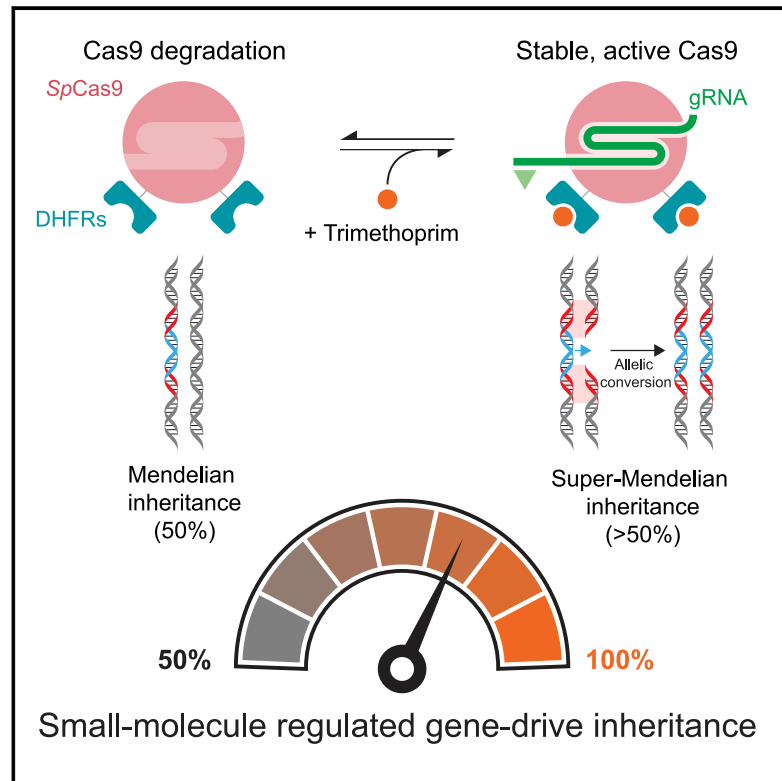


Small-Molecule Control of Super-Mendelian Inheritance in Gene Drives

Graphical Abstract



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In Brief

Gene drives offer potential solutions to fight vector-borne diseases but raise concerns around their safe experimentation. Lopez Del Amo et al. describe a modified Cas9-based gene drive that can be controlled by the addition of a small molecule to the fruit fly diet, adding a layer of safety for laboratory studies.

Highlights

- Trimethoprim leads to *in vivo* Cas9-DHFR activation in fruit flies
- The small molecule can tune a gene drive's super-Mendelian inheritance
- Trimethoprim-induced gene-drive activation can be turned off by removal of the drug
- Drug-controlled gene-drive system can add a safety layer to laboratory experimentation



Report

Small-Molecule Control of Super-Mendelian Inheritance in Gene Drives

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SUMMARY

Synthetic CRISPR-based gene-drive systems have tremendous potential in public health and agriculture, such as for fighting vector-borne diseases or suppressing crop pest populations. These elements can rapidly spread in a population by breaching the inheritance limit of 50% dictated by Mendel's law of gene segregation, making them a promising tool for population engineering. However, current technologies lack control over their propagation capacity, and there are important concerns about potential unchecked spreading. Here, we describe a gene-drive system in *Drosophila* that generates an analog inheritance output that can be tightly and conditionally controlled to between 50% and 100%. This technology uses a modified SpCas9 that responds to a synthetic, orally available small molecule, fine-tuning the inheritance probability. This system opens a new avenue to feasibility studies for spatial and temporal control of gene drives using small molecules.

INTRODUCTION

The Mendelian rule of gene segregation dictates that a given allele has a 50% chance of being transmitted to progeny through sexual reproduction (Figure 1A, top), although CRISPR-based gene drives breach this barrier by increasing the inheritance limit toward 100% (i.e., super-Mendelian inheritance) (Figure 1A, bottom). This increased inheritance rate allows for faster gene transmission and is ushering in an era of active genetics (Esvelt et al., 2014; Gantz and Bier, 2015, 2016). Gene drives have the potential for being applied in areas ranging from basic research (Shapiro et al., 2018) to ecological engineering, including for the management of both insect-borne diseases (Gantz et al., 2015; Hammond et al., 2016; Kyrou et al., 2018) and invasive pest species, as well as in ecosystem restoration (Esvelt and Gemmell, 2017). For example, gene drives have allowed ~100% transmission of antimalarial (Gantz et al., 2015) or infertility (Hammond et al., 2016) genes within *Anopheles* mosquitoes in the laboratory, enabling efficient population modification or suppression, respectively. However, multiple concerns and challenges surround the use of gene drives in both laboratory and ecological settings (Akbari et al., 2015). The unknown consequences of organisms containing engineered gene drives escaping from either

