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Engineering the Composition and Fate of Wild Populations with Gene Drive

Bruce A. Hay,[1,3] Georg Oberhofer,[1] and Ming Guo[2]

[1]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California 91125, USA; email: haybruce@caltech.edu, oberhofer.georg@outlook.com

[2]Departments of Neurology and Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA; email: mingfly@g.ucla.edu

[3]St. John's College, University of Cambridge, Cambridge CB2 1TP, United Kingdom

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Abstract

Insects play important roles as predators, prey, pollinators, recyclers, hosts, parasitoids, and sources of economically important products. They can also destroy crops; wound animals; and serve as vectors for plant, animal, and human diseases. Gene drive—a process by which genes, gene complexes, or chromosomes encoding specific traits are made to spread through wild populations, even if these traits result in a fitness cost to carriers—provides new opportunities for altering populations to benefit humanity and the environment in ways that are species specific and sustainable. Gene drive can be used to alter the genetic composition of an existing population, referred to as population modification or replacement, or to bring about population suppression or elimination. We describe technologies under consideration, progress that has been made, and remaining technological hurdles, particularly with respect to evolutionary stability and our ability to control the spread and ultimate fate of genes introduced into populations.

1. INTRODUCTION

Gene drive occurs when particular genetic elements—genes, gene complexes, or large chromosomal regions—are transmitted to viable, fertile progeny at rates greater than those of competing allelic variants or other parts of the genome. There has long been interest in the idea that genetic manipulation of wild insect populations via gene drive, a form of genetic population management, could be used for beneficial purposes (for reviews, see 25, 33, 86, 94, 170). Transgenes that bring about gain or loss of function, or alleles of endogenous loci, can be linked with a genetic element conferring drive, and the results of modeling and/or lab experiments show that this link can promote their spread, resulting in population modification (e.g., 3–5, 29, 43, 47, 52, 80, 141, 147). Alternatively, drive can result in population suppression or elimination if the spread of the drive element results in a sex ratio bias (e.g., 32, 76, 89, 118, 156) or a fitness cost to carriers (e.g., 32, 83, 108, 156). Field experiments with engineered gene drive have not yet been carried out. Gene drive as a population management tool is in principle species specific and self-sustaining and takes advantage of the ability of engineered individuals to seek out their wild counterparts in areas that are difficult for humans to access on a regular basis with other control strategies. Successful methods need to have multiple features that revolve around the issues of speed and completeness of spread throughout a population; evolutionary stability; species specificity; spatial control over spread to high frequency; and the ability to supplant or reverse an initial modification in favor of some other genotype, including wild type. Our focus in this review is on those gene drive mechanisms that are self-sustaining. For background on the relevant molecular biology underlying the gene drive technologies discussed, the reader is referred to Reference 2 for CRISPR/Cas9 and related RNA-guided nucleases, Reference 67 for modular expression systems, Reference 77 for DNA repair pathways, Reference 66 for microRNA-mediated gene silencing, and Reference 21 for homing endonucleases. For work on non-self-sustaining methods of population management that utilize transient gene drive, the reader is referred to References 34, 63, 85, 112, 137, 142, and 168.

Genetic population management: molecular tools applied to altering the composition or fate of populations or their ability to share genes with wild relatives

Linked: tightly linked genes rarely segregate from each other during meiosis; unlinked genes segregate into different gametes 50% of the time

Fitness: the reproductive success of specific alleles or genotypes relative to the average success of others in the population

Evolutionary stability: the ability of a gene drive to remain active over time in the face of mutation and genetic diversity

Wild type: alleles and chromosome configurations that give rise to the most common phenotypes in a population; typically nontransgenic and not loss of function

