosin Splice-Acceptor Site Rescued the Hemizygous Female Sterility of Homing Strains. Previous modeling had shown that population suppression would be more efficient if homing occurred in both sexes (69). To consistently obtain a drive that was not dominant female sterile, we reasoned that the addition of an optimal SA site directly after the left homology arm could prevent the use of the downstream male exon SA site. The modified homing construct contains the SA site sequence of muscle myosin heavy-chain gene from *D. melanogaster* (70, 71), followed by an in-frame short coding region that includes an HA epitope tag (72) and C-terminal degron sequence (73, 74) (Fig. 1*C*). The latter was included to promote proteolysis of the DSX-HA protein to minimize any potential interference with the DSX^F protein made by the nontargeted *dsx* allele. Following microinjections with the homing construct and Cas9/sgRNA complex, four G_0 produced transgenic offspring. Molecular analyses confirmed the correct integration of the transgene in all four of the dsx^{SA} homing construct (Dsx^{SA}-HC) strains (*SI Appendix*, Fig. S6). In contrast to the sterility observed with the initial homing construct, hemizygous females from all four lines were fertile and comparable to wild type (Fig. 1*D*). The fecundity of a selected line, $23AI1^{SA}$, was comparable to wild type (*SI Appendix*, Table S5). Further, RT-PCR and 3′RACE-PCR analyses confirmed that these hemizygous females were not expressing the male transcript (Fig. 1 *E* and *F*), but the transcript "HA tag-C-degron" was being expressed and correctly spliced (Fig. 1*G* and *SI Appendix*, Fig. S7). Hence, these results demonstrated that myosin splice site presence prevented the male transcript expression and significantly rescued hemizygous female sterility observed with the initial *dsx* knock-in lines. Homozygotes were intersexual, either XY male-like or XX female-like (*SI Appendix*, Fig. S10). Male-like homozygotes did not appear to produce the dsx^M transcript but did efficiently use the myosin SA site.

Homing Gene Drive Efficiency. We tested homing gene drive efficiency by following a split homing gene drive approach (28) using a previously developed *Dmnos-Cas9-NLS* line (#36) that expresses Cas9 in the germline from the *D. melanogaster nanos* promoter (66). Homozygous Cas9 virgin females were crossed with hemizygous males of a homing strain. Their offspring that carried a copy of the Cas9 transgene and the disrupted *dsx* gene with the drive construct were identified and then crossed with wild-type flies (29). The males and females obtained from the initial cross were called "male drive" and "female drive", respectively (Fig. 2*B*). For the dominant sterile female strains, only the male drive homing was assessable, whereas with fertile female strains, both the male and female drive's homing efficiency was measured. This was done by counting the proportion of offspring that showed red fluorescence. If there was no homing, 50% of the offspring would be expected to inherit the disrupted *dsx* allele with the DsRed and sgRNA genes. The female drives of 1C2 and $23AI1^{SA}$ showed drive inheritance of 61% and 58%, respectively (Fig. 2*C*, Dataset S2, Tabs 4 and 6). The homing drive inheritance of the male drives of these strains was significantly higher ($P < 0.0001$, one-way ANOVA) at 71% and 66%, respectively (Fig. 2*D*). The homing drive efficiency of other female fertile lines 1C1 and $6CT1^{SA}$ was found in the same range (Dataset S2, Tabs 3 and 5). For the dominant female sterile strains 3A3 and 15A2, the male drives showed a homing drive inheritance of 57% and 56% (Dataset S2, Tabs 1, 2). We also measured the homing drive efficiency using a *Dmvasa-Cas9-NLS* line (#4) that is active in the germline and soma (66). The male drive showed drive inheritance up to 59 to 65% (*SI Appendix*, Fig. S12 and Dataset S2, Tabs 7-9). The female drive efficiency could not be assessed as females developed a deformed ovipositor structure and were sterile. All of the drive experiments described above were from crosses of groups of males and females (i.e., batch crosses). For the fertile 1C2 line, we also measured drive efficiency with single-drive parents. The individual 1C2 female drive and male drive crosses showed drive inheritance which was comparable to batch crosses with an average of 58% for female drive and 69% for male drive but with more variation between crosses (Dataset S2, Tabs 10, 11).

The *Dmnos-Cas9-NLS* line (#36) used for the above experiments had lower editing efficiency than lines that carried a version of Cas9 with two nuclear localization sequences (NLS-Cas9-NLS), gene promoters from *D. suzukii,* and a viral p10 terminator (66). Unfortunately, we were unable to use these lines for drive experiments as, like the homing construct, they also carried a constitutively expressed red fluorescent protein marker (66). Consequently, with the aim of improving homing efficiency, Cas9 lines were made that expressed NLS-Cas9-NLS from the *D. suzukii nanos* promoter (Fig. 2*A*). The 3′UTR and flanking sequence from the *D. suzukii nanos* gene were used to ensure localization of the Cas9 transcript at the posterior end of the oocyte and translational repression of unlocalized transcripts (75). Preliminary crosses with lines that express sgRNAs for the *yellow* and *white* genes (66) indicated that the NLS-Cas9-NLS lines were all active and produced a high proportion of heritable *yellow* and *white* mutations (*SI Appendix*, Table S9). Homing drive experiments were performed as before with one of the X-linked NLS-Cas9-NLS lines (#27AI1). Homozygous virgin NLS-Cas9-NLS females were crossed with hemizygous Dsx-HC and Dsx^{SA}-HC males, and offspring were collected that had one copy of each transgene. For the dominant female sterile line 3A3, male drive efficiency was high with a homing drive inheritance of 97% (Fig. 2*F*, Dataset S2, Tab 12). Similarly, male and female drives showed a high homing drive inheritance of 97 to 99% with the 1C2 and $23AI1^{SA}$ lines (Fig. 2 *E* and *F*, Dataset S2, Tabs 13-14 and 17). However, fecundity was reduced with the female drives compared to wild type (*SI Appendix*, Table S6), which suggests somatic Cas9 activity and/or maternal Cas9 deposition as Cas9 was inherited from the female parent. Reduced fecundity was observed in *A. gambiae* heterozygous *dsx* drive females (7). Further, the F_2 female offspring from female drives showed a deformed ovipositor and were sterile. Molecular analysis showed the presence of insertion/deletions (indels) at the Cas9 cut site, indicating significant maternal deposition of Cas9 and sgRNA. F_2 females from male drives were fertile. We also performed drive experiments beginning with Cas9 males crossed with hemizygous 1C2 and $23AI1^{SA}$ virgin females. A high frequency of transmission of the DsRed gene was again observed with 99% and 95% of the offspring showing red fluorescence, respectively (*SI Appendix*, Fig. S13 and Dataset S2, Tabs 15, 16).