the laboratory or their intended ecological residence have prompted intense interest in strategies for controlling these genetic elements (Akbari et al., 2015). Furthermore, there are no currently existing methods to precisely control the timing of gene-drive activation, to prevent drives occurring in escaped organisms, and to fine-tune the inheritance probability to a value between 50% and 100%. Such fine-tuning of the inheritance probability would allow for both spatial control of the gene drive in the field as well as fundamental studies on the strengths and limitations of gene drives in laboratory settings. For example, an experimental system could be designed to assess the outcomes of gene drives working at different efficiencies in contained cage trials through the use of fine-tuning, and subsequent computational modeling could identify the key optimization parameters.

CRISPR-based gene drives achieve super-Mendelian inheritance through a Cas9-induced double-strand break on the wild-type allele that is corrected by the homology-directed repair (HDR) pathway which copies the DNA sequence encoding the gene drive from the intact gene-drive allele onto the wild-type one. This process efficiently replaces the wild-type allele with a copy of the engineered gene drive (Figure 1B; Esvelt et al., 2014; Gantz and Bier, 2015, 2016). Controlling the activity of



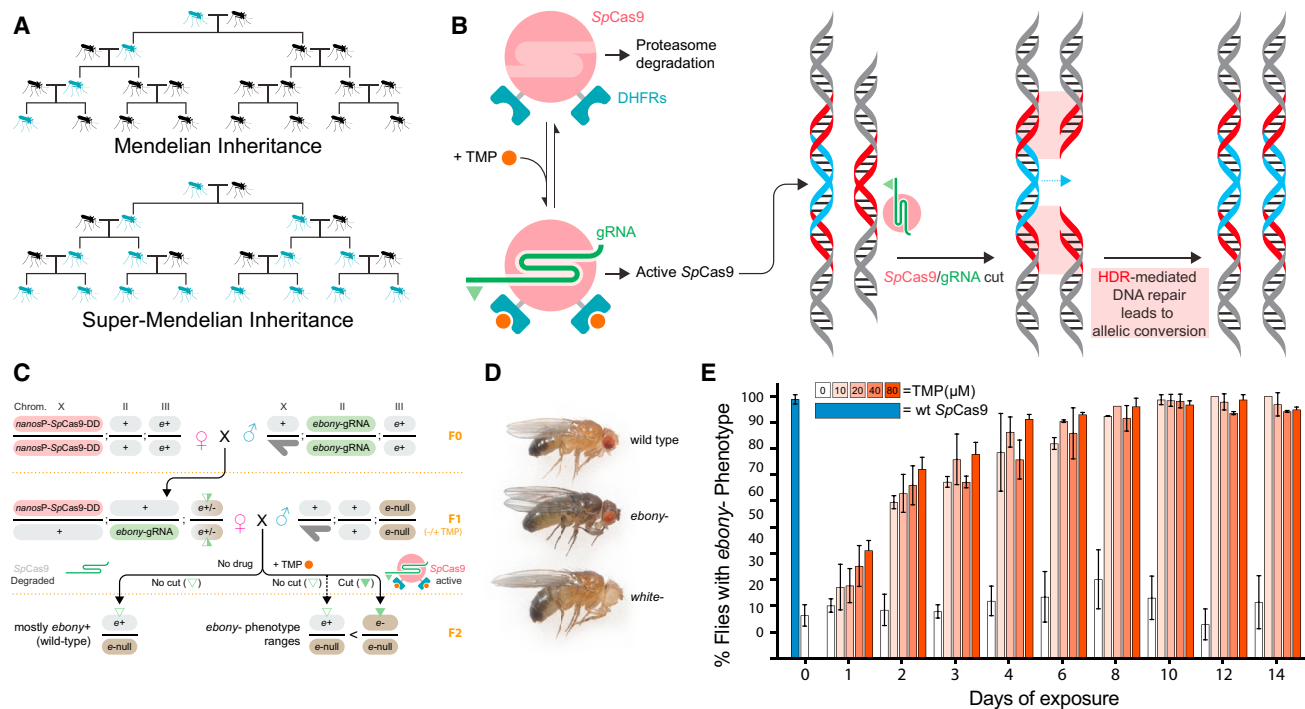


Figure 1. Chemical Control of SpCas9 in *Drosophila*

(A) Super-Mendelian inheritance allows a given genetic trait to propagate exponentially in future lineages. (B) A destabilized-domain (DD) system allows small-molecule-based dosage and temporal control of SpCas9 and subsequent gene drives (DD-SpCas9). In the absence of TMP, DD-SpCas9 is degraded by the proteasome, whereas in the presence of TMP, DD-SpCas9 is active and can induce double-stranded DNA breaks. Repair of the induced cut with the gene-drive-containing template ensures the super-Mendelian transmission of the gene-drive construct to the offspring. (C) Experimental outline for TMP activation of DD-SpCas9 transgenes. F0, females bearing *nanos*-DD-SpCas9 transgenes were crossed with males bearing *U6-gRNA* guides targeting *ebony* (*e*); F1, female progeny with *nanos*-DD-SpCas9 and pFP545 *U6-gRNA* were selected and crossed to *e*⁻/*e*⁻ males and fed on food either in the absence (left) or presence (right) of TMP; F2, progeny were scored visually for mutations in *ebony* that indicated activation of DD-SpCas9 in the germline. A dark-gray half arrow indicates the male Y chromosome. (D) Phenotypes of wild-type fly (top), *ebony* mutant (middle), and *white* mutant (bottom). (E) Dose-dependent TMP activation of DD2-SpCas9 transgenes with *ebony* gRNA. Four days after crossing, flies were transferred to vials of food containing the respective concentration of TMP and were subsequently changed onto fresh food with TMP each day. Offspring were scored for the *ebony* phenotype on the indicated day; *nos*-SpCas9(WT) shown for comparison. Starting on day 2, all values were significant to $p < 0.0001$ relative to 0 μM TMP per day of exposure, as determined through a two-way ANOVA with Sidak multiple comparisons tests. Error bars represent standard deviation.

