Cleave and Rescue, a novel selfish genetic element and general strategy for gene drive

Georg Oberhofer[a,1], Tobin Ivy[a,1], and Bruce A. Hay[a,2]

[a]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125

Edited by James J. Bull, The University of Texas at Austin, Austin, TX, and approved January 7, 2019 (received for review October 2, 2018)

There is great interest in being able to spread beneficial traits throughout wild populations in ways that are self-sustaining. Here, we describe a chromosomal selfish genetic element, *CleaveR* [Cleave and Rescue (*ClvR*)], able to achieve this goal. *ClvR* comprises two linked chromosomal components. One, germline-expressed Cas9 and guide RNAs (gRNAs)—the Cleaver—cleaves and thereby disrupts endogenous copies of a gene whose product is essential. The other, a recoded version of the essential gene resistant to cleavage and gene conversion with cleaved copies—the Rescue—provides essential gene function. *ClvR* enhances its transmission, and that of linked genes, by creating conditions in which progeny lacking *ClvR* die because they have no functional copies of the essential gene. In contrast, those who inherit *ClvR* survive, resulting in an increase in *ClvR* frequency. *ClvR* is predicted to spread to fixation under diverse conditions. To test these predictions, we generated a *ClvR* element in *Drosophila melanogaster*. $ClvR^{tko}$ is located on chromosome 3 and uses Cas9 and four gRNAs to disrupt *melanogaster technical knockout* (*tko*), an X-linked essential gene. Rescue activity is provided by *tko* from *Drosophila virilis*. $ClvR^{tko}$ results in germline and maternal carryover-dependent inactivation of *melanogaster tko* (>99% per generation); lethality caused by this loss is rescued by the *virilis* transgene; $ClvR^{tko}$ activities are robust to genetic diversity in strains from five continents; and uncleavable but functional *melanogaster tko* alleles were not observed. Finally, $ClvR^{tko}$ spreads to transgene fixation. The simplicity of *ClvR* suggests it may be useful for altering populations in diverse species.

gene drive | Cas9 | population replacement | selfish genetic element

Gene drive occurs when particular alleles are transmitted to viable, fertile progeny at rates greater than those of competing allelic variants. Strategies for altering the genetics of populations that incorporate some level of drive to enhance the spread of linked transgenes, but that are not self-sustaining, have been proposed but not yet implemented (1–3). A number of approaches to spreading traits through populations (population replacement/alteration) in ways that are self-sustaining, by linking them with genetic elements that mediate drive, have also been proposed (4–16). Much recent interest has focused on approaches to population alteration that utilize engineered site-specific nucleases that function as homing endonuclease genes (HEGs) (17). A HEG encodes a site-specific nuclease that is inserted within its chromosomal recognition sequence. This prevents cleavage of the homolog within which it resides. If, in a heterozygote, the wild-type allele is cut and homologous recombination (HR) is used as the repair pathway with the HEG-bearing chromosome as the repair template, the HEG heterozygote can be converted into a homozygote (also known as homing), thereby increasing HEG copy number. There is particular interest in HEGs created using the CRISPR/Cas9 endonuclease system, in which the Cas9 endonuclease is targeted to specific sequences through association with one or more independently expressed guide RNAs (gRNAs) (18). Target sequence limitations with Cas9 are modest, and thus Cas9 in conjunction with one or more gRNAs can be used to cleave a gene at multiple positions, making these reagents ideal tools for HEG engineering. Population alteration using HEGs can in principle be achieved in several ways (17, 19). However, all require homing, which requires that cleavage be followed by repair and copying of the intact HEG through high-fidelity HR. While important progress has been made, sustained alteration of a population [as opposed to suppression (20)] to transgene-bearing genotype fixation with a synthetic HEG into artificial or naturally occurring sites remains to be achieved (21–30).

A number of other approaches to bringing about gene drive take as their starting point naturally occurring, chromosomally located, selfish genetic elements whose mechanism of spread does not involve homing (4, 6, 31). Many of these elements can be represented as consisting of a tightly linked pair of genes encoding a *trans*-acting toxin and a *cis*-acting antidote that neutralizes toxin expression and/or activity (TA systems) (4). The general idea is often that toxin expression or activity is repressed in cells that carry the TA pair because they also express the antidote, allowing survival. However, when such a system is present in an organism, those gametes, progeny, or daughter cells that fail to inherit the TA system die because the toxin or effects of toxin activity remain present, while the *cis*-acting antidote is absent: a phenomenon known as postsegregational killing. Examples of such systems where some molecular information is available include the maternal-effect selfish genetic element *Medea* in *Tribolium* (32, 33), the *sup-35*/*pha-1* maternal-effect selfish genetic element in *Caenorhabditis elegans* (34), the *peel-zeel* paternal-effect selfish genetic element in *C. elegans* (35), and the *wtf* gamete/spore killers in yeast (36, 37). Synthetic *Medea* elements generated in *Drosophila* use a similar logic, but with the toxin simply being a maternally expressed miRNA (the toxin) that results in maternal loss of a product normally deposited into the embryo that is essential for

Significance

There is great interest in spreading beneficial traits throughout wild populations in self-sustaining ways. Here, we describe a synthetic selfish genetic element, *CleaveR* [Cleave and Rescue (*ClvR*)], that is simple to build and can spread a linked gene to high frequency in populations. *ClvR* is composed of two components. The first, germline-expressed Cas9 and guide RNAs (gRNAs), cleave and disrupt versions of an essential gene located elsewhere in the genome. The second, a version of the essential gene resistant to cleavage, provides essential gene function. *ClvR* spreads by creating conditions in which progeny lacking *ClvR* die because they have no functional copies of the essential gene. In contrast, those who inherit *ClvR* survive, resulting in an increase in *ClvR* frequency.

Author contributions: G.O., T.I., and B.A.H. conceptualized the study; G.O., T.I., and B.A.H. provided the methodology; G.O. and B.A.H. investigated the study; T.I. provided mathematical modeling; G.O. and B.A.H. wrote the manuscript; G.O. and B.A.H. acquired funding; and G.O., T.I., and B.A.H. wrote the paper

Conflict of interest statement: The authors have filed patent applications on ClvR and related technologies (US Application No. 15/970,728).

This article is a PNAS Direct Submission.

[1]G.O. and T.I. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: haybruce@caltech.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1816928116/-/DCSupplemental.

early embryo development (the consequences of toxin expression). The antidote is a transgene that results in early embryonic expression of a recoded version of this same gene that is resistant to miRNA silencing (the antidote), thereby providing essential gene function in a just-in-time fashion (6, 38, 39). Finally, prokaryotes also contain a number of tightly linked toxin–antidote clusters (including but not limited to type II restriction enzymes and their cognate methyltransferases). While many of these play important roles in cell physiology and defense, there are also multiple lines of evidence showing that some of them act in a selfish manner to increase their representation within populations through postsegregational killing of those that fail to inherit them (and thus the antidote) at cell division as a result of inefficient partitioning, or when in competition with other similar units (plasmids of the same incompatibility group/replicon) that lack them (40, 41). Based on these behaviors bacterial TA systems are sometimes known as addiction modules: the components they encode are fundamentally nonessential (as with the eukaryotic TA systems described above), but once they are acquired, they cannot easily be lost without causing death of the host cell.

The components of naturally occurring TA systems could in principle be adapted to bring about gene drive in other species of interest. While the locus that contains *Tribolium Medea* has been sequenced, the molecular nature of TA components that account for its behavior remains unknown. Toxin and antidotes associated with *C. elegans* maternal- and paternal-effect selfish genetic elements are known (35, 34), as are those associated with gamete/spore killing in yeast (36, 37). However, in these latter cases, it is unclear whether the mechanisms of action and any associated gene regulation required for selfish behavior can be transferred across species. Implementation of synthetic *Medea* was successful in *Drosophila*, but this relied on detailed knowledge of the molecular genetics that underlie maternal and early zygotic control of embryogenesis. Efforts to translate *Medea* to species other than the closely related *Drosophila suzukii* (39) have not yet succeeded. Toxins and antidotes from prokaryotes are well understood at a mechanistic level, and are often likely to be active in eukaryotic systems since many of them target highly conserved processes, such as translation, or promote the degradation of RNA or DNA (40–42). However, the use of these or other gain-of-function toxins and antidotes requires careful titration of the place and time they are transcribed and translated. Achieving such control, as with synthetic *Medea* elements, is likely to require a deep species-specific toolbox of information and reagents, including knowledge of details of development, promoters, and regulators of translation and degradation during key stages of development, such as the maternal-zygotic transition. In sum, while existing TA systems are attractive to consider as a starting point for development of new gene drive systems—since they bring about drive in nature—the available tools do not yet provide a straightforward and general approach to building TA-based chromosomal gene drive methods in diverse species.