2. GENE DRIVE FOR POPULATION MODIFICATION: GENERAL POINTS

2.1. Threshold, Frequency Dependence, and Invasiveness

There are two general mechanisms by which the engineered gene drive elements discussed below promote their spread. In the first mechanism, they increase their representation in viable gametes (e.g., homing and sperm segregation distortion). In the second, they bring about selection against alternative, wild-type alleles in offspring [e.g., various forms of underdominance, *Medea* (*maternal effect dominant embryonic arrest*), and *Cleave and Rescue* (*ClvR*)]. An important metric for classifying drive mechanisms for population modification (and suppression; see below) is by their level of invasiveness: the ability of the drive element to increase in frequency both in the target area and in surrounding areas linked to the target area by various levels of migration, when introduced at various population frequencies and in the presence of drive element–associated fitness costs (**Figure 1**). Low-threshold (including no threshold) gene drive mechanisms require (in deterministic populations) that only a small fraction of individuals in the population carry the drive element in order for spread to occur locally. Low-threshold mechanisms are considered strong if their rate of increase is frequency independent—i.e., high even when they are present at low frequency and when their presence results in fitness costs to carriers. Synthetic homing endonuclease genes (HEGs) provide key examples (32). Low-threshold drive mechanisms are considered weak if the rate is frequency dependent—i.e., very slow at low frequency, fast at intermediate frequencies, and acquiring a threshold when the presence of the element results in a fitness cost to

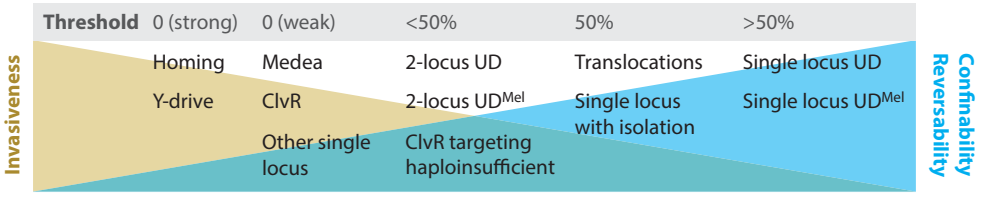


Figure 1

Gene drive thresholds, invasiveness, and relative confinability and reversibility. Invasiveness refers to the overall ability of a drive mechanism to increase in frequency in a target area and surrounding areas connected by migration, when introduced at various frequencies and in the presence of fitness costs. Thresholds are divided into rough categories based on the minimum frequency of transgene-bearing individuals in a population needed to bring about drive in deterministic populations. For those with no threshold, the ability to spread rapidly from low frequency can be strong (frequency independent) or weak (frequency dependent). Confinability and reversibility are, broadly speaking, proportional to drive threshold frequency and inversely proportional to strength at low frequency and invasiveness. The thresholds shown assume elements whose presence results in no other cost to carriers. As costs increase, so do thresholds, except for homing endonuclease genes and Y-drive elements (under most conditions). Discussion of these variables for many drive mechanisms can be found in References 4, 7, 15, 39, 42, 43, 57, 59, 83, 123, 125, 127, 129, 141, and 147.

carriers. *Medea* (20, 165, 166) and *ClvR* (43, 141, 143) are important examples. Both types of low-threshold mechanisms are predicted to be relatively invasive and may be challenging to confine because low levels of migration of drive element–bearing individuals into areas outside a target area in which population modification has already occurred may—depending on the threshold and the migration rate (12, 15–17, 42, 83, 121, 124)—result in these areas being seeded with enough transgene-bearing individuals that drive to high frequency will continue to occur. Low-threshold gene drive mechanisms are attractive when migration rates between the release site and surrounding areas of interest are low and the goal is to spread transgenes over a large area. However, for the same reasons, the low threshold to drive creates challenges to restoring the population to the pretransgenic state, if desired.

High-threshold gene drive mechanisms require, as an intrinsic feature of the genetic mechanisms used to bring about drive, that transgenes make up a much larger fraction of the total insect population before gene drive occurs. Below this frequency, transgenes are actively eliminated from the population. There is no standard nomenclature for what constitutes high threshold (**Figure 1**), although the phrase is usually used in the context of discussions about the ability to confine transgenes to the target population and/or eliminate them if desired. High-threshold drive mechanisms are more confinable than are low-threshold mechanisms in the sense that, once modification has occurred in the target area, spread to high frequency in areas connected to the target area by low levels of migration is less likely (depending on the details of the system and the migration rates) by virtue of the fact that the transgene never reaches the threshold frequency needed for drive (7, 8, 48, 63, 71, 100, 124, 151). Finally, transgenes can, in principle, be eliminated from the population if the release of wild types (or influx through migration) results in the frequency of transgenics being driven below the threshold required for drive, with the caveat that the lower the threshold is for drive into a population, the higher the threshold is for drive out, and vice versa.