the initial Cas9-induced DNA break could start or stop the drive process at will. We hypothesized that a gene drive using a synthetic small molecule that could control Cas9 activity would afford precision control of gene drives for multiple reasons. First, small molecules can provide dosage control of Cas9 activity, and their use in regulating a gene drive would convert the output from singular (i.e., fully on) to analog, wherein the inheritance probability in the population could be fine-tuned to any value between the off state (50% inheritance) and the fully on state (~100% inheritance). Second, the effect of small molecules on the on/off state of Cas9 would be rapid, allowing for precise temporal control of the gene drive by controlling the time the small molecule is made available to the organism. For gene drives meant to propagate lethal or sterile traits to the progeny, temporarily switching off Cas9 could be useful for animal husbandry and population expansion during large-scale studies. Restricting Cas9 activity to a narrow temporal window is also important for proper functioning of the gene drive, as persistent Cas9 activity during development gives rise to drive-resistant alleles or mosaic phe-

notypes formed by non-homologous end-joining (NHEJ) DNA repair processes (Champer et al., 2017, 2018; Gantz et al., 2015; Hammond et al., 2017). Third, by requiring gene-drive inheritance to be contingent on the presence of a synthetic small molecule (as opposed to a naturally occurring small molecule), gene drives can be more easily contained. Finally, small-molecule-based methods are already widely used to control mosquito populations in the developing world, in part because of their high efficacy and low production and administration costs. Because mosquitoes regularly frequent human habitats, many insecticides (e.g., pyrethroids) are administered as mosquito net coatings or as aerosols in homes instead of the mosquito's natural habitat (Zaim et al., 2000). Thus, small-molecule strategies to control gene drives could increase safety and may be adapted for field implementation.

We envisioned building a small-molecule system for controlling gene drives with the following attributes. First, because gene drives are being developed in various organisms from insects to mammals (Grunwald et al., 2019), the controller should

be adaptable to a wide range of body temperatures (e.g., from 25°C in insects to 37°C in mammals). Second, the small molecule should be synthetic and not naturally occurring for efficient containment. Third, turning one of the gene-drive components (e.g., Cas9) into a controller itself does not additionally complicate the gene-drive circuitry. Furthermore, this strategy avoids the use of transcription-based controllers that rely on complex regulation by additional genetic components (e.g., UAS/Gal4 or TetON system). We previously described in mammalian cells a small-molecule-controlled *SpCas9* system built by fusing the structurally unstable *Escherichia coli* dihydrofolate reductase (DHFR) protein to *SpCas9* (DHFR-*SpCas9*-DHFR [DD-*SpCas9*]) (Maji et al., 2017). Upon expression, the DD-*SpCas9* fusion protein is targeted for proteasomal degradation unless the DHFR-binding small molecule trimethoprim (TMP) is added. Because destabilized domains are efficacious in multiple organisms (Armstrong and Goldberg, 2007; Banaszynski et al., 2008; Cho et al., 2013; Herm-Götz et al., 2007), are regulated by a synthetic molecule, and provide superior control of DNA-binding proteins over transcription-based controllers (Shoulders et al., 2013), we reasoned that they could provide an ideal gene-drive controller. Furthermore, as the small molecule is required to stabilize DD-*SpCas9* in this default-off system, there is a reversible dosage control of *SpCas9* nuclease activity and an added level of safety in case of escaped organisms (Figure 1B). The scope of this work was to develop a gene-drive system based on DD-*SpCas9* and proof-of-principle data in the fruit fly that would satisfy the characteristics described above.