Here, we report the creation of a TA-based chromosomally located selfish genetic element whose components are simple and interchangeable, and likely to be generally available across species. Our starting point is the fact that site-specific alteration of DNA in the germline, mediated by Cas9 and gRNAs or other site-specific nucleases, followed by error-prone repair or creation of larger deletions, can be used most simply to disrupt the function of a gene, in our case an essential gene. Site-specific base editing enzymes (43) can be employed toward a similar end. Here, we focus on site-specific nucleases. Novel versions of essential genes that share limited or no nucleotide sequence similarity with the endogenous version, and are thus uncleavable, can rescue the viability and fertility of individuals that otherwise carry only loss-of-function (LOF) versions of the essential gene (44–46). Recombination and gene conversion can occur between a cleaved locus and an uncleaved counterpart located elsewhere in the genome to which it has sequence similarity (47), and this could lead to the creation of functional, cleavage-resistant alleles at the endogenous essential gene locus. Reducing or eliminating sequence similarity between the cleaved version of the essential gene and an uncleavable rescuing version can prevent such events (48). Finally, in the case of diploids, for many essential genes (haplosufficient recessive lethal or sterile), heterozygotes for a LOF allele are, at least to a first approximation, fit (49–51).

Under the above conditions, a cassette that includes germline-expressed Cas9 and gRNAs, designed to cleave *in trans* and thereby disrupt any endogenous wild-type copies of an essential gene, and a recoded version of the essential gene resistant to cleavage and recombination or gene conversion with cleaved versions of the wild-type allele, and therefore able to rescue those who carry it *in cis*, behaves as a selfish genetic element, which we refer to as *CleaveR* [Cleave and Rescue (*ClvR*)] (Fig. 1*A*). The toxin, Cas9 and gRNAs, works *in trans* by creating a permanent, potentially lethal change to the host genome wherever the targeted locus is located. However, this lethality only manifests itself in those who fail to inherit *ClvR* and its *cis*-acting antidote, the Rescue transgene. In contrast, those who inherit *ClvR* and the Rescue transgene contained within it survive, resulting in an increase in the frequency of individuals with *ClvR*-bearing chromosomes compared with those carrying non–*ClvR*-bearing counterparts. (Fig. 1, and other examples in *SI Appendix*, Figs. S1 and S2). This represents a form of postsegregational killing and leads cells, organisms, and populations to become dependent on (addicted to) the *ClvR*-encoded Rescue transgene (the antidote) for their survival. An analogy can be drawn with one strategy used to force the maintenance of a costly, nonessential plasmid in the absence of antibiotic selection. This involves locating an unconditionally essential gene (normally chromosomal) on the plasmid in cells that otherwise lack a functional copy of the essential gene (52). A *ClvR* element simply has the added feature that it provides the mechanism by which the endogenous version of the essential gene is inactivated in addition to the mechanism promoting survival in its absence. In *Results and Discussion*, we consider the specific case of *ClvR* behavior in a diploid animal, *Drosophila melanogaster*, as a model for other species such as mosquitoes, for which there has long been interest in the idea of altering wild populations so that they are unable to transmit diseases such as dengue, yellow fever, chikungunya, or malaria.

Results and Discussion

ClvR and the locus it targets for inactivation can be located on the same chromosome or on different chromosomes. The specific relationship is not important for gene drive since cleavage occurs *in trans*, wherever the target gene is located, while rescue only occurs *in cis*, in those who inherit *ClvR*. *ClvR* behavior is illustrated in Fig. 1 *B–D* for the case in which *ClvR* is located on an autosome and the haplosufficient essential gene targeted for cleavage is located on the X chromosome (see below and Figs. 2–5 for related experiments). Cleavage by Cas9 followed by inaccurate repair creates LOF alleles of the essential gene in the adult female germline (Fig. 1*B*). Diploid germ cells survive because they carry a copy of *ClvR*, which includes the recoded Rescue. In animals, haploid gametes lacking *ClvR* and a functional copy of the essential gene (e.g., some female gametes in Fig. 1 *C* and *D*) will generally survive and be functional because essential gene products utilized during the haploid stage are expressed during the diploid stage and shared between the products of meiosis (53–55). However, in other organisms in which extensive transcription occurs during the haploid stage (e.g., plants and fungi), gametes lacking *ClvR* will be lost if transcription of the targeted essential gene is required during the haploid phase for gamete survival or function (*SI Appendix*, Fig. S1).

Here, we focus on animals. When a heterozygous female mates with a wild-type male, female progeny survive because they inherit a wild-type copy of the essential gene from their father. Some males who inherit the X-linked LOF allele from their mother also survive because they inherit an autosomal copy of *ClvR*, while others die because they inherit the X-linked LOF allele and the wild-type non–*ClvR*-bearing autosomal homolog (Fig. 1*C*). If there is maternal

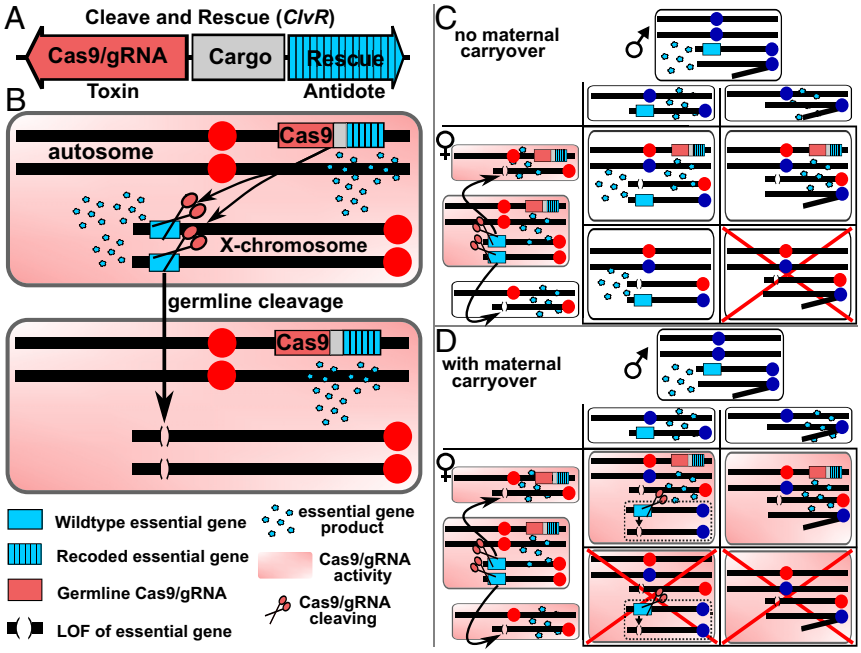

Fig. 1. Basic structure of a *ClvR* element, and its behavior in a diploid, with and without maternal carryover. (*A*) Components of a *ClvR* element. (*B*) Behavior of *ClvR* as implemented for a *ClvR* on an autosome and an essential gene located on X chromosome. The long thick horizontal black bar represents a chromosome with *ClvR* on the right arm of an autosome (see experiments below for an experimental implementation), while the shorter horizontal black bar represents an X chromosome carrying an essential gene. The identity of genes, alleles, and protein and RNA products are indicated. Arrows are drawn from a wild-type allele of the essential gene to the cleaved product resulting from Cas9 activity. (*C*) Results of a cross between a heterozygous *ClvR*-bearing female and a wild-type male, in the absence of maternal carryover of Cas9/gRNA complexes. Arrows indicate conversion from wild-type to LOF allele. (*D*) Same cross as in *C*, but with maternal carryover of Cas9/gRNAs sufficient to convert wild-type alleles of the essential gene inherited from the father into LOF alleles. The dashed boxes highlight the paternal X chromosome before and after cleavage and creation of a LOF allele. Arrows indicate conversion from wild-type to LOF allele. Large red Xs indicate offspring that die because they lack any source of essential gene function. The color of the centromere (large circle) indicates whether the chromosome was inherited from a female (red) or male (blue) parent. The Y chromosome is shown as a short horizontal black bar with an angled segment, and a blue centromere.

carryover of Cas9/gRNA complexes, wild-type alleles of the essential gene inherited from the father can be converted to LOF alleles in the zygote. If this happens in a large fraction of nuclei in the zygote, all progeny not inheriting the *ClvR*-bearing chromosome, and thus lacking a functional copy of the essential gene, die (Fig. 1*D*). Together, these events create conditions in which *ClvR*-bearing parents transmit a potential fitness cost—a nonzero probability of inheriting no functional copies of the essential gene—to progeny. Non–*ClvR*-bearing homologous chromosomes are at risk for this cost, while *ClvR*-bearing chromosomes are not, thereby promoting a relative increase in frequency of the latter (Fig. 1 *C* and *D*).