Potential Resistance Allele Development. In *D. melanogaster* homing drive strains, a lower drive inheritance rate due to resistance allele formation through NHEJ repair pathways has been reported

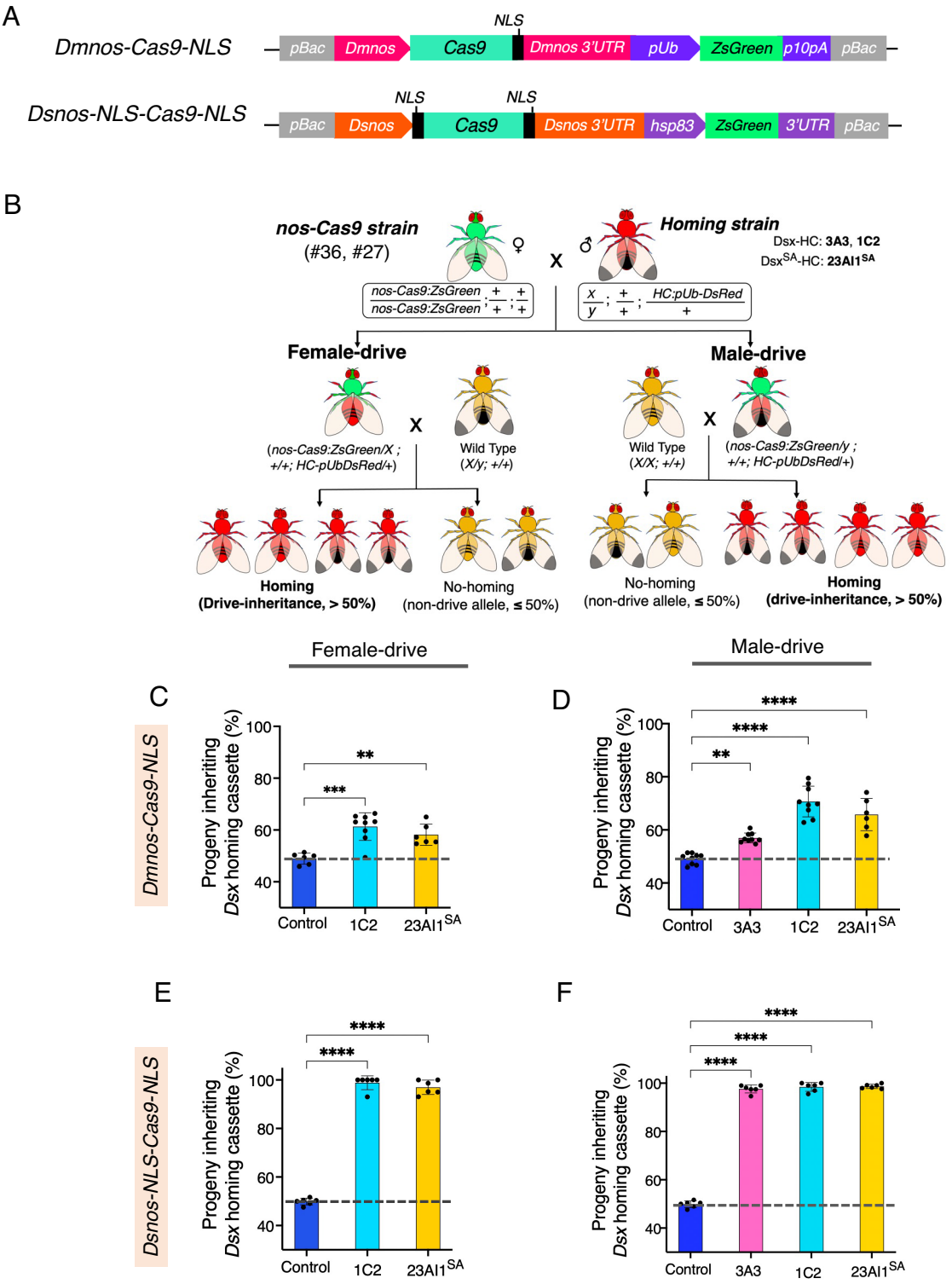

Fig. 2. Split homing drive genetic cross schemes and homing gene drive inheritance. (*A*) Schematic representations of Cas9 expression constructs (not to scale). The *Dmnos-Cas9-NLS* construct uses the *D. melanogaster nos* gene promoter and 3′UTR for expression of Cas9 with a single NLS. The *Dsnos-NLS-Cas9-NLS* construct uses the *D. suzukii nanos* gene promoter and 3′UTR for expression of Cas9 with N and C-terminal NLS. (*B*) Homozygous virgin females of a Cas9 expressing strain (*Dmnos-Cas9* #36 and *Dsnos-NLS-Cas9-NLS* #27AI1) were crossed with hemizygous males of each homing strains (Dsx-HC: 3A3, 1C2 and Dsx-HC^{SA}: 23AI1^{SA}) and their female and male offspring that carried both the Cas9 and homing elements were selected and crossed with wild type. The offspring of the female drive and male drive crosses were scored for the presence of the drive-linked red fluorescent marker gene to assess germline transmission efficiency of the homing gene drive elements. (*C–F*) Plots depicting the drive element inheritance (%) using *Dmnos-Cas9-NLS* (*C* and *D*) and *Dsnos-NLS-Cas9-NLS* (*E* and *F*) lines. Both female drive (*C* and *E*) and male drive (*D* and *F*) are shown for each homing strain compared to control (nondrive crosses). Female drive efficiency could only be assessed in the female fertile strains 1C2 and 23AI^{SA}. Female drive and male drive of each strain showed a significantly higher rate of drive inheritance compared to controls (**P < 0.01, ****P < 0.0001, One-way ANOVA).