RESULTS

Development of a Drug-Stabilized Cas9 Protein for Use in *Drosophila*

We developed a DD-*SpCas9* system for *Drosophila* by testing previously validated clones of DHFR for those that work at lower temperatures (Cho et al., 2013) and optimizing them to our system, testing the effective *in vivo* dose ranges of TMP upon ingestion. We explored several reported clones of DHFR and generated transgenic flies with *nanos*-DD-*SpCas9* constructs containing 2–3 DHFR domains at varied positions: N-terminal, C-terminal, and an internal loop previously described to tolerate a small protein domain insertion (Oakes et al., 2016) (DD1-*SpCas9* through DD4-*SpCas9*; Figure S1). We calibrated TMP-dependent *SpCas9* activation by targeting an easily identifiable dark body phenotype that is produced upon mutation of the recessive *ebony* gene. In these optimization experiments, female flies bearing DD-*SpCas9* (DD1–DD4) were crossed to males bearing a guide RNA (gRNA) targeting *ebony* under the control of the ubiquitous *U6* promoter (Figure 1C). Subsequently, female progeny carrying both DD-*SpCas9* and *U6-gRNA* transgenes were fed at different doses of TMP and crossed to homozygous *ebony* mutant males (Figure 1C). The resulting F2 progeny were scored using the visual phenotyping assay for *SpCas9*-mediated editing of the *ebony* gene (Figure 1D). Although we observed little to no *SpCas9* activity with DHFR fusions for DD1-, DD3-, and DD4-*SpCas9* based on *ebony* mutation rates (Figure S1), we observed a greater TMP activation for DD2-*SpCas9*, which increases gradually over the first 4 days and then plateaus at the

level of constitutively expressed wild-type *SpCas9* by days 6–10 at all TMP concentrations tested (Figure 1E; Figure S1).

A Small Molecule Can Control the Inheritance of a Gene-Drive System in *Drosophila*

We next sought to establish precision control of gene drives using TMP and a modified gRNA-only drive construct called a CopyCat (Xu et al., 2017). This element behaves similarly to a gene-drive construct in the presence of a transgenic source of *SpCas9*, which is itself transmitted in a Mendelian fashion. Briefly, a CopyCat element is composed of a gRNA-expressing gene inserted at the location where the gRNA targets the genome for cleavage. When the CopyCat element is combined with a transgenic source of Cas9, it is capable of copying itself onto the opposing chromosome by HDR. CopyCat elements are unable to spread exponentially in a population and increase only additively each generation based on the initial allele frequency of the Cas9 transgene (Gantz and Bier, 2016). The small-molecule-controlled *Drosophila* system developed herein consisted of two components: (1) a transgenic source of dsRed-marked *SpCas9* (or DD2-*SpCas9*) driven by the *vasa* germline promoter, which was inserted into the *yellow* gene coding sequence; and (2) the GFP-marked CopyCat element containing a gRNA under the control of the *Drosophila* *U6:3* promoter (Figure 2A; Figure S2). We tested the gene-drive-based inheritance bias of our system by inserting a GFP-marked CopyCat element at the *ebony* and *white* genes (see below), for which homozygous mutants are viable and fertile (Figure 1D). This allowed us to measure inheritance rates by following the fluorescent marker and to evaluate failure events (indels) by scoring the visible phenotype (dark body or white eyes without the GFP marker). First, we crossed males carrying the *SpCas9* (or DD2-*SpCas9*) cassette to females containing the *ebony* CopyCat element (F0; Figure 2B). We collected virgin F1 females carrying both the *SpCas9* (or DD2-*SpCas9*) and *ebony* gRNA, which were crossed to wild-type males (Oregon-R) for the F1 germline transmission assessment using a phenotypic analysis of GFP (CopyCat transgene) in the resulting F2 progeny (Figure 2B; Figure S2). This experimental design allowed us to assay the germline inheritance ratios of each single F1 female. Wild-type *SpCas9* showed an ~65% average inheritance for the *ebony* CopyCat element, and as expected, this super-Mendelian inheritance was independent of the presence of TMP (Figure 2D). In contrast, we observed a significant TMP dose-dependent super-Mendelian inheritance for the CopyCat element (Data S1) when combined with the DD2-*SpCas9* construct. In this setup, Mendelian inheritance values of ~50% were seen in the absence of TMP, and these values reached an average inheritance of ~62% at the maximum TMP concentration (80 μM) (Figure 2D).

