Proceedings of the Tenth International Conference on Urban Pests
Rubén Bueno-Marí, Tomas Montalvo, and Wm. H Robinson (editors) 2022
CDM Creador de Motius S.L., Mare de Deu de Montserrat 53-59,
08930 Sant Adrià de Besòs, Barcelona, Spain

CRISPR GENE-DRIVE TECHNOLOGIES FOR URBAN POPULATION ENGINEERING

VÍCTOR LÓPEZ DEL AMO[1], BRITTANY S. LEGER[2], KURT J. COX[3,4,5], SHUBHROZ GILL[3], ALENA L. BISHOP[1], GARRETT D. SCANLON[2], JAMES A. WALKER[2,], VALENTINO M. GANTZ[1,], AND AMIT CHOUDHARY[3,4,5,]

[1]Section of Cell and Developmental Biology, University of California San Diego, La Jolla, CA 92093, USA
[2]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA 02114, USA
[3]Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
[4]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA
[5]Divisions of Renal Medicine and Engineering, Brigham and Women's Hospital, Boston, MA 02115, USA

Abstract By surpassing the 50% inheritance limit of Mendel's law of gene segregation, CRISPR-based gene drives have the potential to fight urban vector-borne diseases or suppress urban pests, but contemporary gene drives can spread unchecked and lack an analog output that modulates inheritance between 50–100%. We describe a generalizable and containable gene drive system in *Drosophila* with an analog output fine-tuned using an engineered Cas9 and a synthetic, orally-available small molecule.

Key words: urban population modification, CRISPR, gene drive, dengue, malaria

INTRODUCTION

The Mendelian rules of random assortment dictate that a given gene has a 50% chance of being transmitted to the progeny through sexual reproduction (Figure. 1a**,** top). CRISPR-based gene drives breach this barrier by increasing the inheritance limit to 100% (i.e., super-Mendelian inheritance), allowing rapid transmission of the gene and ushering in an era of active genetics[1-3] (Figure. 1a, bottom). Gene drives have been applied in basic research[4] and ecological engineering[4,5], including for managing both insect-borne diseases[6,7] and invasive pest species[5] as well as in ecosystem restoration[4,5]. For example, gene drives have allowed ~100% transmission of antimalarial[6] or infertility[7] genes within *Anopheles* mosquito populations 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[8]. Fears of the unknown consequences of organisms containing engineered gene drives escaping the laboratory or their intended ecological residence have prompted intense interest in strategies for the spatial containment of these organisms[8]. Methods that allow for both precise temporal control of gene-drive activation as well as fine-tuning of the inheritance probability to any value between 50% and 100% are highly desirable but do not currently exist. Such fine-tuning of the inheritance probability would allow for both precision control of the output population as well as fundamental studies on the strengths and limitations of gene drives in laboratory settings. Such fine-tuning will allow the design of a controlled experimental system to assess outcomes of gene drives working at different efficiencies in cage trials, and subsequent computational modeling can identify key optimization parameters.

CRISPR-based gene drives can achieve super-Mendelian inheritance through a Cas9-induced double-strand break on the wildtype allele that is repaired by copying from the intact gene drive allele *via* homology-directed repair (HDR), essentially replacing a wildtype allele with the engineered gene and leaving an organism with two copies of the gene to pass on (Figure. 1b)[1-3]. We hypothesized that a gene drive employing a synthetic small molecule as a Cas9 controller 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 can be fine-tuned to any value between the off-state and the fully-on state. Second, small molecules could rapidly switch on/off Cas9, 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 gene drives is necessary for animal husbandry and population expansion of the organism for 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-resistance alleles or mosaic phenotypes formed by non-homologous end-joining (NHEJ) DNA repair processes[6, 9-11]. 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. Since 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[12]. Thus, small molecule control methods for gene drives have a strong foundational basis in field settings.