Population Genetic Behavior of *ClvR*. The behavior of such a *ClvR* element, located on an autosome and targeting a haplosufficient essential gene on the X chromosome (see Figs. 3 and 5 for related experiments), is illustrated in Fig. 2 for a conservative germline cleavage rate of 90% (actual rates, >99%; Fig. 3) and various release percentages and fitness costs, without (Fig. 2*A*) and with (Fig. 2*B*) 90% maternal carryover-dependent cleavage (actual rates, >99%; Fig. 3). *ClvR* is predicted to behave as a low-threshold gene drive mechanism (no deterministic threshold for an element with no fitness cost), spreading to transgene-bearing genotype fixation for a wide range of release percentages and fitness costs. However, in contrast to a HEG, which can spread quickly from low frequency (56), spread of *ClvR* is very frequency dependent: slow when introduced at low frequency, and fast when introduced at high frequency (Fig. 2 *A* and *B*). Maternal carryover-dependent cleavage is not essential for *ClvR*-dependent drive (Fig. 2*A*) but can speed the process and allow the drive element to tolerate larger fitness costs (Fig. 2*B*). Finally, while the behavior of many genes is described as haplosufficient, this designation often reflects the results of characterization under controlled laboratory conditions. Characterization of the same heterozygotes under other environmentally relevant conditions may uncover varying levels of haploinsufficiency (cf. ref. 57). Given that wild populations carrying gene drive elements will experience a variety of biotic and abiotic environmental conditions, it is important to understand how haploinsufficiency would affect *ClvR*-dependent drive. To explore this, we examined the behavior of a *ClvR* located on one autosome, targeting an unlinked locus on a different autosome, with a single functional version of the target gene resulting in some level of haploinsufficiency (Fig. 2*C*). We modeled a two locus autosomal scenario rather than that of an autosomal *ClvR* targeting the X since most essential genes are on autosomes, and to be able to capture the effects of haploinsufficiency in both sexes. Interestingly, *ClvR* is predicted to bring about population alteration under a wide variety of conditions if the essential gene targeted is haploinsufficient (Fig. 2*C*), or even haplolethal (Fig. 2*D*).

Synthesis of *ClvR*^{tko} in *Drosophila melanogaster*. To create *ClvR* in *Drosophila melanogaster*, we first generated a construct carrying a recoded version of *D. melanogaster*'s X-linked *tko* locus, which encodes the conserved, essential, and haplosufficient mitochondrial ribosomal protein *rps12* (58). To minimize homology of the rescue transgene with *D. melanogaster tko*, and thereby limit opportunities for recombination or gene conversion between the two (47, 48), we utilized the *tko* locus from a distantly related species, *Drosophila virilis*. We also introduced six additional silent coding sequence mutations to further reduce homology with the *D. melanogaster* gene (*SI Appendix*, Fig. S3). The *tko* rescue construct (*tko*A) includes a dominant *td-tomato* marker, and an attP recombination site. It was introduced into the *D. melanogaster* genome on the third chromosome, at 68E, using Cas9 mediated HR, generating *tko*A flies (*SI Appendix*, Fig. S4*A*). In a

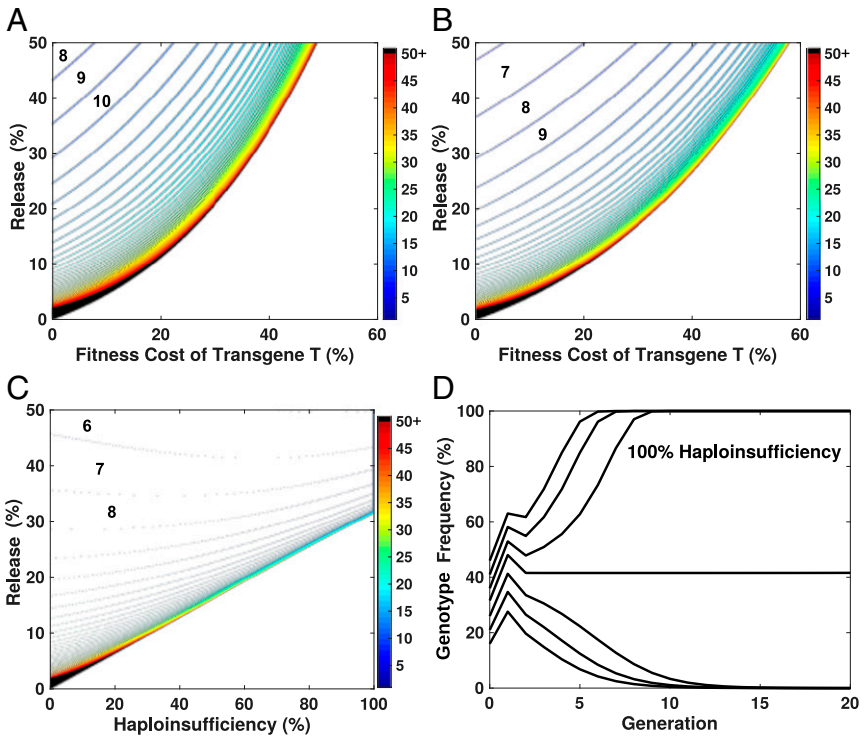

Fig. 2. Population genetic behavior of *ClvR* when targeting a haplosufficient (*A* and *B*) or haploinsufficient (*C* and *D*) essential gene. (*A* and *B*) A discrete generation, deterministic population frequency model of *ClvR* spread in which cleavage occurs in the male and female germline; *ClvR* located on an autosome and the essential gene is located on the X (see data in Figs. 3 and 5) through a single panmictic population, for varying initial release percentages and fitness costs, without (*A*) or with (*B*) maternal carryover-dependent cleavage. The heatmap indicates the number of generations required for the *ClvR*-bearing genotype to approach fixation (i.e., >99% of the total population). (*C*) Heatmap showing the number of generations required for the *ClvR*-bearing genotype to reach fixation (<99% *ClvR*-bearing) for different initial release percentages and haploinsufficient fitness costs (100% = haplolethal), for an autosomal version of *ClvR* targeting a second unlinked autosomal locus, with maternal carryover. (*D*) Individuals traces showing the fate of a *ClvR* from (*C*) targeting a haplolethal gene, for different release percentages. The horizontal line represents an approximation of the unstable equilibrium frequency (~31.5%; genotype frequencies do not change significantly over 20 generations). Genotype frequencies greater than equilibrium, 36%, 41%, and 46%; those below, 26%, 21%, and 16%. Note that the term "Release %" for all heatmaps refers to the percentage of homozygous transgenic males compared with wild-type males and females after a release has occurred (e.g., a 40% release means that 40% of the population is *ClvR*/*ClvR* male, 30% is +/+ male, and 30% is +/+ female). Thus, initial release percentage also equals initial genotype frequency. Note that, for *C* and *D*, *ClvR* itself is assumed to have no fitness cost. Such costs would further increase the minimum release percentages required for drive to occur, as in *A* and *B*.

second step, transgenes expressing Cas9 and four gRNAs designed to recognize and cleave DNA within the *D. melanogaster tko* coding region, but not that of *D. virilis tko*, were integrated into the attP site in *tko*A rescue construct-bearing flies (*SI Appendix*, Fig. S4 *B* and *C*). The gRNAs were each expressed under the control of a U6 polymerase III promoter (59). Cas9 was expressed under the control of *nanos* regulatory sequences, which drive expression in the male and female germline (60). Nanos-driven Cas9 also results in extensive maternal, but not paternal, carryover of active Cas9/gRNA complexes into the zygote (29, 61). The final construct is designated $ClvR^{tko}$ (Fig. 3*A*), and flies that carry it as $ClvR^{tko}$ flies.

Genetic Behavior of $ClvR^{tko}$. Matings between males that carry a LOF mutation for the X-linked eye pigmentation gene *white* (w^{1118}), and that are heterozygous for $ClvR^{tko}$ on the third chromosome (w^{1118}; $ClvR^{tko}$/+), where + indicates a third chromosome that does not carry $ClvR^{tko}$, and homozygous w^{1118}; +/+ females resulted in high levels of progeny viability to adulthood (95.2 ± 2.0%), similar to those for the w^{1118} strain used for transformation (95.9 ± 2.0%). In addition, ~50% (50.1 ± 3.0%) of the adult progeny carried $ClvR^{tko}$, as expected for Mendelian segregation and high $ClvR^{tko}$ heterozygote fitness. Matings among homozygous $ClvR^{tko}$ flies also resulted in high levels of viability to adulthood (95.1 ± 1.7%), indicating that the presence of $ClvR^{tko}$ components (in the likely absence of functional *D. melanogaster tko*; see below) does not result in obvious fitness costs. In contrast, when heterozygous w^{1118}; $ClvR^{tko}$/+ females were mated with homozygous w^{1118}; +/+ males, 53.6 ± 1.3% of progeny did not reach adulthood, and all surviving adults carried $ClvR^{tko}$. On the basis of these results, we infer that the presence of $ClvR^{tko}$ in mothers results in a very high frequency (>99%) of mutational inactivation of the *D. melanogaster tko* locus in the adult female germline and in the zygote through maternal carryover-dependent cleavage of the paternal allele. In consequence, those who fail to inherit $ClvR^{tko}$ die, while those who inherit a single copy of $ClvR^{tko}$ thrive (*SI Appendix*, Table S1 A and B).