Pretransgenic state: a population in which transgenes associated with drive have been removed; not necessarily equivalent to wild population prior to drive

2.2. Gene Drive in Real Populations

Key characteristics of the gene drive mechanisms for modification and suppression discussed in this review, such as threshold, strength, invasiveness, and reversibility, are often described using mathematical models in which populations are very large and well mixed (deterministic

and panmictic), populations are spread across a uniform space, and all individuals participate in reproduction (e.g., 61, 63, 124, 125, 141). This type of model is often used to gain insight into population genetic processes and provides a format that allows comparison of gene drive methods with respect to their basic population genetic features. It provides heuristic guidance that is particularly useful for those working to engineer novel drive mechanisms. However, it is not predictive for any particular species or environment, since it does not include consideration of several other relevant variables, such as stochasticity (finite population size), overlapping generations, the number of reproductively active individuals, and mate choice. Real environments also have substructure and diverse ecologies that change over time, which can lead to shifting population densities and effects on fitness. Population dynamics at borders where transgenics and wild types meet are particularly important, since it is in these environments that drive occurs. Individual dispersal distance, spatial substructure and ecological diversity within and between populations, and density dependence will all influence the fate of a drive element at borders. Our discussion of specific gene drive mechanisms should be understood with these points in mind. For a recent analysis of this literature, the reader is referred to Reference 62.

Introduction threshold: the percent of a population that needs to carry a gene drive element in order for it to spread within that population

3. LOW-THRESHOLD GENE DRIVE FOR POPULATION MODIFICATION

3.1. Toxin–Antidote Drive Elements

Many naturally occurring selfish genetic elements (35), as well as multiple proposed fully synthetic selfish elements (42, 43, 52, 123, 125, 127, 141, 147), can be represented as consisting of a tightly linked pair of genes sitting at a fixed chromosome position, with one gene encoding a toxin and the other encoding an antidote (often referred to as a TA cassette). Expression of these two components in various spatial and temporal patterns results in the death of some or all non-element-bearing gametes or progeny. This leads to a relative increase in the frequency of element-bearing individuals. The *Medea* (5, 28, 52) and *ClvR* (43, 141, 143) selfish genetic elements are paradigmatic of low-threshold versions of this drive strategy in contexts in which there is little cost to individuals that carry the TA cassette.

3.2. *Medea*, the First Synthetic Selfish Genetic Element

Medea elements in nature consist of two tightly linked components. One encodes a maternally expressed gene (the toxin), the product of which (or other consequences of its maternal expression) is inherited by all zygotes. The second encodes a zygotically expressed gene (the antidote), the product of which rescues *Medea*-bearing zygotes from the effects of the maternally provided toxin (5, 19, 20, 22, 52, 117). The combined action of the maternal toxin and zygotic antidote confers a relative advantage to *Medea*-bearing chromosomes by causing the death of offspring of *Medea*-bearing mothers that fail to inherit *Medea* from one or both parents. This behavior is predicted to lead to a relative increase in the population frequency of the *Medea*-bearing chromosome (94, 158, 165, 166). Naturally occurring *Medea* elements in the flour beetle and nematode worm have spread broadly in nature (19, 22, 37), highlighting the potential of engineered *Medea* elements as a method of population engineering, at least when the intrinsic fitness costs to carriers are low.

The *Medea* element in a *Medea*-bearing female does not experience a cost from the killing of non-*Medea*-bearing progeny. Therefore, when the presence of *Medea* results in no other transgene-dependent costs to carriers, *Medea*-dependent killing of non-*Medea* chromosomes results in an increase in the frequency of *Medea*, even when it is introduced into a population at a very low frequency (in other words, it lacks an intrinsic introduction threshold). However, when *Medea*