Before demonstrating small-molecule-based gene drive control in *Drosophila*, a small-molecule controlled Cas9 system had to be developed that would function in the reduced body temperature of these organisms as opposed to the commonly used mammalian cells (i.e. 25°C compared to 37°C)[13] and that exhibited the desired small molecule dose control. We previously described a small molecule-controlled *Sp*Cas9 designed by fusing structurally unstable *E. coli* dihydrofolate reductase (DHFR) to *Sp*Cas9 (DHFR-*Sp*Cas9-DHFR, or DD-*Sp*Cas9)[14]. Upon expression, the DD-*Sp*Cas9 fusion protein is targeted for proteasomal degradation unless the DHFR-binding small molecule, trimethoprim (TMP), is added. The small molecule is required to stabilize the fusion protein in this default-off system and allows reversible dosage control of *Sp*Cas9 nuclease activity in mammalian cells (Figure. 1b). Because of its demonstrated efficacy in mammalian cells, we chose to modify this DD-*Sp*Cas9 system for *Drosophila* to identify appropriate clones of DHFR that work at lower temperatures[13] and to determine the effective *in vivo* dose range of TMP upon ingestion.

RESULTS AND DISCUSSION

We explored a reported clone of DHFR and generated transgenic flies with DD-SpCas9 constructs containing 2 DHFR domains, one at N-terminal and a second one at C-terminal. tolerate a small protein-domain insertion15 (DD2-SpCas9). We calibrated TMP-dependent SpCas9 activation by targeting an easily identifiable dark body phenotype that is produced upon mutation of the recessive ebony gene (Figure. 1d). In these optimization experiments, female flies bearing DD-SpCas9 (DD2) were crossed to males bearing a 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 different doses of TMP and crossed to ebony-/ebony- males (Figure. 1c). The resulting F2We explored a reported clone of DHFR and generated transgenic flies with DD-SpCas9 constructs containing 2 DHFR domains, one at N-terminal and a second one at C-terminal. tolerate a small protein-domain insertion15 (DD2-SpCas9). We calibrated TMP-dependent SpCas9 activation by targeting an easily identifiable dark body phenotype that is produced upon mutation of the recessive ebony gene (Figure. 1d). In these optimization experiments, female flies bearing DD-SpCas9 (DD2) were crossed to males bearing a 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 different doses of TMP and crossed to ebony-/ebony- males (Figure. 1c). The resulting F2 progeny were scored using the visual phenotyping assay for *Sp*Cas9-mediated editing of the *ebony* gene (Figure. 1d). We found strong TMP dose-dependent activation for DD2-*Sp*Cas9 approaching the level of constitutively expressed wildtype *Sp*Cas9 by day 10 at all TMP doses (Figure. 1e). The modified DD2-*Sp*Cas9 was therefore considered highly functional in *Drosophila* and suitable for the desired gene drive experiments.

We next sought to establish precision control of gene drives using TMP and a modified gRNA-only drive construct called a CopyCat[16] element that behaves similarly to a gene drive construct in the presence of a transgenic source of *Sp*Cas9, which is itself transmitted in a Mendelian fashion[16,18]. 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[8]. We used two components: 1) a transgenic source of *Sp*Cas9 (or DD2-*Sp*Cas9) driven by the *vasa* germline promoter marked with DsRed, which is inserted in the *yellow* gene coding sequence; and 2) the CopyCat element marked instead with GFP containing a gRNA under the control of the *Drosophila U6:3* promoter (Figure. 2a). We tested the gene-drive-based inheritance bias by inserting the CopyCat element at the *ebony* gene. Next, we crossed males carrying the *Sp*Cas9 (or DD2-*Sp*Cas9) cassette to females containing *ebony* CopyCat element (F0; Figure. 2b).