To obtain estimates of the rate of female adult germline- and maternal carryover-dependent cleavage and subsequent *D. melanogaster tko* inactivation, we repeated the cross between $ClvR^{tko}$/+ females and wild-type males with larger numbers of individuals (see also *SI Appendix*, Table S5, for additional experiments of this type with genetically diverse strains). All but one of 3,736 progeny that survived to adulthood (cleavage rate of >99.9%) carried $ClvR^{tko}$ (Fig. 3*B* and *SI Appendix*, Table S2). To estimate male germline cleavage rates, we carried out a cross between $ClvR^{tko}$/+ males and females that carried a lethal *tko* LOF allele [tko^3, a frameshift mutation at amino acid 108 that introduces a premature stop codon (62)] *in trans* to the balancer chromosome FM7 (the balancer prevents meiotic recombination between X chromosomes), which is wild type for *tko* and carries a dominant mutation in the Bar gene, B^1. Female progeny that inherit the

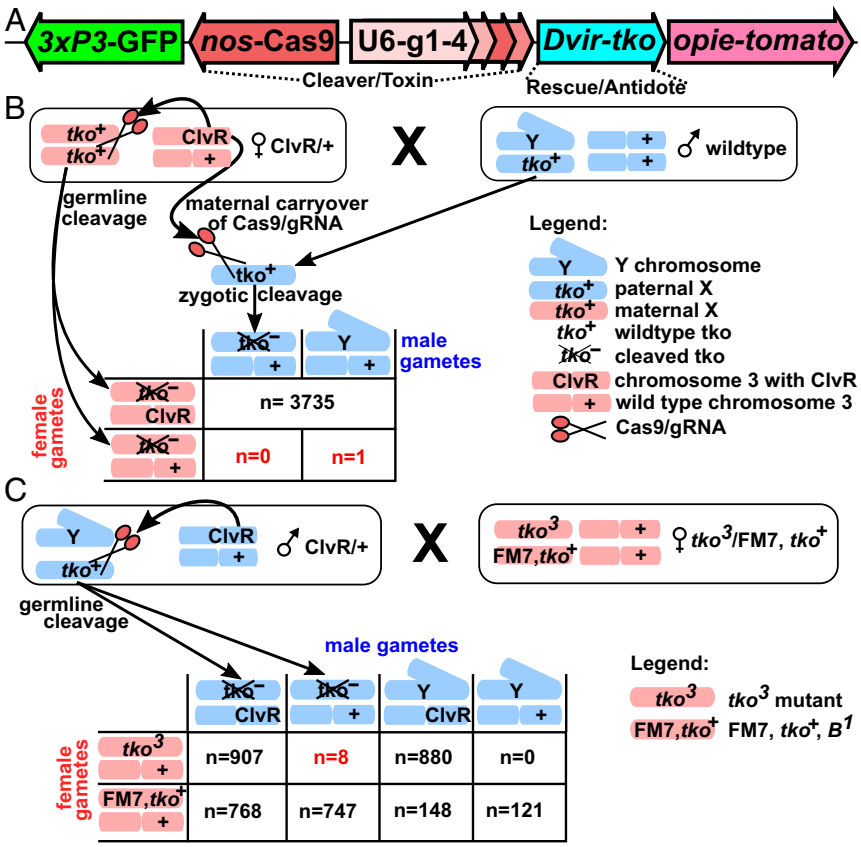

Fig. 3. Components of *ClvR* and its behavior in females and males. (*A*) Component genes and their arrangement in *ClvR*^{tko}. (*B*) The behavior of *ClvR*^{tko} when present in a *ClvR*^{tko}/+ adult female. Female progeny inherit an X from their mother (red) and one from their father (blue). Male progeny inherit an X from their mother. One non–*ClvR*^{tko} -bearing male survived, while all other 3,735 male and female progeny inherited *ClvR*^{tko}, for a cleavage rate of >99.9%. (*C*) The behavior of *ClvR*^{tko} when present in a *ClvR*^{tko}/+ male. When *ClvR*^{tko}/+ males are crossed to *tko*^{3}/FM7,*B*^{1} females (the FM7 balancer chromosome is wild type for *tko*), non-FM7,*B*^{1} female progeny carry *tko*^{3}, a homozygous recessive lethal allele of *tko*) and an X chromosome from their father. In total, 907 of these carry *ClvR*^{tko}, while only 8 (which may not represent independent events; *SI Appendix*, Fig. S5 and Table S3) do not, for a cleavage rate of >99%. Individuals carrying the FM7,*B*^{1} balancer, particularly males, are much less fit than others, and were not considered in the calculations.

maternal *tko*^{3} allele (identified by their failure to carry the dominant *B*^{1} marker), and that lack *ClvR*^{tko} (and therefore lack the *td-tomato* and GFP markers), should die if *D. melanogaster tko* was inactivated in the parental male germline and survive if it was not. Eight females carrying the *tko*^{3} allele and lacking *ClvR*^{tko} were recovered compared with 907 that carried *tko*^{3} and *ClvR*^{tko}, for a minimum male germline cleavage rate of >99% (Fig. 3*C* and *SI Appendix*, Fig. S5 and Table S3). *ClvR*^{tko}-dependent rescue of the *tko*^{3} mutant phenotype is indicated by the large numbers of *tko*^{3}/Y; *ClvR*^{tko}/+ progeny (880), compared with none for *tko*^{3}/Y; +/+ (Fig. 3*C*).

X Chromosomes in Which a *tko* LOF Allele Was Not Created Following Exposure to *ClvR*^{tko} Remain Sensitive to Cleavage by *ClvR*^{tko}. We sequenced the *D. melanogaster tko* locus from each of the nine X chromosomes above, in which a *tko* LOF allele was not created (escapers) following exposure to maternal or paternal *ClvR*^{tko}. In the single escaper coming from a *ClvR*^{tko}/+ mother, all four gRNA target sites were unaltered. For seven escapers coming from the *ClvR*^{tko}/+ father, there was a common 3 bp in-frame deletion within the gRNA1 target site, and the remaining three target sites were unaltered. For escaper M3, a mixed sequencing signal, which may be indicative of nuclear mosaicism, was obtained. When each of the above escaper chromosomes was isolated in a male and the male crossed to *ClvR*^{tko}/+ females, all surviving progeny inherited the *ClvR*^{tko} *td-tomato* and GFP markers, showing that the *D. melanogaster tko* locus remained sensitive to cleavage (*SI Appendix*, Fig. S5 and Table S4).

***ClvR*^{tko} Functions in Diverse Genetic Backgrounds.** To alter wild populations, a gene drive mechanism must be able to function in diverse genetic backgrounds. To begin to explore this topic with *ClvR*, we crossed *ClvR*^{tko}/+ females to males from Global Diversity Lines (GDL) isolated from five different continents (63), and used in previous work investigating Cas9 function in the context of engineered HEGs (27). After each generation, we scored the frequency of *ClvR*^{tko} flies, collected 30 virgins, and backcrossed them again to males from each of the GDL lines. Results are summarized in *SI Appendix*, Table S5. All offspring were *ClvR*^{tko}-bearing for each of six generations (7,882 progeny scored). While these results do not preclude the existence of unlinked genetic variants and/or gRNA target polymorphisms in wild populations that would result in decreased rates of cleavage and LOF mutation creation, they show that the system is not specific to a common laboratory strain [*SI Appendix*, Table S6, shows all gRNA target site polymorphisms in strains from the 1000 fly genomes project (64)].

Molecular Nature of Mutations Created in *D. melanogaster tko* Created Following Exposure to *ClvR*^{tko}. To analyze the mutations in *D. melanogaster tko* created by *ClvR*^{tko} we selected 2 *ClvR*^{tko}-bearing male progeny from each of nine individual single crosses (18 total flies) between heterozygous *ClvR*^{tko} females and *w*^{1118} males

(from Fig. 3*B*). Sequencing results from the region of the *D. melanogaster tko* locus spanning the gRNA-binding sites are summarized in *SI Appendix*, Table S7A (alignments in *SI Appendix*, Fig. S6 *A* and *B*). The gRNA1 target site contained indels of varying size in all 18 individuals. The gRNA2 target site contained a likely preexisting polymorphism in four individuals (also observed in roughly half of the 1000 Fly Genome Project strains (64)), and a 2 bp deletion in 3. The gRNA3 target site was unaltered in all individuals, and the gRNA4 target contained indels in nine individuals. Somewhat surprisingly, larger deletions between target sites were not observed. This raises the possibility, suggested by others (65), that close juxtaposition of multiple target sites—in our case, four target sites within a 250-bp region of the *tko* ORF—limits Cas9's ability to simultaneously interact with and/or cleave multiple nearby target sites as a consequence of Cas9-dependent DNA supercoiling.

One implication of such a model is that mutations should accumulate at additional target sites over time, as the target sites first cleaved by Cas9 are rendered nonfunctional for further Cas9 binding due to mutation within the gRNA target site. To explore this possibility, and the general question of whether all gRNA target sites can be cleaved, we sequenced the melanogaster *tko* locus from a homozygous *ClvR*^{tko} stock that had been inbred for three generations (*SI Appendix*, Fig. S6 *C* and *D*, and Table S7B). Among the 12 analyzed males, all 12 had mutations at the gRNA1 target site. The gRNA2 target site was mutated in five, unaltered in one individual, and carried the suspected common polymorphism in the remaining six. The gRNA3 target site was mutated in 1 fly, and the gRNA4 target site was mutated in all 12 flies. Thus, cleavage events accumulate over time, and all sites can be cleaved, although cleavage efficiencies differ (from 100% for gRNA1 in generation 1 to 8% for gRNA3 after three generations).