(28, 34). As the female exon of *dsx* is essential for function, most deletion mutations would simply be lost from the population. However, it is possible that some small in-frame deletion or insertion mutations would be resistant to Cas9 cleavage but produce a functional DSX protein. Consequently, we performed deep sequencing and Sanger sequencing of genomic DNA from

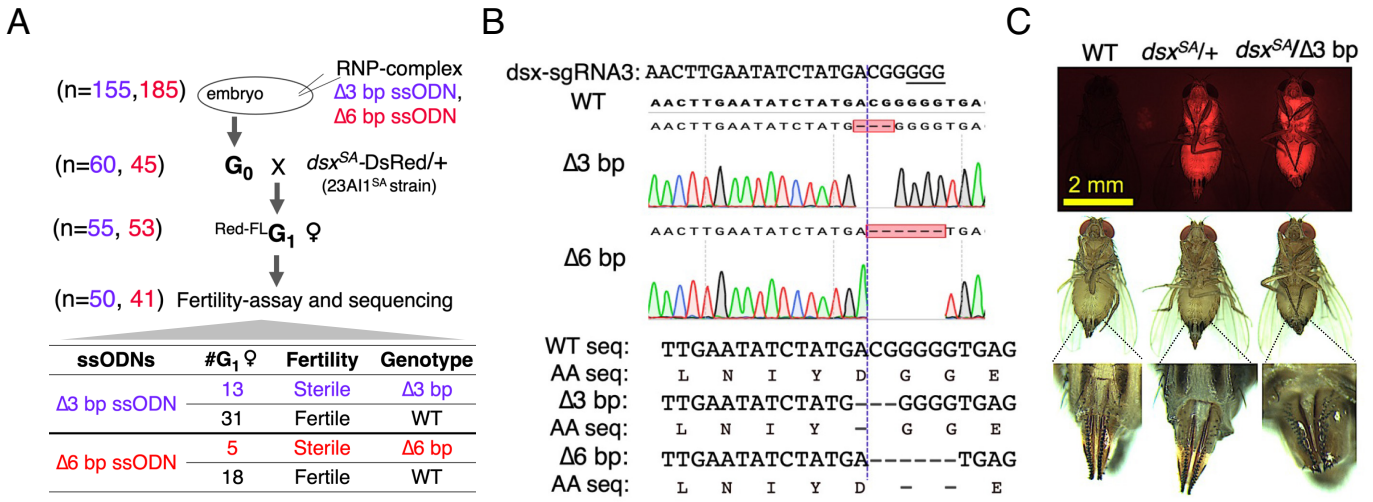

Fig. 3. In-frame deletion mutations are resistant to cleavage but are not functional. (*A*) Crossing scheme used to obtain individual flies that carried one *dsx* allele with a deletion mutation. *D. suzukii* embryos were injected with a mix of ssODN carrying the desired deletion and Cas9/sgRNA complex. The table shows the number of G_1 individuals that had the desired 3 bp or 6 bp deletion and their fertility. (*B*) DNA sequences of G_1 individuals showing the presence of the desired deletion and the affected amino acids in DSX^F. (*C*) The G_1 females with the 3-bp deletion show a deformed ovipositor.

nondrive flies, the flies that did not inherit the DsRed fluorescent protein gene. The flies will carry one wild-type copy of *dsx* from the wild-type parent and one potentially mutant *dsx* allele from the drive parent. The analysis of nondrive flies from the crosses with the *Dmnos-Cas9-NLS* #36 line revealed that approximately 90 to 99% were wild type (*SI Appendix*, Fig. S11), which is consistent with the low activity of the Cas9 line. Of the modified alleles, likely loss-of-function (frameshift) and potentially functional in-frame deletion mutations were identified (Dataset S3, Tabs 1-3). From the drive experiments with the *Dsnos-NLS-Cas9-NLS* line, a higher proportion of the nondrive flies showed indels, which is consistent with higher Cas9 activity of the line. From the female drive crosses, 100% of the nondrive flies showed modified *dsx* alleles. About 50% of the nondrive flies from the male drive crosses were wild type (Dataset S3, Tab 4).

We next evaluated whether some of the mutations would prevent in vitro cleavage of DNA templates by Cas9/sgRNA complex. While single bp changes within the dsx-sgRNA3 target did not prevent DNA cleavage, templates with 3 bp and 6 bp in-frame deletions were not appreciably digested by Cas9 (*SI Appendix*, Fig. S1). To determine whether the DSX protein produced from a *dsx* mutant allele was functional, we injected *D. suzukii* embryos with Cas9/dsx-sgRNA3 complex and long single-stranded oligonucleotides (ssODNs) with the deletion mutations. If the ssODN was used as a template for HDR, the *dsx* allele should have the desired in-frame 3-bp or 6-bp deletion. Individual G_0 were crossed with hemizygous $23AI1^{SA}$ flies and their female offspring collected and individually assessed for fertility and then genotyped (Fig. 3, Dataset S3, Tab 5). All G_1 females that carried a *dsx* allele with either the 3bp or 6bp deletion were sterile and had a deformed ovipositor (Fig. 3). Thus, the DSX^F proteins produced by the mutant alleles do not appear to be functional.