The Drug-Regulated *Drosophila* Gene-Drive System Can Be Efficiently Shut Off after Activation

To demonstrate that our approach is generalizable to another locus, we generated a second system using a CopyCat construct that targeted the *white* gene, causing a lack of pigmentation in the fly eye when disrupted (Figure 1D). We followed a similar experimental approach by crossing males expressing *SpCas9* or DD2-*SpCas9* to females carrying the *white* CopyCat

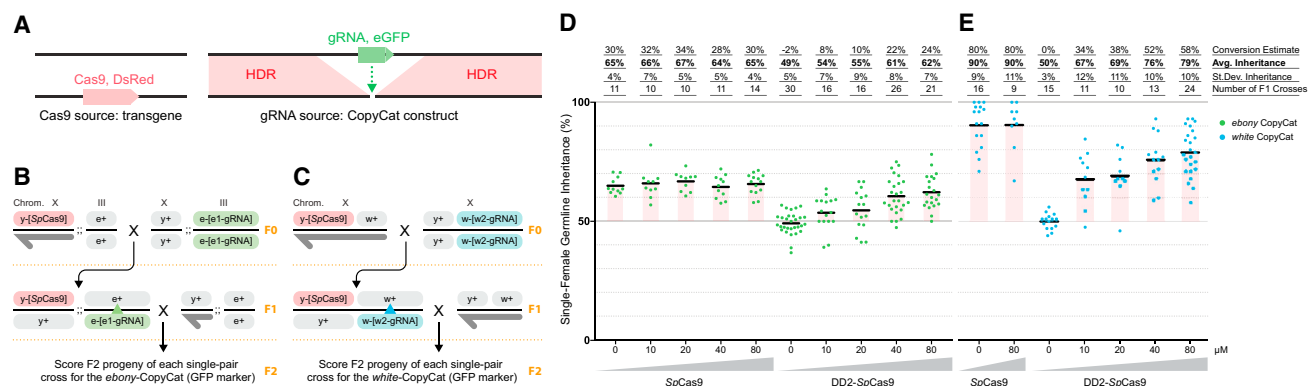


Figure 2. A Small-Molecule-Contingent Gene Drive

(A) Schematic of the CopyCat drive system. The dsRed-marked Cas9 is a static transgene providing Cas9 for mobilizing the GFP-marked CopyCat element by allelic conversion driven by the surrounding homology.

(B and C) Cross schemes of males expressing *SpCas9* or *DD2-SpCas9* were crossed to virgin females carrying the *ebony* (B) and *white* (C) CopyCat construct. Collected virgin females (Cas9-dsRed + gRNA-GFP) were crossed to wild-type males to score germline transmission rates by screening the GFP marker in the F2 progeny. A dark-gray half arrow indicates the male Y chromosome.

(D and E) Assessment of gene-drive activity in the germline of F1 females by phenotypically scoring the F2 progeny of the crosses (B, C) for either the *ebony* (D) or *white* (E) CopyCat constructs. Our control line (wild-type *SpCas9*), which is not regulated by TMP, displayed super-Mendelian inheritance independent of the presence of TMP. *DD2-SpCas9* showed Mendelian inheritance rates in the absence of TMP (~50%), whereas TMP treatment triggered an increasing inheritance bias of the gRNA drive element that correlated with TMP concentration for both the *ebony* (D) and *white* (E) CopyCat constructs. Our measurements of inheritance rates allowed us to estimate the allelic conversion rates for our CopyCat constructs, reported on top of the graph along with the inheritance average (also as black bars on the graph) standard deviation (St. Dev.) and the number of F1 crosses performed (n).