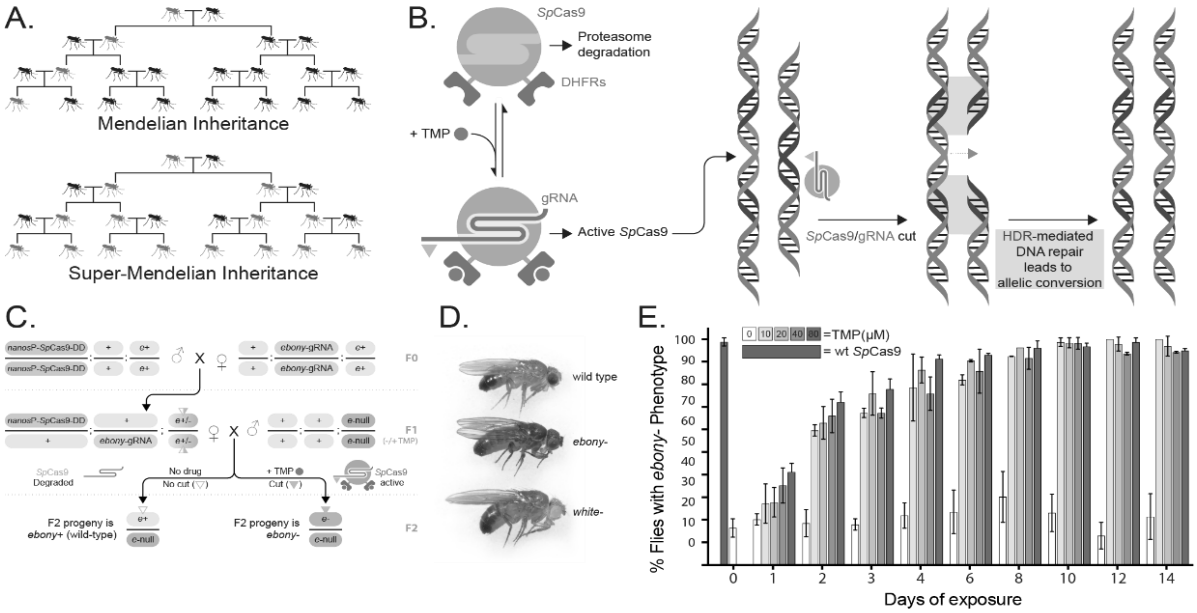

Figure 1 | Chemical control of *Sp*Cas9 in Drosophila. (a) Super-Mendelian inheritance allows a given genetic trait to propagate exponentially in future lineages. **(b)** A destabilized-domain system allows small-molecule based dosage and temporal control of *Sp*Cas9 and subsequent gene drives (DD-*Sp*Cas9). In the absence of TMP, DD-*Sp*Cas9 is degraded by the proteasome, but in the presence of TMP, DD-*Sp*Cas9 is active and can induce double-stranded DNA breaks. Repair of the induced cut with the gene drive-containing template insures the super-Mendelian transmission of the gene-drive construct to the offspring. **(c)** Experimental outline for TMP-activation of *DD-Sp*Cas9 transgenes. F0: Females bearing *nanos*-DD-*Sp*Cas9 transgenes were crossed with males bearing *U6-gRNA* guides targeting *ebony (e)*. F1: Female progeny with *nanos-DD-Sp*Cas9 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-*Sp*Cas9 in the germline. **(d)** Phenotypes of wildtype flies (top), *ebony* mutant (middle), and *white* mutant (bottom). **(e)** Dose-dependent TMP-activation of *DD2-Sp*Cas9 transgenes with *ebony* gRNA. Four days after crossing, flies were transferred to vials containing food with 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-Sp*Cas9*(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 two-way ANOVA with Sidak's multiple comparisons tests.

We collected virgin F1 females carrying both the *Sp*Cas9 (or DD2-*Sp*Cas9) and *ebony* gRNA, which were crossed to wildtype males (Oregon-R) for germline transmission assessment using a phenotypic analysis of GFP (CopyCat) in the F2 progeny (**Figure. 2b**). This experimental design allowed us to assay the germline inheritance ratios of each single female. Wildtype *Sp*Cas9 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. 2c**). In contrast, we observed significant TMP dose-dependent super-Mendelian inheritance for the gRNA element using the DD2-*Sp*Cas9 construct, with Mendelian inheritance values of ~50% in the absence of TMP that reached an average inheritance of ~62% at the maximum concentration (80 μM TMP) (**Figure. 2c**). To the best of our knowledge, these findings are the first example of gene drives with an analog output wherein the inheritance probability can be fine-tuned by an input controller.