Mathematical Simulations. We next modeled discrete generation lab cage population suppression experiments similar in design to those we used previously with a *D. suzukii* fsRIDL strain (61). We were interested in determining what release ratios would be needed to drive the cage populations to extinction. As before, the starting population would consist of 300 pairs of a North Carolina wild-type strain of *D. suzukii*. The released males would carry one copy of the X-linked NLS-Cas9-NLS gene used above and one copy of a *dsx* homing drive transgene. To be successful, the released males would need to be competitive with the wild-type males for mating with virgin females. Consequently, we next performed mating competition experiments with wild-type males, drive males, and wild-type virgin females in a 1:1:1 ratio. The results show that the drive males are fully competitive with wild-type males (*SI Appendix*, Table S10). Nevertheless, the model incorporated a fitness cost of 5% for the Cas9 transgene. The model also included a reduced fecundity for hemizygous $23AI1^{SA}$ drive females (approximately 63% relative to wild type, *SI Appendix*, Table S6) and 100% sterility for female offspring of hemizygous drive females (*SI Appendix*, Fig. S14). Repeated releases of males at relatively low release ratios (1:4) with either dominant or recessive female sterile drive led to suppression within 10 generations (Fig. 4). In contrast, single releases with release ratios as high as 10:1 (transgenic: wild type) were much less successful in driving the cage populations to extinction (*SI Appendix*, Fig. S15). We also modeled a fully autonomous drive using the same fitness/fecundity values and homing frequencies as for the split drive. We found that a single release of the dominant female sterile drive is ineffective at low release ratios (*SI Appendix*, Fig. S16), consistent with earlier modeling that found that an HEG drive that caused dominant female sterility did not invade from rare (76). In contrast, a single release of the recessive female sterile drive showed a much higher likelihood of causing extinction (*SI Appendix*, Fig. S16).

Discussion

In this study, we developed a CRISPR/Cas9-based split homing gene drive that targets *dsx,* a gene essential for sexual development. A gene drive targeting *dsx* was found to be highly efficient for suppression of *A. gambiae* cage populations (7). Similarly, our homing gene drive targeted the highly conserved female-specific exon of *dsx* in *D. suzukii*. In this study, the sgRNA was fully within the female exon, whereas for *A. gambiae,* the sgRNA spanned the intron–female exon boundary. This was thought to be advantageous as there appears to be strong selection for the nucleotide sequence around the splice site, likely reflecting sequence requirements for sex-specific alternative splicing. In *D. suzukii*, it was not possible to design a sgRNA that spanned the exon–intron boundary due to a lack of a suitable PAM site. The most similar would be a sgRNA that is mostly in the female exon but with a PAM in the intron (sgRNA6, SI Appendix, Fig. S1 *A* and *B*). Nevertheless,

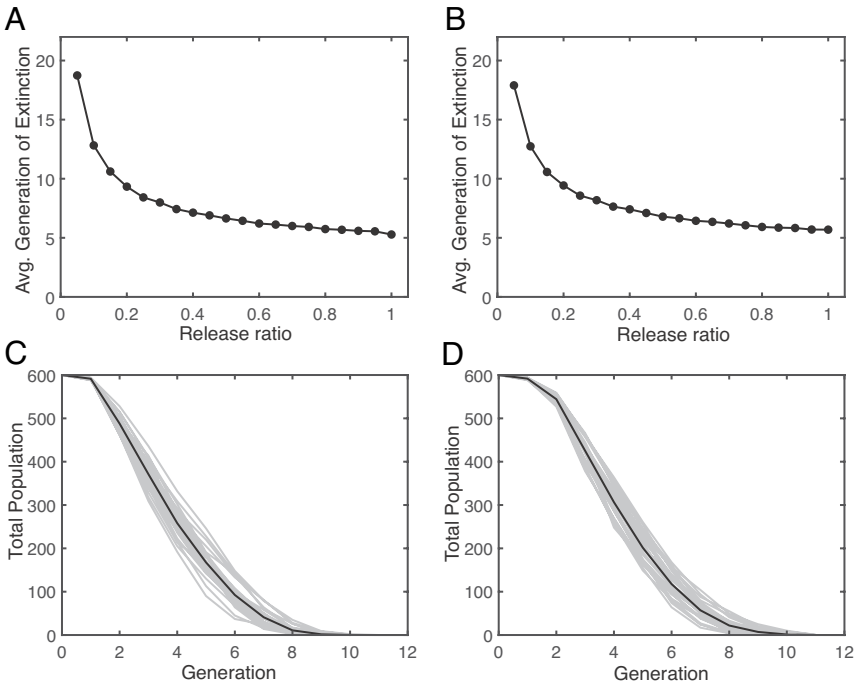

Fig. 4. Modeling results of small cage experiments. Each cage contains 300 wild-type pairs. The *Top* row shows the average generation of extinction versus release ratio for a (*A*) dominant and (*B*) recessive female sterile drive. Releases occur every generation and ratios vary between 5% (1:20) and 100% (1:1). Each data point is the average generation of extinction for 100 replicates. The plots in the *Bottom* row show the mean total population of 30 individual simulations for a (*C*) dominant and (*D*) recessive female sterile drive assuming a 1:4 release ratio each generation. In both figures, trajectories for the individual replicates are plotted in gray.

the sequence we targeted does appear to be functionally constrained. We found that *dsx* alleles carrying either a 3 bp or 6 bp in-frame deletion did not produce a functional DSX^F protein. Both mutations were detected in nondrive flies and do prevent in vitro cleavage of DNA templates by Cas9/sgRNA. However, since the DSX^F protein is not functional, it would not produce resistance to drive. Of course, it is possible that other mutations in the targeted sequence could lead to functional resistance to drive. These could be uncovered by performing gene drive experiments with cage populations of *D. suzukii*. The potential for resistance allele formation could also be reduced by modifying the homing drive to express more than one sgRNA such as dsx-sgRNAs 3 and 4 or 6 (77, 78).