(Figure 2C; Figure S2) and raising the progeny on 0 to 80 μ M of TMP. The *white* gRNA element driven by *SpCas9* displayed an ~90% average inheritance in both the presence and absence of TMP, reinforcing the conclusion that the presence of TMP does not affect *SpCas9* function. We also observed that the components or location of the *white* CopyCat results in a greater copying efficiency than the ~65% of their *ebony* counterparts (Figure 2E). As was the case with the *ebony* construct, *DD2-SpCas9* combined with the *white* CopyCat element presented Mendelian inheritance rates of ~50% in the absence of TMP (Figure 2E) and, in the presence of TMP, displayed increasing super-Mendelian inheritance rates of 67% (10 μ M), 69% (20 μ M), 76% (40 μ M), and 79% (80 μ M), as scored by the GFP phenotype in the F2 progeny (Figure 2E; Data S1). This demonstrates that the TMP small molecule provides the desired fine-tuning of the super-Mendelian inheritance rate, which could be used to control gene-drive systems based on Cas9. We observed only minimal cutting in the absence of TMP that was phenotypically evaluated using F2 males of the *DD2-SpCas9 white* CopyCat experiment (0 μ M versus 80 μ M). This analysis was possible because the *white* gene targeted for conversion was located on the same chromosome where the Cas9 was inserted (within the yellow gene coding sequence), and the quantification is given in Figure S2. Lastly, we assessed the reversibility of our system by scoring the GFP inheritance of our *white* CopyCat in a four-generation experiment in which generations F1 and F2 were treated with TMP, and generations F3 and F4 were kept on regular food (Figure S2; Data S2). Importantly, we observed complete reversibility of our system over subsequent generations. The TMP-treated F0-F1 displays super-Mendelian inheritance in the F2 progeny, whereas removal of TMP leads to the F4 progeny showing ~50% inheritance of the *white* CopyCat

element (Figure S2; Data S2). To the best of our knowledge, these findings are the first example of gene drives with an analog output wherein the super-Mendelian inheritance can be controlled by the presence of a small molecule.

DISCUSSION

We herein report a split gene-drive system controlled by a synthetic, orally available small molecule that can tune the inheritance probability of the genetic element. Our first-generation small-molecule controllers should be amenable to further improvements. For example, TMP is non-toxic in humans and is used as an antibiotic for treating bacterial infections (Dubbs and Sommerkamp, 2019). However, we observed a developmental delay when flies were fed on TMP food (data not shown), which could be due to toxicity to the insect's microbiome. Such toxicity could be averted using a pro-drug strategy wherein inactive pro-TMP is rendered active by the removal of a "pro-moiety" by an enzyme expressed only in the germline of the engineered organism. Multiple orthogonal enzyme-substrate pairs have been reported that are compatible with TMP, including an esterase (Tian et al., 2012) and a nitroreductase (Gruber et al., 2018). Additionally, TMP is used in combination with sulfonamides for malaria prophylaxis (Hobbs et al., 2017). The use of this antibiotic for controlling gene drives in *Anopheles* mosquito applications could, therefore, contribute to developing resistance by the malaria parasite and impact prophylaxis efforts. A pro-drug strategy restricting TMP activity to the insect germline would also be able to address such concerns. An alternative option would be using non-antibiotic, small-molecule analogs of TMP, which are equally able to regulate the DHFR domain activity, such as the recently described 14a compound (MCC8529) (Peng et al., 2019).

We observed a minimal *SpCas9* activity in the “off” state of our system, which could result in the accumulation of resistant mutations, therefore potentially reducing the gene-drive spread when activated. Future research will focus on solutions to avoid the leakiness observed in the system, perhaps by optimizing the DHFR domains to obtain higher Cas9 degradation in the absence of TMP. A possible limitation of our approach could be that mutations conferring increased stability to the DD-*SpCas9* could result in a fully “on” gene drive, which would no longer be controlled by the small molecule. However, because our system incorporates two DHFR domains to control Cas9 degradation, the chances of acquiring mutations on both domains will be reduced. Large population studies would allow evaluation of dynamics of this system and the rise of such mutations.

Furthermore, the use of destabilized domains to control Cas9 represents a new tool for spatiotemporal control of Cas9 activity for the *Drosophila* community. Importantly, this method should be generalizable to other organisms wherein a *SpCas9*-based gene drive has already been demonstrated, such as mosquitoes (Gantz et al., 2015; Hammond et al., 2016) and mice (Grunwald et al., 2019). Lastly, these first-generation controllers could also be modified to control multiple orthogonal gene drives in a single organism. Such controllers could drive two synergistic or antagonistic genes, with the activity of each decided by the dose of the appropriate small molecule. Because our method is extendable to other Cas nucleases (e.g., SaCas9) and additional orthogonal destabilized-domain-small-molecule pairs are readily available (Banaszynski et al., 2006; Iwamoto et al., 2010; Miyazaki et al., 2012), the independent control of two gene drives should be a feasible technology.