is rare, the selection against non-*Medea* alleles is very weak (there is very little killing of the non-*Medea*-bearing chromosomes in the population), so that the fate of *Medea* approximates that of a new mutation (121, 124, 165, 166), which is usually lost from the population even if it is beneficial (93) (**Figure 1**). As the frequency of *Medea* increases, there is more killing of non-*Medea*-bearing chromosomes, and *Medea* spreads rapidly to genotype or allele fixation (166). This behavior has two important implications. First, accidental release of a few *Medea*-bearing individuals is unlikely to result in spread through drive (121). Second, whenever the presence of *Medea* or its associated cargo results in a fitness cost to carriers, *Medea* will have an introduction threshold below which it is lost (because the fitness cost to carriers outweighs the frequency-dependent fitness cost of *Medea*-dependent death to noncarriers) and above which it spreads (however, References 158 and 165 document an exceptional case of family-level selection in which *Medea*-dependent killing of noncarrier siblings provides a positive fitness benefit to those carrying *Medea* due to increased resource availability). However, *Medea* should still be seen as a relatively invasive gene drive mechanism because, once established in a target area, low levels of migration for a wide range of fitness costs are sufficient for it to cross the threshold for spread in neighboring populations (12, 124) [e.g., even with a 30% fitness cost and an approximately 1% migration rate per generation, *Medea* is predicted to spread to high frequency (124)]. The dynamics of *Medea* behavior in more complex populations that include space and stochastic effects are only beginning to be explored through modeling (99, 111, 124). Finally, strategies for bringing about population suppression with *Medea* subsequent to population modification have also been proposed (5). These involve use of cargo transgenes that result in an environmental condition-dependent female sterility or lethality (e.g., in response to seasonal diapause, temperature, or an otherwise benign chemical).

Fixation: at genotype fixation, all individuals carry at least one copy of an allele; at allele fixation, all alleles are identical

In the synthetic *Medea* elements created to date, the toxin is a gene that encodes multiple maternally expressed microRNAs (miRNAs). These are designed to bind—at multiple positions—transcripts (mRNAs) of a maternally expressed gene whose product is essential for embryogenesis but not oogenesis. Interactions between an miRNA and an mRNA, which are based on sequence complementarity, promote transcript degradation and/or inhibit translation of the encoded protein (66, 178), thereby creating a condition that could cause the death of all progeny of a *Medea*-bearing mother. The antidote is a recoded version of the same essential gene (unable to interact with the miRNA), expressed just in time in the embryo, thereby rescuing normal development of *Medea*-bearing progeny of *Medea*-bearing mothers, regardless of whether they inherit *Medea* from the mother or the father (**Figure 2*a*,*b***). Three *Medea* elements, $Medea^{myd88}$, $Medea^{dab}$, and $Medea^{o\text{-}fut1}$, each targeting a distinct gene, have been created in *Drosophila melanogaster*, and these spread to genotype fixation in lab cage populations (5, 52). Strategies for bringing about cycles of modification with *Medea* that replace old elements with new ones have also been articulated (52, 95). However, while a *Medea* element has been generated in the closely related fruit pest *Drosophila suzukii* (28), it has not yet been possible, using RNAi or miRNAs, to generate the maternal-effect killing needed to bring about drive in mosquitoes (24). This may be due to saturation of the miRNA loading and effector machinery with endogenous small RNAs, insufficient strength of the maternal promoters used to drive miRNA expression, or differences in the maternal requirements for the genes being targeted in mosquitoes as compared with *Drosophila*. It will be interesting to see if maternally expressed mRNAs essential for embryonic development, or mRNAs expressed and required in the early zygote (through maternal carryover of the toxin), can be targeted using other approaches, such as site-specific RNA cleavage mediated by members of the CAS family of RNA-guided RNA endonucleases (1) or through effects mediated by Pumilio/fem-3 RNA binding domain (PUF) (177) or pentatricopeptide repeat (PPR) (130) family RNA-binding proteins, which have a modular structure and can be engineered to bind specific RNA sequences. Finally, given the generally low threshold for drive by *Medea*, restoration to the