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 lack of pigmentation in the fly eye when disrupted. We followed a similar experimental approach by crossing males expressing *Sp*Cas9 or DD2-*Sp*Cas9 to females carrying the *white* CopyCat (**Figure. 2**) and raising the progeny on 0 or 80 μM of TMP. The *white* gRNA element driven by

*Sp*Cas9 displayed an ~90% average inheritance in both the presence and absence of TMP, reinforcing the conclusion that presence of TMP does not affect *Sp*Cas9 function and suggesting that the components or location of the *white* CopyCat result in a greater copying efficiency than the ~65% of their *ebony* counterparts (**Figure. 2e**). As is the case of the *ebony* construct, DD2-*Sp*Cas9 combined with the *white* CopyCat showed the normal Mendelian inheritance of ~50% in the absence of TMP (**Figure. 2e**), while 80 μM of TMP triggered a super-Mendelian inheritance rate of 79% as scored by the GFP phenotype in the F2 progeny (**Fig. 2e**). The combined results from the two constructs confirm the use of small-molecule control of DD2-*Sp*Cas9 in *Drosophila* gene drives.

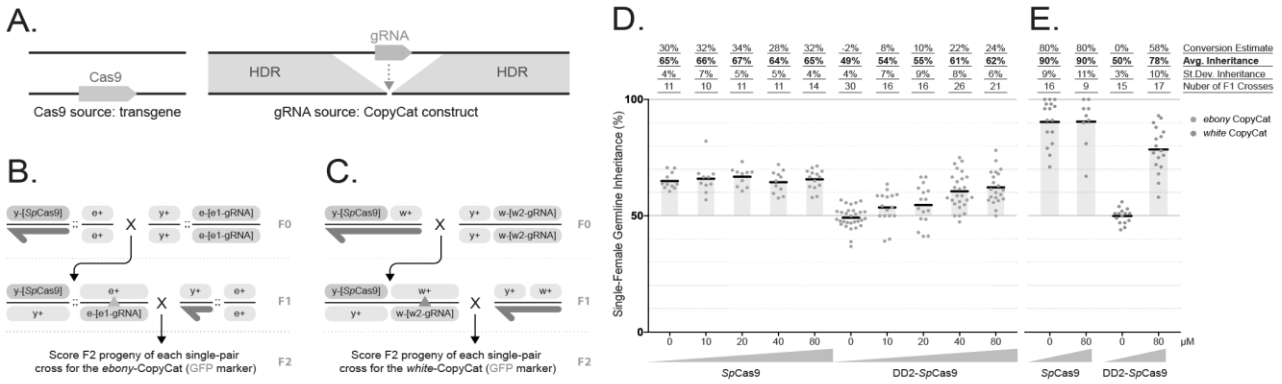

Figure 2 | A small molecule-contingent gene drive. (a) Schematic of the CopyCat drive system. The Cas9 marked with DsRed is a static transgene providing the Cas9 for the CopyCat element allelic conversion process driven by the surrounding homology. **(b,c)** Males expressing *Sp*Cas9 or DD2-*Sp*Cas9 (fluorescent red marker, DsRed) were crossed to virgin females carrying the CopyCat construct. Collected virgin females (Cas9-DsRed + gRNA-GFP) were crossed to wildtype males to score germline transmission rates by screening the GFP marker in the F2 progeny. **(d,e)** Assessment of gene-drive activity in the germline of F1 females by phenotypically scoring the F2 progeny. Our control line (wildtype *Sp*Cas9), which is not regulated by TMP, displayed super-Mendelian inheritance independent of the presence of TMP. DD2-*Sp*Cas9 showed Mendelian inheritance rates in the absence of TMP, while TMP treatment triggered an increasing inheritance bias of the gRNA drive element that correlated with TMP concentration.

CONCLUSIONS

We herein report the first examples of gene drive elements with analog output controlled by a synthetic, orally-available small molecule. Depending on the timing of small molecule administration to *Drosophila*, temporal control of gene-drive activation was achieved. In addition, the synthetic nature of TMP can contain a gene drive by requiring a deliberate application of the molecule, and genes will be inherited only in a Mendelian fashion in the absence of the small molecule. The destabilized domains are transportable to multiple organisms, suggesting that our technology is generalizable to other organisms wherein an *Sp*Cas9-based gene drive has already been demonstrated, such as mosquitoes[6, 7] and mice[17]. Our first-generation small-molecule controllers are also amenable to further modifications. For example, while TMP is non-toxic in humans, it is an antibiotic that can potentially affect an organism's microbiome as well as the surrounding environment. Such toxicity can be averted using a prodrug strategy wherein TMP is rendered inactive by appending a "pro-moiety" that can be removed by an enzyme expressed in the germline of the engineered organism. Multiple orthogonal enzyme-substrate pairs have been reported that can be implemented with TMP, including an esterase[18] and a nitroreductase[19]. These first-generation controllers can also be modified to orthogonally control multiple gene drives in a single organism that could drive two synergistic or antagonistic genes, with the activity of each decided by the dose of the appropriate small molecule. Since our destabilized-domain methodology is extendable to next-generation Cas nucleases (e.g., SaCas9) and multiple orthogonal destabilized-domain/small molecule pairs are readily available[20-22], the independent control of two gene drives is now feasible technology. This gene drive system, which is activated by the deliberate application of a synthetic molecule, represents a new method that could be combined with existing strategies (physical, genetic, ecological) to provide an additional layer of safety for gene-drive containment. For future urban applications, next-generation small-molecule-regulated