The controller reported here should be transportable to other systems and compatible with current gene-drive configurations. Because our gene-drive system is activated by the deliberate application of a synthetic molecule, it provides a potential new method for increasing the safety of laboratory experimentation (Akbari et al., 2015). This system can be combined with existing strategies (physical, genetic, and ecological) (Akbari et al., 2015) to provide an additional layer of safety for gene-drive containment in the laboratory. Although our gene-drive experiments were performed under laboratory conditions, future improvements in the system that bypass the observed TMP toxicity and leakiness could evaluate the applicability of this method to control the spread of gene-drive elements in the wild. We envision that developments of this system could be adapted to restrict the spread of a gene drive in a circumscribed locale by, for example, vaporizing the drug inside homes as it is currently done for mosquito-repellent small molecules or pesticides.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.107841>.

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AUTHOR CONTRIBUTIONS

A.C. conceived the project. B.S.L., K.J.C., S.G., G.D.S., J.A.W., and A.C. designed and/or optimized the DD-*SpCas9* system in *Drosophila*. V.L.D.A., A.L.B., V.M.G., and A.C. designed the gene-drive constructs and experiments, which were performed by V.L.D.A., A.L.B., and V.M.G. V.L.D.A., B.S.L., K.J.C., S.G., J.A.W., V.M.G., and A.C. wrote the manuscript, which was edited by all the authors.

DECLARATION OF INTERESTS

A.C. is a co-inventor on International Patent Application no. PCT/US2015/067177 and PCT/US2017/040115, “CRISPR Having or Associated with Destabilization Domains” filed by the Broad Institute, which relates to the destabilization domains used in this manuscript. V.M.G. has equity interests in Synbal, Inc. and Agragene, Inc., companies that may potentially benefit from the research results, and also serves on both companies’ Scientific Advisory Board and on the Board of Directors of Synbal, Inc. 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.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
<i>nos</i> -DD1- <i>SpCas9</i>	This manuscript	N/A
<i>nos</i> -DD2- <i>SpCas9</i>	This manuscript	N/A
<i>nos</i> -DD3- <i>SpCas9</i>	This manuscript	N/A
<i>nos</i> -DD4- <i>SpCas9</i>	This manuscript	N/A
<i>vasa</i> - <i>SpCas9</i>	This manuscript	Gene Bank: MN551085
<i>vasa</i> -DD2- <i>SpCas9</i>	This manuscript	Gene Bank: MN551086
<i>ebony</i> -CopyCat	This manuscript	N/A
<i>white</i> -CopyCat	This manuscript	N/A
Experimental Models: Fruit fly lines		
<i>nos</i> - <i>SpCas9</i>	Port et al., 2015	BDSC: 54591

RESOURCE AVAILABILITY

Lead Contact

Any request will be fulfilled by the Lead Contact, vgantz@ucsd.edu Amit Choudhary (achoudhary@bwh.harvard.edu).

Materials Availability

The reagents that were previously generated and used in this work are available for the general public and their references are provided below. Reagents generated in this work are available upon request and should be directed to the Lead Contact. All reagents require a completed Materials Transfer Agreement.

Data and Code Availability

This work did not include any datasets or code. All raw counting data from the gene drive experiments is provided as supplemental data.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks were raised at 18°C with a 12/12 hour day/night cycle on regular cornmeal molasses medium. Experimental flies were kept at 25°C with a 12/12 hour day/night cycle. We used shatterproof polypropylene vials with 2.5 × 9.5 mm (diameter × height) size from Genesee Scientific Corporation (Cat# 32-120) for both fly stock maintenance and experiments. The *ebony* gene-editing stocks (Figure 1; Figure S1) were maintained in a BSL-1 facility at the Massachusetts General Hospital. All gene-drive work presented here (Figure 2; Figure S2) followed procedures and protocols approved by the Institutional Biosafety Committee from the University of California San Diego, complying with all relevant ethical regulations for animal testing and research. Gene-drive experiments were performed in a high-security Arthropod Containment Level 2 (ACL2) barrier facility.

METHOD DETAILS

Plasmid construction

The *nanos*-DD-*SpCas9* transgenic constructs were made using the pnos-Cas9 (Addgene# 62208) plasmid. The rest of the constructs were built by Gibson assembly using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Cat. # E2621) described in Gibson et al. (2009). Specific features included in each construct are depicted in Figure S1. Portions of the engineered Cas9 constructs were synthesized using GenScript Inc. DNA Synthesis services. Sequence information for the plasmids is available upon request.