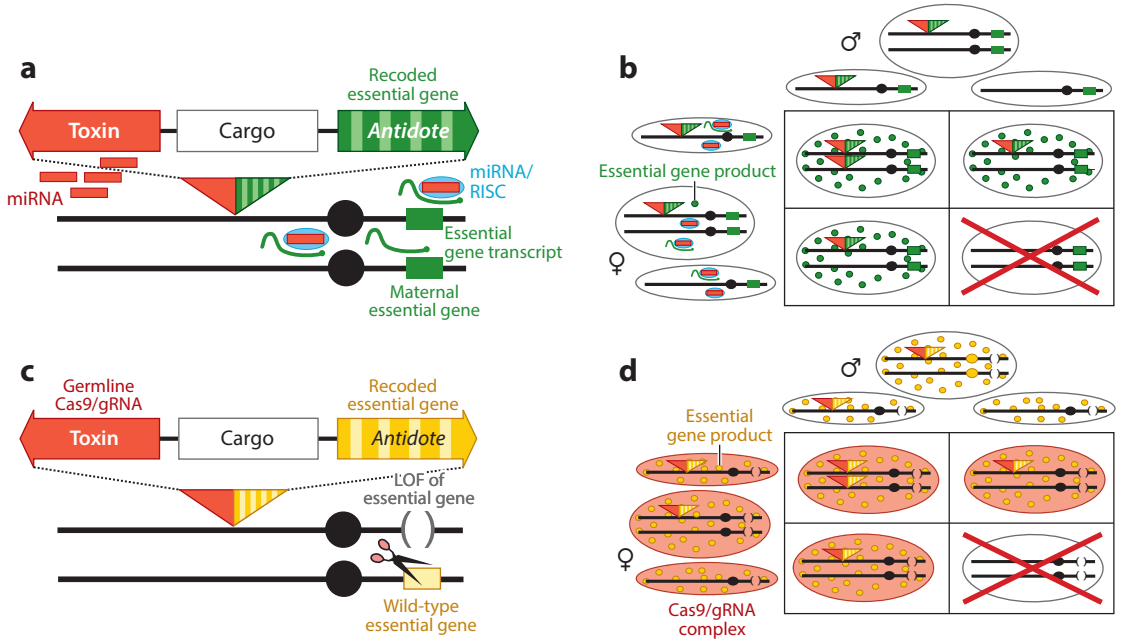


Figure 2

Schematic outlining the molecular basis of *Medea* and *Cleave and Rescue* (*ClvR*) gene drive. (*a*) A synthetic *Medea* element. The toxin is composed of maternally expressed microRNAs (miRNAs). These are loaded into the RISC complex, whereupon they silence expression from a transcript whose product is essential for early embryogenesis (maternal essential gene). The antidote is a version of this same essential gene whose transcript is recoded so as to be resistant to miRNA-dependent silencing and that is expressed in the zygote early enough to restore the essential function lost through maternal silencing (zygotic recoded essential gene). (*b*) Progeny of *Medea*-bearing mothers who fail to inherit *Medea* die because they lack the essential gene product, while those who inherit *Medea* from one or both parents survive because they express it just in time, under the control of an early zygotic promoter. (*c*) A *ClvR* element. Cas9 and guide RNAs (the toxins) are expressed in the male and female germline and in the zygote as a result of maternal carryover. They cleave wild-type copies of a gene needed for viability or some other critical process such as flight or fertility (*yellow rectangle*). Inaccurate repair creates loss-of-function (LOF) mutations (*parentheses*) in the essential gene. The antidote is a recoded version of the essential gene resistant to cleavage, expressed under the control of its own promoter (*darker yellow rectangle with vertical stripes*). (*d*) Individuals who fail to inherit *ClvR* die because they lack a source of the essential gene product, while those inheriting *ClvR* survive because they carry one or two copies of the recoded essential gene. Note that, because *ClvR* now provides the sole source of the essential gene product, populations of *ClvR*-bearing individuals are now dependent on—addicted to—*ClvR* for their survival.

pretransgenic state through dilution with wild types will be challenging unless the fitness cost to carriers is high or rescue with one copy of the antidote is incomplete (166).

3.3. *Cleave and Rescue*

Cas9 family endonucleases have target sites determined by an approximately 20-nucleotide protospacer sequence within an independently expressed guide RNA (gRNA). Because sequence limitations are modest, Cas9 and/or other RNA-guided nucleases can uniquely target most positions in any genome (for a review, see 2). Multiple sites can be targeted for cleavage simply by expressing multiple gRNAs. The double-strand DNA breaks generated by Cas9 or any other nuclease can be repaired through homologous recombination (HR), using the uncleaved sister chromatid, the homologous chromosome, or other homologous sequences located elsewhere as a template. Alternatively, repair can occur through error-prone processes such as non-homologous end joining

(NHEJ), which creates base changes and insertions and deletions (indels) of various sorts (for a review, see 77).