The mutations we observe presumably arise initially from error-prone repair by nonhomologous end-joining (NHEJ) or microhomology-mediated end-joining pathways (Fig. 4). However, we note that *ClvR* elements may also utilize HR and homing to create new LOF alleles when the *ClvR*-bearing individuals introduced into the wild population carry (as the above results indicate they will) uncleavable LOF indels in the targeted essential gene. For example, if *ClvR*-bearing individuals carrying LOF indels in the essential gene mate with wild-type, *ClvR*-bearing progeny will be heterozygous for chromosomes that carry the LOF indels and the wild-type version of the essential gene. In the germline of these individuals, the LOF indel-bearing chromosome (which is uncleavable) can serve as a template for HR-dependent repair of cleaved wild-type alleles, converting them to the LOF sequence (Fig. 4). Such behavior in cleavage heterozygotes was recently described in yeast (66). Further implications of homing-dependent alteration of the essential gene locus are discussed below.

***ClvR*^{tko} Spreads to Genotype Fixation in *D. melanogaster*.** Our combined results show that *ClvR*^{tko} results in a very high frequency of germline and maternal carryover-dependent mutational inactivation of the *D. melanogaster tko* locus (>99% per generation); the lethality caused by this loss can be efficiently rescued using the *D. virilis* transgene; the high frequency of *ClvR*^{tko}-dependent mutational inactivation of *D. melanogaster tko* and rescue by *D. virilis tko* is robust to genetic diversity; and cleaved but functional *D. melanogaster tko* alleles resistant to further cleavage, which could limit drive, were not observed. These observations predict that *ClvR*^{tko} will spread to genotype fixation. To test this prediction, we initiated two drive experiments. In one experiment, w^{1118}; *ClvR*^{tko}/+ heterozygous males were mated with w^{1118}; +/+ females, creating a progeny population used to seed the first generation in which *ClvR*^{tko} was present in one-half of the individuals, at a total population allele frequency of 25%. In a second experiment, homozygous w^{1118}; *ClvR*^{tko} males and w^{1118}; +/+ males were premated with equal numbers of w^{1118}; +/+ females, which were then combined and used to seed the first generation (25% *ClvR*-bearing individuals), also resulting in an initial *ClvR*^{tko} allele frequency of 25%. This level of introduction, although substantial, is not unreasonable as it is substantially lower than that used in earlier nontransgenic insect population suppression programs (67). As a control, we carried out similar drive experiments utilizing flies that carry the Rescue-only *tko* construct, *tko*A, and that are wild type at the endogenous *tko* locus (w^{1118}; *tko*A). *tko*A carries the *td-tomato* marker and the Rescue transgene, but lacks gRNAs and Cas9, and is thus expected to show Mendelian transmission. w^{1118}; *tko*A/+ males were mated with w^{1118}; +/+ females (also wild type for *tko*), creating a progeny population used to seed the first generation in which *tko*A was present in one-half of the individuals, at a total population allele frequency of 25%. For the first drive experiment, five replicate population cages were followed for 18 generations (drive 1, Fig. 5*A*). For the second drive experiment, four replicate populations were followed for 16 generations (drive 2, Fig. 5*B*). For the control, four *tko*A populations were followed for 10 generations. In both *ClvR*^{tko} drive experiments *ClvR*^{tko} spread to genotype fixation between six and nine generations for all replicates. In contrast, the control transgene, *tko*A, remained near its introduction frequency in all populations. As expected based on modeling, wild-type (+) alleles at the third chromosome locus into which *ClvR*^{tko} was inserted were still present in the five drive 1 populations (Fig. 5*D* and *SI Appendix*, Table S8), but since

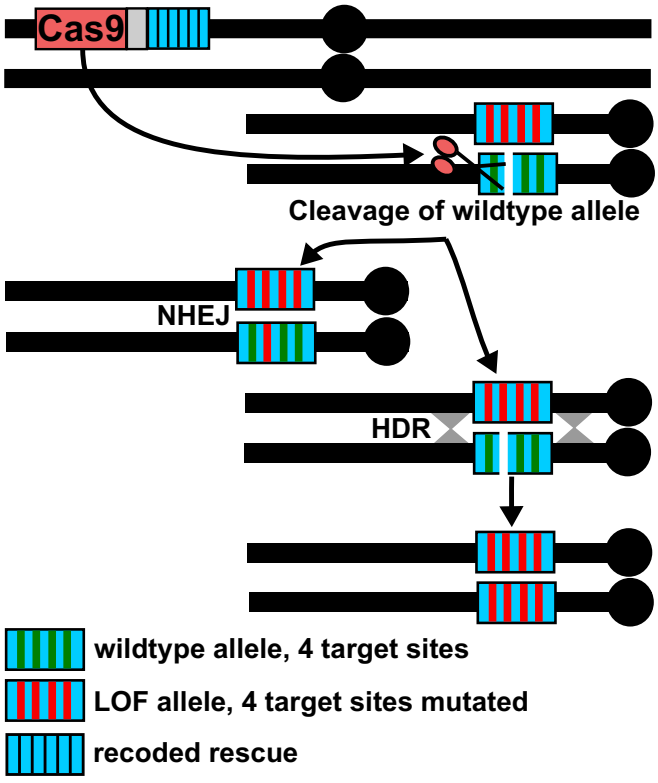

Fig. 4. LOF alleles can be created via cleavage followed by NHEJ, or via cleavage followed by HDR using an existing uncleavable LOF allele as a template for repair. The figure illustrates the germline of a female heterozygous for ClvR, and heterozygous for a LOF allele of the essential gene mutated at all four target sites, and a wild-type allele. Cleavage followed by error-prone repair (NHEJ) results in the creation of a new LOF allele mutated at one target site. Alternatively, cleavage can be followed by repair using the uncleavable LOF allele as a template, thereby resulting in conversion of the wild-type allele into a LOF allele in which all four target sites are mutated.

wild-type alleles of *D. melanogaster tko* are eliminated by *ClvR*^{tko} (*SI Appendix*, Fig. S6 and Table S7), these chromosomes are trapped in *ClvR*^{tko}/+ heterozygotes.

Strategies for Maintaining *ClvR* Functionality over Time. In any gene drive-based strategy for altering the makeup of a population, the cargo and drive mechanism are subject to separation, mutational inactivation, and loss of efficacy. Resilience, an ability to respond to these forces in ways that maintain and/or restore the ability to alter populations over time, is essential. Mutation of cargo genes or loss of effectiveness as a result of evolution of the host, or of other species such as pathogens on which they are meant to act, requires that strategies be available for removing an old element from the population and replacing it with a new one. This can be achieved using an approach analogous to that proposed for synthetic *Medea* selfish genetic elements (6, 68), in which a second-generation *ClvR*, *ClvR*$^{n+1}$, is located at the same site as the first-generation element, *ClvR*^{n}, with *ClvR*$^{n+1}$ targeting essential gene^{n+1}, while also carrying the original rescuing copy of essential gene^{n}. Because progeny carrying *ClvR*^{n} are sensitive to loss of essential gene^{n+1}, only those carrying *ClvR*$^{n+1}$ survive, regardless of their status with respect to *ClvR*^{n} (*SI Appendix*, Fig. S7). Opportunities for physical separation of Cargo from a functional Rescue can also be minimized, as with *Medea* (6), by interleaving Cargo and Rescue transgenes in various ways (*SI Appendix*, Figs. S8–S10).

Cleavage is required for *ClvR* selfish behavior, and can fail as a result of mutation within target sites or Cas9/gRNAs. Mutations within the target sites that create uncleavable, but functional alleles of the target locus (resistant alleles), can lead to loss of *ClvR* from the population if its presence is associated with a fitness cost. Resistant alleles can arise from de novo mutations, from preexisting natural variation in the population, and as a result of error-prone NHEJ or microhomology-mediated end-joining pathways. Error-prone repair is likely to be the most important because the mutation rate per nucleotide/per generation is low, ~10^{-8} to 10^{-9} (69), and high-frequency preexisting mutations that produce target site resistance to cleavage can be avoided through sequencing of the target population. In contrast, NHEJ-mediated creation of resistance alleles following cleavage can occur frequently [>10^{-3} per generation (27, 70)], although use of targets sites that cannot easily mutate to resistance and high fitness may be able to reduce this frequency dramatically (20). Modeling suggests that the probability of completely resistant alleles emerging with a multiplex of gRNAs is approximately equal to that of the probability of resistant alleles emerging at all gRNA target sites simultaneously, that is, p^{n}, where p is the probability of a single site mutating to resistance and n is the number of gRNAs/target sites (71). Thus, even for a high rate of single target site mutation to resistance of 10^{-2} to 10^{-3}, resistant alleles at all target sites might be predicted to arise only infrequently (~10^{-8} to 10^{-12}) with a four-gRNA *ClvR*. However, this calculation assumes no standing variation in the population at any of these sites, that all gRNAs work equally well, and that ectopic gene conversion between the Rescue transgene and the cleaved allele can be completely prevented by recoding.