Unlike in *A. gambiae* (7, 24), *D. suzukii* females with a single copy of the *dsx* homing construct were found to be sterile (except 1C lines, see below). The dominant female sterility was not completely unexpected as naturally occurring insertion mutations within the *D. melanogaster dsx* female-specific exon can cause dominant sterility (46). The mutations in the female exon interrupt regular *dsx* pre-mRNA alternative splicing generating both the male and female isoforms causing intersexual development (44, 46, 79). That is, apparently, an insertion mutation can disrupt the enhancement of the weak female-specific SA site by the TRA/TRA2/RBP1 complex bound to the *dsx* 3′UTR, and consequently, the spliceosome makes use of the downstream male exon SA site. In the homing drive strains, the wild-type *dsx* allele in hemizygous females would continue to produce the female-specific transcript. Consequently, XX flies would have both dsx^M and dsx^F transcripts, likely causing the observed sterility (46, 80). Although hemizygous sterile females appeared to have a normal ovipositor and contained ovaries, they did not lay eggs, which was possibly caused by altered *dsx* neuronal function affecting female reproductive behaviors (81). The exception was the 1C hemizygous females (1C1 and 1C2), which were fertile. This could be due to differences in genetic background between the strains. We also considered that this could be due to nucleotide polymorphisms in the *dsx* gene as alternative splicing follows a complicated regulatory process that is influenced by presence of exonic and intronic mutations (82). However, there were very few nucleotide differences in the 6.7-kb region between the transgene and the downstream *dsx* male exon and none that were unique to the 1C2 strain. There were a few nucleotide differences between the 1C2 strain and the sterile strains in a 1.07-kb region upstream of the transgene. It is possible that these few specific nucleotides are important for the correct splicing of *dsx* transcripts. The nucleotide polymorphisms unique to 1C2 were in the first intron about 500 to 600 bp upstream of exon 2 (*SI Appendix*, Fig. S9). 1C2 males and females did produce an unusual minor transcript that had a duplication of exon 2. This exon duplication could be due to trans-splicing, which is rare in *Drosophila* (83). A study of RNA splicing in rat liver and kidney concluded that exon repetition is rare, allele specific, and determined in cis (84). If so, the nucleotide polymorphisms unique to 1C2 in the first intron could be influencing this unusual splicing event. It also possible that 1C2 has an extra copy of exon 2, but if so, it must well upstream of the female exon. As epigenetic mechanisms can influence alternative splicing (85), another possibility is that the local chromatin environment around the *dsx* gene in the 1C lines somehow influences splicing such that the splicing machinery does not use the male exon SA site. This may explain why we did not detect splicing of exon 2 to either the male exon or the cryptic SA site in the polyubiquitin promoter of the DsRed marker gene in the 1C2 strain. Both splicing events were found in all of the dominant sterile strains. It is possible that the protein encoded by the transcript that uses the cryptic SA site could bind to the DSX^F protein produced by the wild-type allele and interfere with function as DSX functions as a dimer (86). If so, the observed sterility would be due to production of DSX^M and the dominant-negative DSX protein. Last, with regard to splicing, we found that all males produced the dsx^{M2} transcript (Fig. 1 and *SI Appendix*, Table S8), which was recently identified

in *D. melanogaster* and found to have masculinizing activity (87). This male-specific transcript is similar to dsx^F but retains the female intron, which contains an in-frame translation stop codon near the beginning of the intron. Homozygous 1C2 and $23AI1^{SA}$ developed as sterile intersexes with XY more male-like and XX more female-like. It is not clear why XY and XX intersexes showed different morphologies as we did not detect either dsx^F or dsx^M transcripts and both XY and XX appear to produce dsx^{M2} (*SI Appendix*, Fig. S10).

Since prior modeling suggested that population suppression would be more efficient if drive was not limited to one sex (69), we modified the homing construct by adding a myosin SA site to reduce the use of the male exon acceptor site. The construct also included an in-frame carboxy-terminal degron motif that could destabilize the protein, but this remains to be shown. All lines carrying the modified homing construct were fertile and did not show any evidence of male splicing. Whereas the dominant female sterile strains are maintained by crossing transgenic males with wild-type virgin females each generation, the dsx^{SA} strains are easily maintained with only the occasional need to remove nontransgenic flies. For rearing at a larger scale, it could be advantageous to make a balancer chromosome with the wild-type *dsx* gene within a chromosomal inversion and carrying a recessive lethal mutation. Such "balancer" chromosomes are widely used for maintenance of *D. melanogaster* lines (88).

Drive conversion efficiencies with the Cas9 lines that express a version of Cas9 with N and C-terminal NLS were 89 to 99% for female drive and 95 to 97% for male drive. Drive efficiencies were lower with our original lines that express Cas9 with a single C-terminal NLS. This was consistent with our previous report that NLS-Cas9-NLS lines showed higher editing efficiency (66). Cas9 carrying multiple NLSs has been reported to show higher editing efficiencies in mammalian cells (89, 90). However, it is also possible that using the promoter and 3′UTR from the *D. suzukii nos* gene for Cas9 expression has improved editing efficiency.

To determine release ratios that could be successful in future lab cage suppression experiments, we modeled repeated releases of males hemizygous for Cas9 and a *dsx* homing drive construct. The modeling showed that repeated releases of hemizygous males at low release ratios (e.g., 1:4) could suppress a population in less than 10 generations. This compares favorably to the large releases required for SIT [typically 10:1 or higher (56, 91)] and the 3:1 ratio used previously with fsRIDL males in a discrete generation experiment (61). Interestingly, releasing males carrying either the recessive or dominant female sterile gene drives produced similar results (Fig. 4 *C* and *D*). With the dominant female sterile drive, suppression is due to introgression of the disrupted *dsx* gene and homing gene drive in males. The results are similar to coupling fsRIDL to gene drive, which was previously modeled to be very efficient for population suppression (92). We also modeled single releases of fully autonomous dominant and recessive female sterile drives. Consistent with previous modeling of a HEG homing drive that caused dominant female sterility (76), low release ratios of the dominant female drive were unlikely to cause extinction of the cage populations. With drive limited to males, at low release ratios, the drive allele does not accumulate to a high enough level to place a significant load on the cage population. Of course, these modeling predictions remain to be confirmed experimentally. For a future field release, the split drive systems developed in this study have the advantage that the drive is reversible and largely confinable, particularly if the population is isolated (26, 33). For larger-scale field releases, performing a cross of Cas9 and *dsx* homing lines would be a challenge and would require development of methods for mass separation of sexes. This is also a challenge for the *A. gambiae dsx* homing strains and for implementation of the pgSIT approach in *D. suzukii* (6, 7). Fluorescence-based machine sorting could provide a means for separating sexes at scale (93).