A *ClvR* element (141) [also referred to as toxin antidote recessive embryo (TARE) in a related implementation (43)] has two components. The first is a DNA sequence–modifying enzyme such as Cas9 and a gRNA (or any other enzyme that site-specifically modifies DNA) (e.g., 9, 109, 134). These constitute the toxin or *Cleaver*, which is expressed in the germline and acts in *trans* to disrupt the endogenous version of an essential gene, thereby creating loss-of-function (LOF) alleles. The second is a recoded version of the essential gene resistant to cleavage that acts in *cis* to guarantee the survival of those who carry it (the antidote or *Rescue*). Cas9 and gRNAs create potentially lethal LOF alleles of the essential gene in the germline, wherever the gene is located. The lethal LOF phenotype manifests itself in those who fail to inherit *ClvR* and have no other functional copies of the essential gene. In contrast, those who inherit *ClvR* always survive [providing that a haplosufficient gene (one copy is sufficient for a wild-type phenotype) has been targeted] because they inherit the *Rescue*, a tightly linked, recoded copy of the essential gene. In this way, as with *Medea*, *ClvR* spreads by killing those who lack it. An important difference is that, while Cas9 and gRNAs represent the toxin in this TA system, the toxic activity created, the LOF alleles that actually mediate drive, exist independently of the toxin. Thus, once a *ClvR* element has spread to transgene fixation, all functional wild-type alleles of the essential gene will have been eliminated through cleavage and LOF allele creation, with the only source of essential gene function coming from *ClvR* itself. As a result, every member of the population now requires the presence of *ClvR* (because it carries the *Rescue*) for survival, a state of permanent transgene fixation. The population will remain in this state indefinitely, even when fitness costs are present and the *ClvR* (and its associated cargo) is not at allele fixation. This feature works to maintain population modification over time, even if Cas9 and/or gRNAs are subsequently lost through mutation in many individuals—because the ubiquitous LOF alleles previously created by active Cas9 or gRNAs continue to select against any individuals that lack *ClvR* in LOF homozygotes (141, 142).

The predicted dynamics of *ClvR* are very similar to those of *Medea*, although *ClvR*-dependent drive is a bit stronger. Drive is frequency dependent and lacks a threshold in the absence of fitness cost, but comes with a threshold when the presence of *ClvR* results in a fitness cost to carriers. When drive occurs, transgenes also spread to genotype or allele fixation (42, 43, 141, 143). Given these characteristics, drive of *ClvR* from a source population in which it is fixed into a neighboring population by migration will also have characteristics similar to those of *Medea*—which is to say, low threshold and invasive if associated fitness costs are modest. Interestingly, targeting an essential gene that is haploinsufficient or haplolethal (a fitness cost or death, respectively, occurring when only one functional copy of the gene is available) does not constitute an insurmountable barrier to drive. Higher thresholds for drive are created, but when the threshold is surpassed, spread to transgene and allele fixation still occur (141). The ability to create *ClvR*s with higher intrinsic introduction thresholds, while taking advantage of a common drive mechanism architecture (cleavage of an essential gene coupled with rescue of those who inherit the element), may be useful for creating drive elements with reduced levels of invasiveness and increased potential for confinement and removal through dilution with wild types.

Four independent *ClvR*-type elements have been generated in *Drosophila*. Three of these, located at a common position on the third chromosome, target a haplosufficient essential gene located on either the X (*technical knockout*), 2nd (*dribble*), or 3rd chromosome (*Transcription factor-IIA-S*) with Cas9 and four gRNAs. These are expressed in the male and female germline, in which they cleave and mutate the essential gene to LOF at high frequency. Cas9–gRNA complexes are also carried over from the maternal germline into the zygote, where they cleave and create LOF mutations in paternal copies of the essential gene. These are complete elements, and each also

carries a recoded and cleavage-resistant version of the essential gene being targeted, derived from another *Drosophila* species. All elements spread to transgene fixation in genetically diverse wild-type laboratory populations (141, 143). The fourth (TARE) element is a split element composed of gRNAs, a *Rescue*, and cargo and provides a proof-of-principal demonstration of the same points when tested in populations in which germline-expressed Cas9 is provided from an independent locus (43). *ClvR* has also been shown to drive cycles of population modification using a version of the strategy originally proposed for *Medea* (52), providing the first demonstration that genetic modification of a population with gene drive can be overwritten with new content, an important point of control (143). Finally, two-locus versions of *ClvR*, which provide self-limiting drive, have also been created (142). Together, these results suggest that *ClvR* elements provide a way to modify populations of many species.