gene drives could be adapted to control the spread of these elements in a circumscribed locale by, for example, vaporizing the drug inside homes as it is currently done for mosquito-repellent small molecules or pesticides.

METHODS

Fly rearing and maintenance for phenotype experiments. Flies 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. For food containing TMP, we used Formula 4-24 Instant Drosophila Food (Carolina Biological Supply Company) reconstituted by adding water or water containing different TMP concentrations (10, 20, 40, 80 μM). Flies were anesthetized to select individuals for crossing and phenotyping and were phenotyped by viewing with a Zeiss Stemi 2000 microscope for gene editing studies and a Leica M165 FC Stereo microscope with fluorescence for gene drive experiments. For gene editing studies as detailed in Figure 1, only flies with the full *ebony* phenotype were scored as *ebony*; all intermediate phenotypes to non-phenotypic flies were scored as wildtype. All these experiments were carried out in a BSL-1 facility at the Massachusetts General Hospital. For the gene drive experiments as detailed in Figure 2, we used the GFP marker as an indicator of successful conversion. We also tracked the scored mosaic phenotype in the eyes (see Supplementary Table S1). All gene drive experiments were carried out in a BSL-2 room built for gene drive purposes at the Biological Sciences Department, University of California San Diego.

Transgenic line generation and genotyping for phenotype experiments. All injections to generate transgenic flies were performed by BestGene Inc. or Rainbow Transgenic Flies Inc. Transgenic lines for gene editing experiments (Fig.1) were generated using site-specific φC31 integration at the ZH2A attB site (2A3) on the X chromosome site using *yw ZH-2A*. For wildtype Cas9 under the *nos* promoter, we used y[1] M{w[+mC]=nos-Cas9.P}ZH-2A w[*] from the Bloomington Stock Center[23]. The *ebony* sgRNA line was pFP545 (ref: [24]). For gene drive experiments, all constructs were injected into an isogenized Oregon-R (OrR) strain from our laboratory to keep a homogeneous background in all our experiments. All the Cas9 lines were inserted at the same location (*yellow* gene) to ensure comparable Cas9 expression levels. The CopyCat elements flanked by specific homology arms and marked with GFP were inserted in *ebony* and *white* genes. After construct injection, we received the G0 flies in the larval state (80–120 larvae). Once they eclosed, we distributed all G0 adults in different tubes (5–6 females crossed to 5–6 males). Then, G1 progeny were screened for the presence of the specific fluorescent marker in their eyes, which was indicative of the marker insertion. Flies positive for the marker were crossed individually to OrR flies (same used for injection) to make a homozygous stock for each transgenic line. Finally, we sequenced the final stocks to confirm the correct integration of the cassette in each line.

Plasmid construction. Standard molecular biology techniques were used to build all the constructs analyzed in this work, and sequence information is uploaded on NCBI

Statistical analysis. We used GraphPad Prism 7 to perform all statistical analyses. The two-way ANOVA and Sidak's multiple comparison tests were used (see Supplementary Table S1 and S2).

ACKNOWLEDGEMENTS

This work was supported by the Burroughs Wellcome Fund (Career Award at the Scientific Interface to A.C.), DARPA (Brdi N66001-17-2-4055), NIH (R21AI126239 to A.C. and DP5OD023098 to V.M.G). This work is dedicated to Professor Ronald T. Raines on the occasion of his 60th birthday.