Phenotyping experiments

For gene-editing experiments, crossing schemes are detailed in Figure 1C. For food containing TMP, we used Formula 4-24 Instant *Drosophila* Food (Carolina Biological Supply Company Cat.#: 173210) reconstituted by adding water or water containing different TMP concentrations (10, 20, 40, 80 μM). Only adult flies with the full *ebony* phenotype were scored as *ebony*; all intermediate phenotypes to non-phenotypic flies were scored as wild-type. For gene-drive experiments, crossing schemes are shown in Figures 2B

and 2C. Additionally, a graphical representation is available in [Figure S2](#). A 100 mM stock solution of TMP dissolved in DMSO was premixed with the water for food reconstitution to a final concentration of 0, 10, 20, 40, or 80 μ M. TMP vials were prepared by adding 1 g of Formula 4-24 Instant *Drosophila* Food (Carolina Biological Supply Company Cat.#: 173210) and reconstituted with 3 mL of the water-TMP mixture. F0 crosses were performed in pools (3–5 individuals of each sex) of *SpCas9* or DD2-*SpCas9* males (DsRed – red eyes) and females containing the gRNA-only drive (GFP – green eyes). All crosses were kept for four days in vials containing different TMP concentrations, after which the parents were discarded. Virgin F1 females carrying both the Cas9 construct (either *SpCas9* or DD2-*SpCas9*) and gRNA elements were single-pair crossed to wild-type males, keeping the same TMP conditions as for the corresponding F0 crosses. After four days, the F1 cross individuals were discarded and the resulting F2 progeny was scored for the presence of the GFP marker as an indicator of successful allelic conversion. Additionally, we also tracked the scored mosaic phenotype in the eyes (see Data S1). Gene-editing experiments scoring the *ebony* phenotype ([Figures 1](#) and [S1](#)), were carried out in a BSL-1 facility at the Massachusetts General Hospital. Gene-drive experiments from [Figures 2](#) and [S2](#) were performed in an Arthropod Containment Level 2 (ACL2) facility built for gene-drive purposes at the Biological Sciences Department, University of California San Diego. All gene-drive flies from this facility are frozen for 48 hours before being removed from the room, autoclaved, and discarded as bio-hazardous waste.

Transgenic line generation and genotyping

All injections to generate transgenic flies were performed by BestGene Inc. or Rainbow Transgenic Flies Inc. For gene-editing experiments ([Figure 1](#)), transgenic lines were generated using site-specific ϕ C31 integration at the ZH2A attB site (2A3) on the X chromosome site using *yw ZH-2A*. For wild-type Cas9 under the *nos* promoter, we used $y[1] M\{w[+mC] = nos-Cas9.P\}ZH-2A w[*]$ from the Bloomington Stock Center ([Port et al., 2014](#)). The *ebony* sgRNA line was pFP545 ([Port et al., 2015](#)). For gene-drive experiments ([Figure 2](#)), all constructs were injected into the same Oregon-R (OrR) strain to maintain a homogeneous genetic background. All the Cas9 lines were inserted at the same location (*yellow* gene coding sequence) to ensure comparable Cas9 expression levels. The CopyCat elements flanked by specific homology arms and marked with GFP were inserted in *ebony* and *white* gene coding sequences. For the establishment of all transgenic lines, we received the injected generation 0 (G0) flies in the larval stage (80–120 larvae). Once they eclosed, we distributed all G0 adults in different tubes (5–6 females crossed to 5–6 males). Then, the G1 progeny were screened for the presence of the specific fluorescent marker in their eyes, which was indicative of transgene insertion. Positive F1 individuals for the fluorescent marker were crossed individually to OrR flies (same used for injection). To generate homozygous stocks for each transgenic line marker-positive F2 flies were intercrossed, and F3 flies displaying both the fluorescence and the expected phenotypes (*yellow*, *ebony* or *white*) were pooled. Correct integration of the transgene was confirmed by PCR amplification and subsequent sequencing of the whole construct using primers landing outside of the homology arms used for transgenesis.

Microscopy

For *ebony* gene-editing experiments, adult flies were anesthetized to select individuals for crossing and phenotyping using a Zeiss Stemi 2000 microscope. Gene-drive experiments were performed using a Leica M165 FC Stereo microscope with fluorescence to track the inheritance of the transgenes.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used GraphPad Prism 7 to perform all statistical analyses and graphical representations. The two-way ANOVA and Sidak's multiple comparison tests were used (see [Data S1](#) and [S2](#)). Error bars indicate mean \pm standard deviation. For gene-drive dot plots ([Figures 2](#) and [S2](#)), mean and standard deviation are also depicted on top of the figures.