The *dsx* gene was thought to be an excellent target site for gene drive by previous research (7), one that was highly conserved with the potential to be used in a wide variety of insect species. Our finding of dominant sterility potentially complicates the use of this gene in some species, but our use of a SA suggests a way that this could likely be rectified, preserving *dsx* as a candidate target in diverse species.

Materials and Methods

Homing and Cas9 Constructs Design and Assembly. The highly conserved small protein-coding part of the *dsx* female-specific exon was targeted to make a split homing drive strain that would disrupt female fly development. First, the *dsx* gene sequence was retrieved from the SWD fly base (http://spottedwingflybase.org) and used to design sgRNAs using ChopChop (https://chopchop.cbu.uib.no). The most efficient sgRNAs were selected through in vitro testing (*SI Appendix*, Fig. S1), and a synthetic DNA fragment designed with left and right homology arms sequences to either side of the Cas9 cut site for one of the gRNAs (sgRNA5) (*SI Appendix*, Table S1).

All the plasmids used in this study were made following standard cloning techniques and confirmed by DNA sequencing. First, for the homing constructs, the pCFD3 vector (Addgene, #49410) that contains the *D. melanogaster* U6:3 promoter and terminator was digested with *BbsI* and then ligated with annealed oligos sgRNA3SS: GTCGAACTTGAATATCTATGACGG and sgRNA3AS: AAACCCGTCATAGATATTCAAGTT (*SI Appendix*, Table S11) to make the pCFD3-*dsx*-sgRNA3 construct. Next, the *U6:3-dsx-sgRNA3* region (968 bp) from this plasmid was amplified using primers cfd.F1 and cfd.R1 (*SI Appendix*, Table S11) and then cloned into NotI/NcoI digested pUC57-DsxHDR plasmid to make pUC57-DsxHDR-U6:3-dsx-sgRNA3, an intermediate construct. The pUC57-DsxHDR plasmid contains a 2,102-bp synthesized fragment (GenScript, Piscataway, NJ) that consists of *dsx* left (1,071 bp) and right (929 bp) homology arms along with a multiple cloning site (MCS-102 bp). To facilitate cloning of the fluorescent protein marker gene PUb-DsRed, an Age-I site was added to the pPUb-DsRed-attB plasmid (94) using the Q5site directed mutagenesis kit (NEB, #E0554S). The DsRed gene was then excised using Asc-I/Age-I and ligated with pUC57-DsxHDR-sgRNA3 that had been digested with the same enzymes to make the final homing construct "pUC57-DsxHDR-U6:3dsx-sgRNA3-pUbDsRed" (Dsx homing construct, 8.9 kb). To make the *dsx* homing construct with a SA site, a fragment was synthesized that contains the *D. melanogaster* myosin gene SA followed by a short coding region for a HA tag followed by C-terminal degron and translation stop codon and then the *D. melanogaster hsp70* 3′UTR and polyA site (*SI Appendix*, Table S1). The fragment was excised by digestion with PspOMI/PacI and cloned into "pUC57-DsxHDR:U6:3-sgRNA3-pUbDsRed" cut with PspOMI and PacI to generate pUC57-DsxSA:U6:3dsxsgRNA3-pUbDsRed (DsxSA homing construct, 9.5 kb).

For the Cas9 expression construct, we first made an intermediate plasmid pBAC2[Dshsp83-Kozak-NLS-ZsGreen-3′Dshsp83], by amplifying fragments containing the *D. suzukii hsp83* promoter, NLS-ZsGreen, and *D. suzukii hsp83* 3′UTR and 3′ flanking using the primers listed in *SI Appendix*, Table S11 with plasmid DNA templates. The three fragments were purified and sequentially assembled with a linearized pBAC2 vector (NcoI/SacII) (95) using NEBuilder HiFi DNA assembly kit (NEB#E2621). We next amplified fragments containing the *Dsnos* promoter, Cas9 with two NLSs (96), and *Dsnos* 3′ UTR and flanking using primers listed in *SI Appendix*, Table S11, with plasmid DNA templates. The purified fragments were sequentially assembled with a linearized pBAC2[*Dshsp83-Kozak-NLS-ZsGreen-3′Dshsp83*] vector (NotI/XhoI) using the NEBuilder HiFi DNA assembly kit. The resulting plasmid was confirmed by DNA sequencing and named pB-Dsnos-NLS-SpCas9-NLS hsp83-ZsGreen.

Microinjections and Homing Strains Establishment. Plasmid DNA was purified from *E. coli* cultures using the Zymo Midi Kit (Cat#D4201), ethanol-precipitated, and dissolved in injection buffer (sodium phosphate buffer supplied with 300 mM of KCl) to a final concentration of ~1,000 ng/μL. Prior to microinjection, the plasmid DNA was filtered through a 0.45-μm filter (MilliporeSigma,

Cat#UFC30HV25). To make the RNP complexes for use with the homing constructs, either synthetic "crRNA:tracrRNA" duplex (IDT synthesized) or sgRNA made by in vitro transcription using the MEGAshortscript T7 transcription kit (Invitrogen, Cat#AM1354). For crRNA:tracrRNA, the dsx-crRNA (72 ng/μL) and tracrRNA (134 ng/μL) were mixed in nuclease-free duplex buffer (IDT) and incubated at 95 °C for 5 min and then allowed to cool to room temperature for duplex formation. The ribonucleoprotein complexes (RNPs) were preassembled by incubating crRNA:tracrRNA duplex (134 ng/μL:72 ng/μL) or synthesized sgRNA (150 ng/μL) with Cas9 protein (NEB #M0646T) in injection buffer supplemented with 300 mM KCl at 37 °C for 20 to 30 min. The final injection mix consisted of plasmid DNA at 500 ng/μL and Cas9 RNP complex at a final concentration of 322 to 500 ng/μL for Cas9 and was centrifuged at max speed for at least 10 min at 4 °C prior to injection. For making in-frame deletion mutations, ssODNs were synthesized (IDT) and mixed with Cas9 RNP complex to a final concentration of 2 to 10 μM. *D. suzukii* precellular embryos were microinjected, and the G_0 adult flies were individually crossed with wild-type counter flies and the offspring screened for red fluorescence to identify any positive transformants as described previously (61, 67). In one of the injections, ligase 4-dsRNA (200 ng, MegaScrib RNAi kit used for dsRNA synthesis) was included in the injection mix with the aim of promoting the HDR repair pathway for the homing construct knock-in.