REFERENCES

1. **Gantz, V.M. & Bier, E**. Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* **348**, 442-444 (2015).

2. **Gantz, V.M. & Bier, E.** The dawn of active genetics. *BioEssays* **38**, 50-63 (2016).

3. **Esvelt, K.M., Smidler, A.L., Catteruccia, F. & Church, G.M**. Concerning RNA-guided gene drives for the alteration of wild populations. *Elife* **3** (2014).

4. **Shapiro, R.S. *et al.*** A CRISPR-Cas9-based gene drive platform for genetic interaction analysis in Candida albicans. *Nat. Microbiol.* **3**, 73-82 (2018).

5. **Esvelt, K.M. & Gemmell, N.J.** Conservation demands safe gene drive. *PLoS Biol.* **15**, e2003850 (2017).

6. **Gantz, V.M. *et al.*** Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc. Natl. Acad. Sci. U S A* **112**, E6736-6743 (2015).

7. **Hammond, A. *et al.*** A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nat. Biotechnol.* **34**, 78-83 (2016).

8. **Akbari, O.S. *et al*.** BIOSAFETY. Safeguarding gene drive experiments in the laboratory. *Science* **349**, 927-929 (2015).

9. **Champer, J. *et al.*** Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. *PLoS Genet.* **13**, e1006796 (2017).

10. **Champer, J. *et al*.** Reducing resistance allele formation in CRISPR gene drive. *Proc. Natl. Acad. Sci. U S A* **115**, 5522-5527 (2018).

11. **Hammond, A.M. *et al.*** The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genet.* **13**, e1007039 (2017).

12. **Zaim, M., Aitio, A. & Nakashima, N**. Safety of pyrethroid-treated mosquito nets. *Med. Vet. Entomol.* **14**, 1-5 (2000).

13. **Cho, U. *et al.*** Rapid and tunable control of protein stability in Caenorhabditis elegans using a small molecule. *PLoS One* **8**, e72393 (2013).

14. **Maji, B. *et al*.** Multidimensional chemical control of CRISPR-Cas9. *Nat. Chem. Biol.* **13**, 9-11 (2017).

15. Oakes, B.L. *et al*. Profiling of engineering hotspots identifies an allosteric CRISPR-Cas9 switch. *Nat. Biotechnol.* **34**, 646-651 (2016).

16. **Xu, X.S., Gantz, V.M., Siomava, N. & Bier, E.** CRISPR/Cas9 and active genetics-based trans-species replacement of the endogenous Drosophila kni-L2 CRM reveals unexpected complexity. *Elife* **6** (2017).

17. **Grunwald, H.A. *et al.*** Super-Mendelian inheritance mediated by CRISPR/Cas9 in the female mouse germline. *bioRxiv*, 362558 (2018).

18. **Tian, L. *et al*.** Selective esterase-ester pair for targeting small molecules with cellular specificity. *Proc. Natl. Acad. Sci. U S A* **109**, 4756-4761 (2012).

19. **Gruber, T.D. *et al*.** Cell-Specific Chemical Delivery Using a Selective Nitroreductase-Nitroaryl Pair. *ACS Chem. Biol.* **13**, 2888-2896 (2018).

20. **Banaszynski, L.A., Chen, L.C., Maynard-Smith, L.A., Ooi, A.G. & Wandless, T.J**. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**, 995-1004 (2006).

21. **Iwamoto, M., Bjorklund, T., Lundberg, C., Kirik, D. & Wandless, T.J.** A general chemical method to regulate protein stability in the mammalian central nervous system. *Chem. Biol.* **17**, 981-988 (2010).

22. **Miyazaki, Y., Imoto, H., Chen, L.C. & Wandless, T.J**. Destabilizing domains derived from the human estrogen receptor. *J. Am. Chem. Soc.* **134**, 3942-3945 (2012).

23. **Port, F., Chen, H.M., Lee, T. & Bullock, S.L**. Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc. Natl. Acad. Sci. U S A* **111**, E2967-2976 (2014).

24. **Port, F., Muschalik, N. & Bullock, S.L**. Systematic Evaluation of Drosophila CRISPR Tools Reveals Safe and Robust Alternatives to Autonomous Gene Drives in Basic Research. *G3 (Bethesda)* **5**, 1493-1502 (2015).