The results reported herein, using laboratory and global diversity strains (0 resistant alleles out of more than 11,000 progeny scored; Fig. 3 and *SI Appendix*, Table S5), along with other recent work on HEGs (29, 61), provide experimental support for the idea that multiplexing of gRNAs can prevent the creation of cleavage-resistant, but functional alleles. Use of target sites that cannot easily mutate to a cleavage-resistant but high-fitness genotype have also been used toward a similar end (20). Targeting highly conserved housekeeping genes such as *tko* supports both strategies. Nonetheless, given that drive in very large populations has not yet been attempted, we briefly consider a "worst-case" scenario involving resistant alleles, *ClvR*, and a panmictic population, to gain some feeling for the consequences of resistant alleles on *ClvR* lifetime. We suppose that alleles that are completely resistant to four gRNAs, and with high fitness, arise at a high frequency of 10^{-6} per generation, that the presence of *ClvR* results in a significant fitness cost of 20% when homozygous (10% when heterozygous), and that *ClvR* is introduced at a low (10%) or a high (50%) frequency. Under these conditions, *ClvR*-bearing individuals constitute ≥99% of the population for 456 generations when introduced at a frequency of 10%, and 713 generations when introduced at a frequency of 50%. If homing of resistant alleles into cleaved wild-type alleles in heterozygotes carrying *ClvR* is now included (Fig. 6*A*), *ClvR* lifetime at high frequency (≥99% transgene-bearing) is modestly reduced to 409 generations for a 10% introduction frequency (Fig. 6*B*) and 707 generations for a 50% introduction frequency (Fig. 6*C*). The effect of homing is limited because it requires the presence

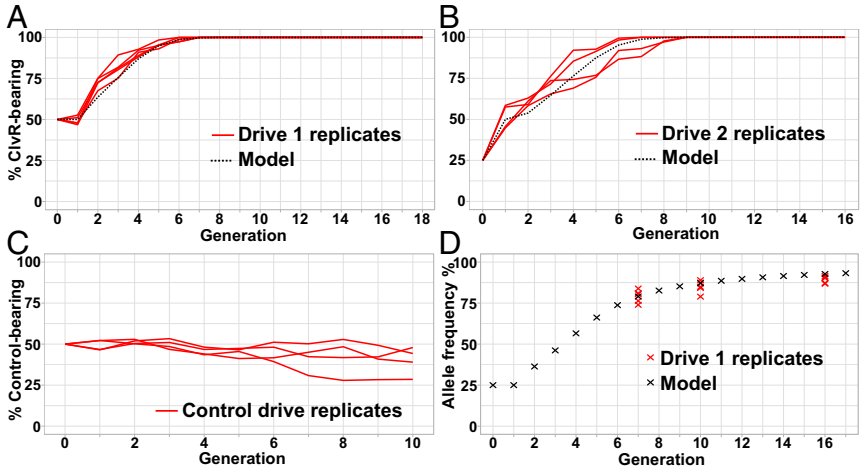

Fig. 5. *ClvR* spreads to genotype fixation in *Drosophila*. The frequency of *ClvR*-bearing individuals (*ClvR*/+ and *ClvR*/*ClvR*) is indicated on the *y* axis and the generation number on the *x* axis. Drive replicates in red; predicted drive behavior in dotted black lines. (*A*) Drive 1: ♂*ClvR*^{tko}/+ XX ♀w^{1118} as generation 0. (*B*) Drive 2: ♂*ClvR*^{tko}/*ClvR*^{tko} XX ♀ w^{1118} and ♂w^{1118} XX ♀w^{1118} at a 1:1 ratio as generation 0. (*C*) Control drive: ♂tkoA/+ XX ♀w^{1118} as generation 0. For the control drive, we used flies carrying construct tkoA (*Methods*) that had only the rescue and the *td-tomato* marker, but no Cas9 and gRNAs. (*D*) Allele frequency of *ClvR*^{tko} in drive 1. Replicates coming from drive 1 in red. Model (black) is the predicted allele frequency inferred from modeling of the drive using parameters estimated from the data in Fig. 3, and assuming no fitness cost to carrying *ClvR* (see *SI Appendix*, Table S8, for counts).

of a specific genotype, a *ClvR*-bearing mother carrying both a wild-type and a resistant allele at the essential gene locus (Fig. 6*A*), and the speed at which *ClvR* elements transform wild-type alleles into LOF alleles works to limit the frequency of such individuals.

With respect to mutational inactivation of Cas9 and gRNAs, *ClvR*-dependent drive of Cargo into a population is predicted to be remarkably insensitive to loss of these components when *ClvR* is introduced area-wide, even when inactive versions of Cas9/gRNAs are present at significant frequencies (5% of the *ClvR*-bearing individuals) in the initial population, and carriers of these mutant versions are more fit than those carrying intact Cas9 (Fig. 6 *D*–*F*). Drive is robust because so long as active *ClvR* elements are present, the population is rapidly driven toward Rescue- and thus Cargo-bearing genotype fixation by the ongoing loss of endogenous wild-type copies of the essential gene. Once all endogenous alleles of the essential gene are rendered nonfunctional, the population is locked into a Rescue—and thus Cargo-bearing—state regardless of whether Cas9 and gRNAs are still active. These points notwithstanding, we note that *ClvR* dynamics in the presence of resistant alleles at the target site or inactive Cas9 are likely to be more complicated in spatially structured populations that also include migration of wild types, a topic that remains to be explored. Strategies for further constraining the ability of Cas9/gRNAs to mutate to inactivity that involve forcing Cas9 and gRNAs to bring about transcription of the rescue as well as cleavage of the essential gene can also be envisioned (Fig. 6*G*). In one such strategy, a Cas9-VPR fusion protein is utilized. Cas9-VPR mediates cleavage at full length target sites. Cas9-VPR can also bind truncated gRNA target sites and drive transcription of a nearby gene, but is unable to cleave these sites (72). In this way, the same gRNAs and Cas9 are used

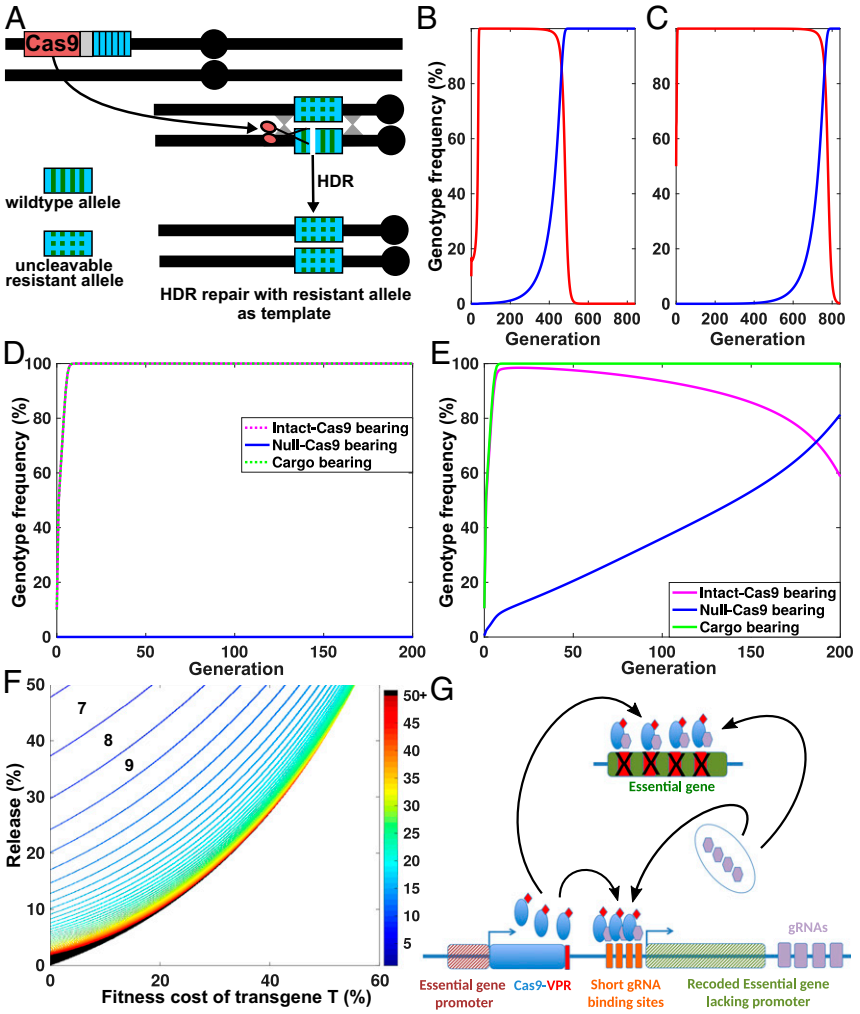

Fig. 6. The consequences of target site/Cas9/gRNA inactivation for the spread of cargo, and ways of selecting against inactivation. (*A*) Illustration of how repair using HR and the resistant allele as a template can result in an increase in frequency of a resistant allele. (*B*) Genotype frequencies of *ClvR* (red line) and a resistant allele (blue) for an element that is introduced at a 10% genotype frequency (release of homozygous *ClvR* males), and that carries a 20% fitness cost, with 100% homing. (*C*) Same as in *B*, but for a 50% introduction frequency. (*D*) Behavior of *ClvR* for an element that is introduced at a 10% genotype frequency (release of homozygous *ClvR* males), and that carries a 10% fitness cost. No inactive versions of *ClvR* (dead Cas9) are present (0% null). (*E*) Same as in *D*, but with versions of *ClvR* that lack active Cas9 introduced, so as to make up 5% of the initial *ClvR*-bearing population. The fitness cost of dead Cas9-bearing *ClvR* elements is assumed to be half that (5%) of the fully functional element. (*F*) Heatmap showing number of generations needed for Cargo to reach transgene-bearing genotype fixation (>99%) for different release percentages and fitness costs, in which versions of *ClvR* that lack active Cas9 are introduced so as to constitute 5% of the *ClvR*-bearing individuals, for each release percentage. Fifty percent of the fitness cost associated with *ClvR* is assumed to be due to Cas9 activity, with the rest being due to Cargo. Thus, the wild-type, non-*ClvR* chromosome always has the highest fitness. Compare with Fig. 2*B*. (*G*) A hypothetical circuit that selects against mutation of Cas9/gRNAs to inactivity in which Cas9 activity is made essential for Rescue function.

for cleavage of the endogenous version of the essential gene and transcription of the Rescue transgene.