Confirmation of Homing Constructs Integration into the *dsx* Gene. To confirm successful knock-in of the homing constructs into *dsx* target region, genomic DNA was extracted from adults, and PCR was performed using a primer (*SI Appendix*, Table S11) from the homing cassette and a primer from the neighboring genomic region. PCR was performed on both sides of the knock-in. Further, the PCR products were cloned into pGEM-T easy vector (Promega) followed by Sanger sequencing.

RT-PCR and RACE-PCR of *dsx* Transcripts. RT-PCR and 3′ RACE-PCR were performed to analyze *dsx* transcripts in the female flies of established lines, with wild-type flies used as control. To perform RT-PCR on these lines, total RNA was isolated as previously described (97) from hemizygous virgin females, hemizygous males, and homozygous XY intersex (n = 12 to 20) followed by cDNA synthesis using SuperScript III (Invitrogen, Cat#18080400) following the manufacturer's recommendations. cDNAs were used as templates to amplify the *dsx* male and female isoforms using specific primer pairs (*SI Appendix*, Table S11) and OneTaq 2X master mix with standard buffer (NEB, #M0482S). RT-PCR products were purified and cloned into the pGem-T easy vector (Promega, Cat#A1360) followed by Sanger sequencing (*SI Appendix*, Table S8). 3′ RACE-PCR on RNA from 3A3, 1C2, and 23AI1^{SA} lines was carried out according to the kit manual (SMARTer RACE 5′/3′ kit from Clontech, cat#634859) with RACE ready cDNA synthesized from the virgin hemizygous females. Amplification was performed using the provided universal primer mix along with *dsx* gene-specific-primer1 (GSP1), followed by a nested PCR with *dsx* GSP2 (*SI Appendix*, Table S11). The nested 3′-RACE-PCR products were cloned into the pRACE vector, and plasmid DNA was sequenced using Sanger sequencing (*SI Appendix*, Table S7).

Sequencing to Detect SNPs (Single Nucleotide Polymorphisms). From the fertile (1C2) and sterile strains (3A3, 15A2, and 30B1), a 6.7-kb region from the transgene to the downstream *dsx* male exon was amplified from genomic DNA using a 1:1 mixture of Q-5 High-Fidelity (M0492S) and OneTaq (M0482S) DNA polymerases with primer pair pUbF4_CTAATTCACAAGACCCTGAAGCA and DsxMTR4_GAGTAATACTTACACAAAGAGGACAGC (*SI Appendix*, Table S8). The PCR products were cloned into pGem-T easy vector (Promega), and the complete sequence of the plasmid DNA was determined by Illumina sequencing (Next Generation Sequencing) at the MGH, CCIB DNA core facility (Cambridge, MA). The 1.07-kb region upstream of the transgene was amplified from genomic DNA (Dsx-HC and Dsx-HC^{SA} strains), cloned into pGem-T easy vector (Promega, Cat#A1360), and the DNA sequence determined by Sanger sequencing. All of the obtained sequences were analyzed for any SNP among the strains using MacVector [version 18.2.3 (33)].

Transgenic Female Fertility, Egg-Laying, and Male Competition Assays. Fertility of female fertile lines was assessed by crossing single 4 to 5-d-old females with three wild-type males. Twenty replicates were performed for each line. For sterile female lines 3A3, 15A2, and 30B1, batch crosses were set with 10 females and 10 wild-type males. Vials were monitored for any offspring for at least 2 wk after setting the crosses (*SI Appendix*, Table S3). For the egg-laying assay, 6 to 7-d-old hemizygous females (n = 15 to 20) were mated with the same number and age of wild-type males and left for 24 h on plates (60 × 15 mm) containing fresh diet. Flies were then transferred to fresh egg-laying plates for a further 24 h. Both sets of plates were examined for deposited eggs (*SI Appendix*, Table S4). All the crosses were set in triplicate. The wild-type NC strain was used as control.

To assess male competitiveness, five wild-type males, five transgenic males, and five wild-type virgin females were placed in a vial and observed for 1 h. The flies were all 5 d old. Any copulating pairs were removed from the vial by aspiration. The genotype of the males was determined by fluorescence microscopy.

Fly Rearing and Split Homing Gene Drive Crosses. All transgenic lines and wild-type fly stocks were raised on cornmeal-yeast-agar diet at room temperature (20 to 22 °C) in the open laboratory as described previously (62). The wild-type colony was established from infested fruit collected from a field in North Carolina in 2011 (98) and has been periodically augmented with flies collected in North Carolina (99). Males from sterile *dsx* homing lines (3A3, 15A2, and 30B1) were maintained by crossing males with wild-type virgin females. To measure the editing activity of the *Dsnos-NLS-Cas9-NLS* lines, virgin females were crossed with males from the *yellow* sgRNA line ex2 and *white* sgRNA line wex2 (66). Virgin female offspring were crossed with wild-type or *yellow* males and the phenotype of the male offspring scored for yellow body or white eye phenotypes.