Resistant allele: DNA sequence alterations in targets that prevent gene drive activity but do not compromise the fitness of carriers

3.4. Homing

Selfish genetic elements known as HEGs encode a site-specific nuclease and are located at the same site in the genome as their target site (actually within it, thereby disrupting it). When a HEG is present in heterozygotes, the wild-type allele, which contains an intact target site, is subject to cleavage. If this DNA break is repaired through HR using the HEG-bearing chromosome as the template (this process is also known as gene conversion), then the wild-type allele is converted to a HEG allele, thereby bringing about an increase in the HEG copy number (for a review, see 81). In 2003, Austin Burt (32) proposed that HEGs and other sequence-specific designed nucleases (referred to in this review generally as HEGs) could be used in several ways to modify or suppress wild populations. In this section, we discuss population modification.

Population modification using HEGs can, in principle, be achieved in several ways. In the first approach, one or more cargo genes are located internal to the homology arms that mediate HEG copying into the cleaved target site, and therefore, the cargo genes travel with the HEG (**Figure 3*a***). If the homing rate is high, and the fitness cost to carriers is low, then such a HEG is predicted to lack an introduction threshold and have a fast rate of spread even when it is rare. This is because, early during drive, most HEG-bearing individuals are heterozygotes, for which the rate of increase is determined only by the homing frequency, which can approach 1. In the simplest version of this approach, the HEG targets a nonessential sequence, thereby avoiding the decrease in population fitness that would come with targeting an essential gene (32). In a more recently proposed two-locus version (transcomplementing gene drive), Cas9 and gRNAs are split into two different transgenic lines (cargo can be associated with either or both). The gRNAs target wild-type alleles at the Cas9 locus and the gRNA locus. Thus, when the components are separate, neither component displays gene drive activity. However, when combined through a genetic cross, the two sets of transgenes reconstitute the properties of a full HEG, resulting in drive of both elements (116). The challenge with these approaches is that homing into sequences not strongly constrained by selection permits the creation, through NHEJ, of uncleavable but functional alleles (resistant alleles) at the target site (**Figure 3*a***), which block drive (for examples, see 45, 46, 80, 90, 92, 105, 106, 112, 116, 146). One approach seeks to solve this problem by having homing occur, at a population level, into multiple independent neutral positions (72). A second approach involves homing into an essential gene (in which sequences important for function are constrained), with the homology arms bringing in recoded portions of the essential gene needed to restore activity (73, 138). Recent experiments in *Drosophila* (47) and mosquito (3) lab populations suggest that this approach can succeed. Another variant that takes advantage of sequence constraints associated with gene essentiality is known as an integral gene drive (IGD). With an IGD, two components undergo homing. One, encoding Cas9 and gRNAs, homes into (and thus comes under the transcriptional