Conclusions. Our findings demonstrate that the genetic composition of a population can be rapidly altered using the relatively simple toolkit of components that make up a *ClvR* gene drive/selfish genetic element: a site-specific DNA-modifying enzyme such as Cas9 and the gRNAs that guide it to specific targets, sequences sufficient to drive gene expression in the germline (which need not be germline-specific), an essential gene to act as target, and a recoded version of the essential gene resistant to sequence modification and able to rescue the LOF condition. Highly conserved housekeeping genes such as *tko* that participate in universal cellular processes required for cell survival or maintenance of basic cellular functions are good candidates for use in implementation *ClvR* in diverse species since they are essential in most if not all species (44–46). Importantly, modeling shows that drive and the alteration of populations to transgene-bearing genotype fixation can be achieved regardless of whether the essential gene being targeted is haplosufficient or haploinsufficient. This is likely to be important since haploinsufficiency may be more common than appreciated, and the fitness of individuals heterozygous for a LOF allele, under conditions present in the wild, is rarely known in advance. Finally, in the case where LOF alleles in the essential gene are created as a result of cleavage (as opposed to cleavage-independent base editing), *ClvR* does not require utilization of a specific repair pathway.

An important feature of *ClvR* is that the rate at which it spreads is frequency dependent (Fig. 2), very slow when introduced at low frequency, and fast when introduced at high frequency. In consequence, *ClvR* is likely to be most useful when it can be introduced area-wide, rather than from a point source within a larger area of interest. More detailed modeling that takes into account features such as density dependence, migration, and spatial structure is required to fully understand *ClvR* behavior. There are several other important unknowns. First, it is unclear what the costs and consequences are of long-term expression of DNA sequence-modifying enzymes such as Cas9, and if selection for alleles at other loci that result in decreased expression and/or activity may occur. A related unknown is the extent to which diversity in genome sequence in wild populations at the target site or elsewhere will thwart cleavage at the target locus. Our failure to identify cleavage-resistant, but functional *tko* alleles among >11,000 progeny from crosses of heterozygous *ClvR*-bearing females to wild-type males from a laboratory strain and GDL strains from five continents are promising in this regard, but the level of diversity tested likely pales beside that present in wild populations of some species of interest (73, 74). The problem of sequence diversity is also faced by other drive mechanisms designed to alter populations, such as synthetic *Medea* (6), some versions of underdominance (15, 16), and HEG-based homing (17, 19), which rely on the recognition of specific nucleotide sequences for their mechanism of action. Only further work in genetically diverse populations of species of interest, in facsimiles of wild environments, will suffice to determine whether synthetic selfish genetic elements able to thrive in the wild can be created.

Methods

Target Gene Selection and gRNA Design. We selected the *tko* gene on the X chromosome as the target for the *ClvR* system. It encodes an essential mitochondrial ribosome protein and is recessive lethal and haplosufficient (58). We used the benchling software suite to design gRNAs targeting the exonic regions of the gene at four positions, selected based on on-target activity ranking (75). An additional criteria was that the gRNAs have a mutated PAM in the rescue construct to avoid any potential off-target cleavage therein (see below).

Cloning of *ClvR* Constructs and Fly Germline Transformation. All plasmids used in this work were assembled with standard molecular cloning techniques and Gibson assembly (76). All restriction enzymes, enzymes for Gibson Assembly mastermix, and Q5 polymerase used in PCRs were from NEB; gel extraction kits and JM109 cells for cloning were from Zymo Research. The DNA extraction kit was from Qiagen (DNeasy). The gRNA cassette and Cas9 were based on pCFD3(4)-dU6:3gRNA and pnos-Cas9-nos, which were a gift from Simon Bullock, Division of Cell Biology, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom (77) (Addgene; #49410 and #62208) and modified as described previously (61). Construct A (*SI Appendix*, Fig. S4*A*) was inserted into the fly germline via Cas9-mediated HR. Construct B (*SI Appendix*, Fig. S4*B*) was integrated into an attP landing site within flies carrying construct A using the phiC31 site-specific integration system. Detailed procedures can be found in *SI Appendix*, *Supplementary Methods*. Construct sequence fasta files can be found in Dataset S1.

Fly Crosses and Husbandry of *ClvR*^{tko} Flies. Fly husbandry and crosses were performed under standard conditions at 26 °C. Rainbow Transgenics carried out all of the fly injections. Containment and handling procedures for *ClvR*^{tko} flies were as described previously (61), with G.O. and B.A.H. performing all fly handling. Details are in *SI Appendix*, *Supplementary Methods*.

Data Availability. All data are available in the main text or *SI Appendix*. *ClvR*^{tko} flies are available on request to labs that will meet or exceed containment guidelines outlined in ref. 61.

ACKNOWLEDGMENTS. We thank Marlene Biller and Alexander Sampson for technical assistance, and Jackson Champer and Andrew G. Clark for providing the GDL *Drosophila* strains. Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH Grant P40OD018537) were used in this study. This work was carried out with support to B.A.H. from the US Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) Specialty Crop Initiative, under USDA NIFA Award 2012-51181-20086. G.O. was supported by a research fellowship from the Deutsche Forschungsgemeinschaft (OB428/1-1). T.I. was supported by NIH Training Grant 5T32GM007616-39.

1. Gould F, Huang Y, Legros M, Lloyd AL (2008) A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. *Proc Biol Sci* 275:2823–2829.
2. Noble C, et al. (2016) Daisy-chain gene drives for the alteration of local populations. bioRxiv:10.1101/057307. Preprint, posted June 7, 2016.
3. Burt A, Deredec A (2018) Self-limiting population genetic control with sex-linked genome editors. *Proc R Soc B* 285:20180776.
4. Burt A, Trivers R (2008) *Genes in Conflict: The Biology of Selfish Genetic Elements* (Belknap Press, Cambridge, MA), 1st Ed.
5. Braig HR, Yan G (2001) The spread of genetic constructs in natural insect populations. *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*, eds Letourneau DK, Burrows BE (CRC Press, Boca Raton, FL), pp 251–314.
6. Chen CH, et al. (2007) A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* 316:597–600.
7. Marshall JM, Hay BA (2011) Inverse *Medea* as a novel gene drive system for local population replacement: A theoretical analysis. *J Hered* 102:336–341.
8. Marshall JM, Pittman GW, Buchman AB, Hay BA (2011) *Semele*: A killer-male, rescue-female system for suppression and replacement of insect disease vector populations. *Genetics* 187:535–551.
9. Marshall JM, Hay BA (2012) General principles of single-construct chromosomal gene drive. *Evolution* 66:2150–2166.
10. Gould F, Schliekelman P (2004) Population genetics of autocidal control and strain replacement. *Annu Rev Entomol* 49:193–217.
11. Davis S, Bax N, Grewe P (2001) Engineered underdominance allows efficient and economical introgression of traits into pest populations. *J Theor Biol* 212:83–98.
12. Altrock PM, Traulsen A, Reeves RG, Reed FA (2010) Using underdominance to bistably transform local populations. *J Theor Biol* 267:62–75.
13. Altrock PM, Traulsen A, Reed FA (2011) Stability properties of underdominance in finite subdivided populations. *PLoS Comput Biol* 7:e1002260.
14. Akbari OS, et al. (2013) A synthetic gene drive system for local, reversible modification and suppression of insect populations. *Curr Biol* 23:671–677.
15. Gokhale CS, Reeves RG, Reed FA (2014) Dynamics of a combined *Medea*-underdominant population transformation system. *BMC Evol Biol* 14:98.
16. Reeves RG, Bryk J, Altrock PM, Denton JA, Reed FA (2014) First steps towards underdominant genetic transformation of insect populations. *PLoS One* 9:e97557.
17. Burt A (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proc Biol Sci* 270:921–928.
18. Esvelt KM, Smidler AL, Catteruccia F, Church GM (2014) Concerning RNA-guided gene drives for the alteration of wild populations. *eLife* 3:e03401.
19. Noble C, Olejarz J, Esvelt KM, Church GM, Nowak MA (2017) Evolutionary dynamics of CRISPR gene drives. *Sci Adv* 3:e1601964.
20. Kyrou K, et al. (2018) A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nat Biotechnol* 36:1062–1066.
21. Windbichler N, et al. (2011) A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature* 473:212–215.