To set split homing drive crosses (28), the previously characterized *Dmnos-Cas9-NLS* #36 (also called Nos.Cas9.G#36) and *Dmvasa-Cas9-NLS* #4 (also called Vasa.Cas9.G#4/10) Cas9 lines (66) were crossed with *dsx* homing lines. Homing drive crosses were set in 2 to 3 batches with 10 pairs. For "Cross-I", virgin Cas9 females and homing males were crossed, and the trans-heterozygous (having both Cas9 and homing cassette) F_1 offspring were collected. The males and virgin females, called "male drive" and "female drive", respectively, were then crossed with wild-type flies of the opposite sex in "Cross-II". The F_2 offspring from Cross-II were scored for the inheritance of the red fluorescent protein marker using an M205FA microscope (Leica Microsystems, Buffalo Grove, IL) with the DsRed filter [ex545/25, em 595/50 nm]. The F_2 offspring that did not show red fluorescence (i.e. non-drive) flies were saved at −80 °C for molecular analysis. In addition to the batch crosses, individual male or female 1C2 flies were crossed with wild-type flies (1:3) to evaluate drive inheritance (Dataset S2, Tabs 10, 11). All "Cross-I" crosses were set in triplicate. Similarly, the split homing drive crosses were set with the X chromosome-linked *Dsnos-NLS-Cas9-NLS* line #27AI1 and the Dsx-HC (3A3, 1C2) and Dsx^{SA}-HC (23AI1^{SA}) lines. The gene drive experiments were performed in an ACL2 containment facility.

Resistance Assessment. Genomic DNA was extracted from nondrive flies that lacked red fluorescence (~28 to 150) using organic extraction as described previously (97) or by using a Qiagen DNeasy Blood and Tissue kit (Cat#69504) following the manufacturer's instructions. The DNA was amplified using OneTaq polymerase (NEB#M0484S) and the primer pair DsxcutF1_GGGTAAGTGAACTGATCATTGAAAC and DsxcutR1_ GAAGATAATCCAAGTTGCGCTTTAG that flank the Cas9 cut site and the 406-bp DNA fragment sequenced using Illumina DNA sequencing (Azenta Life Sciences). The deep sequencing data were analyzed using CRISPResso2 software, version 2.2.8. (100). Paired-end reads with a minimum average read quality 30 (parameter -q 30) were analyzed for "indels" on the target site. Frameshift analysis was performed (using parameter -c) on female exon's coding sequence. For nondrive flies obtained from crosses with the *Dsnos-NLS-Cas9-NLS* line #27AI1, individual flies were homogenized in 50 squash buffer (10 mM Tris pH 8.2, 1 mM EDTA pH8, 25 mM NaCl, and 200 μg/mL proteinase K) and then incubated at 37 °C for 45 min and 95 °C for 2 min. PCR was performed as above with 2 μL of genomic DNA. The PCR products were sequenced using Sanger sequencing and analyzed using the Synthego ICE Analysis Tool v3 (Synthego). For the experiments with ssODNs, DNA from individual flies was obtained by homogenization in squash buffer, amplified, and sequenced using Sanger sequencing. The DNA sequences were analyzed using SnapGene (SnapGene, version 6.1.1).

Statistical Analysis. Statistical analyses were mostly performed using GraphPad Prism version 9.1.2 (225). At least three replicate data were used for the comparisons. For the gene drive inheritance analysis, one-way ANOVA, Tukey's multiple comparison test, was performed. Drive inheritance between female drive and male drive was analyzed using Student's *t* test (in Excel). The female fertility, egg laying, and female fecundity data were analyzed using one-way ANOVA, Dunnett's multiple comparison test. The male mating competitiveness data were analyzed in SAS (version 9.4, Cary, NC). A two-sided z-test was run for each line comparing the MCI to the null hypothesized proportion of 0.5.

Modeling. We used a discrete-time stochastic model with nonoverlapping generations and conversion efficiencies based on sex. No relative fitness cost associated with the *dsx* transgene was assumed, although a (multiplicative) fitness cost of 5% affecting hatching probability was assumed for the Cas9 transgene. Resistant-allele evolution was ignored. We used this model to simulate a recessive female sterile split drive based on the 23AI1^{SA} line, a dominant female sterile split drive, and fully autonomous versions of each. In all cases, flies homozygous for the *dsx* transgene are sterile.

For the recessive female sterile drive, the conversion efficiencies used are 97.36% and 93.88% in males and females, respectively. In addition, hemizygous females have a fecundity of 62.74% relative to wild-type females. Strong maternal deposition is included so that all female offspring of hemizygous drive (one copy each Cas9 and gRNA transgenes) are sterile. For the dominant female sterile drive, the male conversion efficiency used was 95.03%.

We simulated the spread of either gene drive in a small cage experiment using a population of 600 flies. The cage experiment is based on the approach outlined in Li et al. (61). Specifically, each generation is initialized with N=300x(T/C) male and female flies. Here, T is the average number of female offspring produced in a test bottle, and C is the average number of female offspring produced in a control (i.e., drive-free) bottle. C remains fixed throughout the experiment, but T will change as the drive allele becomes more abundant. This experimental setup ignores density-dependent dynamics in the fly population.

The number of released transgenic males is constant and is equal to a proportion of the original 300 males. For example, releasing one transgenic male fly for every four wild-type male flies corresponds to a release ratio of 0.25 and adds 75 transgenic males each generation. For both drives, released males are hemizygous for the Cas9 and *dsx* transgenes. The number of eggs produced by each pairing is determined by a Poisson distribution with mean 50. The genotype of the father and subsequent eggs are determined by their respective multinomial distributions. Sex is binomially distributed.

To initialize the next generation, N male and female eggs each are selected randomly from those produced in the previous generation. Hatching probability depends on genotype and is binomially distributed. Survival to adulthood is assumed constant across genotypes.

Data, Materials, and Software Availability. The data that support the findings of this study are available in the main manuscript and in the supporting information. The NCBI accession numbers for pUC57-DsxHDR-U6:3dsxgRNA3-pUbDsRed, pUC57-DsxSA:U6:3dsxgRNA3-pUbDsRed, and pB-Dsnos-NLS-SpCas9-NLS hsp83-ZsGreen plasmids are OQ130035—OQ130037. The code used to perform the mathematical modeling is available online at https://github.com/cbutler112358/suzukii_pop_supp_code (101).

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