22. Chan YS, Huen DS, Glauert R, Whiteway E, Russell S (2013) Optimising homing endonuclease gene drive performance in a semi-refractory species: The *Drosophila melanogaster* experience. *PLoS One* 8:e54130.
23. Chan YS, et al. (2013) The design and in vivo evaluation of engineered I-OnuI-based enzymes for HEG gene drive. *PLoS One* 8:e74254.
24. Thyme SB, et al. (2014) Reprogramming homing endonuclease specificity through computational design and directed evolution. *Nucleic Acids Res* 42:2564–2576.
25. Simoni A, et al. (2014) Development of synthetic selfish elements based on modular nucleases in *Drosophila melanogaster*. *Nucleic Acids Res* 42:7461–7472.
26. Gantz VM, Bier E (2015) Genome editing. The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations. *Science* 348:442–444.
27. Champer J, et al. (2017) 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.
28. Gantz VM, et al. (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proc Natl Acad Sci USA* 112:E6736–E6743.
29. Champer J, et al. (2018) Reducing resistance allele formation in CRISPR gene drive. *Proc Natl Acad Sci USA* 115:5522–5527.
30. Champer J, et al. (2019) Molecular safeguarding of CRISPR gene drive experiments. *eLife* 8:e41439.
31. Yan G, Braig HR (2001) The spread of genetic constructs in natural insect populations. *Genetically Engineered Organisms* (CRC Press, Boca Raton, FL), pp 251–314.
32. Beeman RW, Friesen KS, Denell RE (1992) Maternal-effect selfish genes in flour beetles. *Science* 256:89–92.
33. Lorenzen MD, et al. (2008) The maternal-effect, selfish genetic element *Medea* is associated with a composite Tc1 transposon. *Proc Natl Acad Sci USA* 105:10085–10089.
34. Ben-David E, Burga A, Kruglyak L (2017) A maternal-effect selfish genetic element in *Caenorhabditis elegans*. *Science* 356:1051–1055.
35. Seidel HS, et al. (2011) A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in *C. elegans*. *PLoS Biol* 9:e1001115.
36. Nuckolls NL, et al. (2017) *wtf* genes are prolific dual poison-antidote meiotic drivers. *eLife* 6:e26033.
37. Hu W, et al. (2017) A large gene family in fission yeast encodes spore killers that subvert Mendel's law. *eLife* 6:e26057.
38. Akbari OS, et al. (2014) Novel synthetic *Medea* selfish genetic elements drive population replacement in *Drosophila*; a theoretical exploration of *Medea*-dependent population suppression. *ACS Synth Biol* 3:915–928.
39. Buchman A, Marshall JM, Ostrovski D, Yang T, Akbari OS (2018) Synthetically engineered *Medea* gene drive system in the worldwide crop pest *Drosophila suzukii*. *Proc Natl Acad Sci USA* 115:4725–4730.
40. Mruk I, Kobayashi I (2014) To be or not to be: Regulation of restriction-modification systems and other toxin-antitoxin systems. *Nucleic Acids Res* 42:70–86.
41. Harms A, Brodersen DE, Mitarai N, Gerdes K (2018) Toxins, targets, and triggers: An overview of toxin-antitoxin biology. *Mol Cell* 70:768–784.
42. Hernández-Arriaga AM, Chan WT, Espinosa M, Díaz-Orejas R (2014) Conditional activation of toxin-antitoxin systems: Postsegregational killing and beyond. *Microbiol Spectr,* 2.
43. Eid A, Alshareef S, Mahfouz MM (2018) CRISPR base editors: Genome editing without double-stranded breaks. *Biochem J* 475:1955–1964.
44. Kachroo AH, et al. (2015) Evolution. Systematic humanization of yeast genes reveals conserved functions and genetic modularity. *Science* 348:921–925.
45. Kachroo AH, et al. (2017) Systematic bacterialization of yeast genes identifies a near-universally swappable pathway. *eLife* 6:e25093.
46. Hamza A, et al. (2015) Complementation of yeast genes with human genes as an experimental platform for functional testing of human genetic variants. *Genetics* 201: 1263–1274.
47. Gloor GB, Nassif NA, Johnson-Schlitz DM, Preston CR, Engels WR (1991) Targeted gene replacement in *Drosophila* via P element-induced gap repair. *Science* 253: 1110–1117.
48. Tham KC, Kanaar R, Lebbink JH (2016) Mismatch repair and homeologous recombination. *DNA Repair (Amst)* 38:75–83.
49. Cook RK, et al. (2012) The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. *Genome Biol* 13:R21.
50. Simmons MJ, Crow JF (1977) Mutations affecting fitness in *Drosophila* populations. *Annu Rev Genet* 11:49–78.
51. Lindsley DL, et al. (1972) Segmental aneuploidy and the genetic gross structure of the *Drosophila* genome. *Genetics* 71:157–184.
52. Hägg P, de Pohl JW, Abdulkarim F, Isaksson LA (2004) A host/plasmid system that is not dependent on antibiotics and antibiotic resistance genes for stable plasmid maintenance in *Escherichia coli*. *J Biotechnol* 111:17–30.
53. Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD (1989) Genetically haploid spermatids are phenotypically diploid. *Nature* 337:373–376.
54. Hime GR, Brill JA, Fuller MT (1996) Assembly of ring canals in the male germ line from structural components of the contractile ring. *J Cell Sci* 109:2779–2788.
55. Niedenberger BA, et al. (2018) Dynamic cytoplasmic projections connect mammalian spermatogonia *in vivo*. *Development* 145:dev161323.
56. Beaghton A, et al. (2017) Requirements for driving antipathogen effector genes into populations of disease vectors by homing. *Genetics* 205:1587–1596.
57. Ohnuki S, Ohya Y (2018) High-dimensional single-cell phenotyping reveals extensive haploinsufficiency. *PLoS Biol* 16:e2005130.
58. Royden CS, Pirrotta V, Jan LY (1987) The tko locus, site of a behavioral mutation in *D. melanogaster*, codes for a protein homologous to prokaryotic ribosomal protein S12. *Cell* 51:165–173.
59. Wakiyama M, Matsumoto T, Yokoyama S (2005) *Drosophila* U6 promoter-driven short hairpin RNAs effectively induce RNA interference in Schneider 2 cells. *Biochem Biophys Res Commun* 331:1163–1170.
60. Van Doren M, Williamson AL, Lehmann R (1998) Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr Biol* 8:243–246.
61. Oberhofer G, Ivy T, Hay BA (2018) Behavior of homing endonuclease gene drives targeting genes required for viability or female fertility with multiplexed guide RNAs. *Proc Natl Acad Sci USA* 115:E9343–E9352.
62. Toivonen JM, et al. (2001) Technical knockout, a *Drosophila* model of mitochondrial deafness. *Genetics* 159:241–254.
63. Grenier JK, et al. (2015) Global diversity lines—a five-continent reference panel of sequenced *Drosophila melanogaster* strains. *G3 (Bethesda)* 5:593–603.
64. Lack JB, Lange JD, Tang AD, Corbett-Detig RB, Pool JE (2016) A Thousand Fly Genomes: An expanded *Drosophila* genome nexus. *Mol Biol Evol* 33:3308–3313.
65. Farasat I, Salis HM (2016) A biophysical model of CRISPR/Cas9 activity for rational design of genome editing and gene regulation. *PLoS Comput Biol* 12:e1004724.
66. Gorter de Vries AR, et al. (December 5, 2018) Allele-specific genome editing using CRISPR-Cas9 is associated with loss of heterozygosity in diploid yeast. *Nucleic Acids Res*, 10.1093/nar/gky1216.
67. Dyck VA, Hendrichs J, Robinson AS (2005) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management* (Springer, Dordrecht, The Netherlands).
68. Hay BA, et al. (2010) Engineering the genomes of wild insect populations: Challenges, and opportunities provided by synthetic *Medea* selfish genetic elements. *J Insect Physiol* 56:1402–1413.
69. Haag-Liautard C, et al. (2007) Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature* 445:82–85, and erratum (2008) 453:128.
70. Hammond AM, et al. (2017) The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genet* 13: e1007039.
71. Marshall JM, Buchman A, Sánchez C HM, Akbari OS (2017) Overcoming evolved resistance to population-suppressing homing-based gene drives. *Sci Rep* 7:3776.
72. Kiani S, et al. (2015) Cas9 gRNA engineering for genome editing, activation and repression. *Nat Methods* 12:1051–1054.
73. Dickson LB, et al. (2017) Exon-enriched libraries reveal large genic differences between *Aedes aegypti* from Senegal, West Africa, and populations outside Africa. *G3 (Bethesda)* 7:571–582.
74. *Anopheles gambiae* 1000 Genomes Consortium (2017) Genetic diversity of the African malaria vector *Anopheles gambiae*. *Nature* 552:96–100.
75. Doench JG, et al. (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* 34:184–191.
76. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345.
77. Port F, Chen H-M, Lee T, Bullock SL (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. *Proc Natl Acad Sci USA* 111: E2967–E2976.