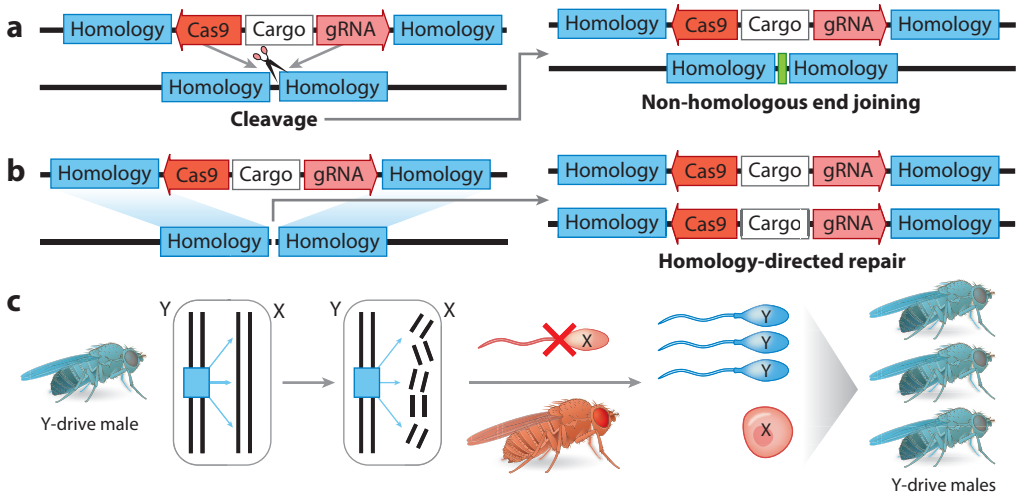


Figure 3

Strong drive with no threshold: homing and Y-drive. (*a*) A homing endonuclease gene (HEG) carrying a cargo gene. Regions of homology to the wild-type chromosome flank the HEG. In heterozygotes, cleavage of the wild-type chromosome occurs. If repair occurs through error-prone non-homologous end joining, then insertions, deletions, or base changes (indels) of various sorts are created (*thin green bar*). (*b*) If repair occurs using the homology arms, then copying of the HEG occurs, resulting in conversion of a germline HEG heterozygote into a germline homozygote. Note that the sequence of the wild-type chromosome labeled "Homology" can include any genomic location that contains one or more target sites. These include (but are not limited to) a neutral locus (e.g., 172), an essential gene (e.g., 47), and a gene required for female but not male fertility (e.g., 108). The outcome of homing—modification or suppression—depends on the nature of the target gene, other components incorporated into the HEG, and sometimes the activity of transgenes located elsewhere in the genome (e.g., 32). (*c*) In Y-drive, a Y chromosome carries a site-specific nuclease (*blue box*) that cleaves the X chromosome at multiple positions during spermatogenesis. If this leads to loss of X-bearing sperm, then Y-drive males give rise only to more Y-bearing males (*blue individuals*) when mated with wild-type females (*pink individuals*).

control of) a germline-expressed essential gene. The second, a cargo–gRNA cassette (using Cas9 from the first cassette), homes into (and is under the transcriptional control of) an essential gene expressed in a somatic tissue relevant to cargo gene function. For both sets of transgenes, copying occurs in such a way that the function of the essential gene is not disrupted (98, 135). Multiplexing of gRNAs can be used to reduce the rate of resistance allele formation (44, 49, 140). However, each of these approaches still requires complete copying of one or more elements to move the cargo. Since cargo genes do not contribute to homing (no endogenous HEGs carry a cargo), it seems likely that partial homing events (in which only some sequences are copied) will result at some frequency, particularly if HEGs contain multiple cargo genes. This would lead to the creation and spread of HEGs that lack a functional cargo, as occurs with transposon mobilization (36, 131). The likelihood that DNA synthesis fidelity is dramatically lower during homing or gene conversion than during normal chromosomal DNA replication only adds insult to injury in this regard (97, 150).

Multiplexing: an increase in copy number and/or sequence diversity of a gene drive component to decrease the rate of failure

In an alternative approach, which seems much more robust, the drive element includes components located at two different loci (32). The first component is a HEG with no cargo, which is designed to insert itself into a highly conserved gene required for viability or fertility, thereby disrupting its function (e.g., 3, 90, 108, 140). The second element, which is unlinked (located elsewhere in the genome), consists of a recoded version of the essential gene, resistant to cleavage and

Natural selection: alleles and genotypes that promote or decrease an organism's survival and reproduction (fitness) spread or are lost through natural selection

able to rescue LOF mutants at the endogenous locus (as with *ClvR* elements). Tightly linked to the rescue transgene are one or more cargo genes. As the HEG drives itself into the population, genotypes that include the recoded version of the essential gene and the cargo are selected for because they restore viability or fertility to homozygotes for the HEG insertion, which would otherwise be dead or sterile. Therefore, they spread as the HEG spreads. A particularly appealing feature of this two-locus configuration is that, because the cargo genes do not need to be copied during homing, they can be arbitrarily large and are replicated with high fidelity, providing for longer functional lifetimes (17).

What is the state of the field? Gene drive–mediated population modification by a HEG that targets an endogenous site was demonstrated in populations of yeast, in the mitochondrial genome, in 1985 (103). Early work in animals showed that a recoded version of this same HEG, when placed in *trans* to an engineered target site located at the same genomic position in the nuclear genome, could bring about significant levels of homing in *Anopheles gambiae* (172). Homing into artificial target sites was subsequently observed in *Drosophila* using HEGs (50, 51, 162), engineered zinc finger nucleases, and transcription activator-like effector nucleases (157). However, engineering challenges associated with targeting endogenous sites, construct stability, and a high frequency of NHEJ events that created resistant alleles initially limited further development.

Given these observations, the ability of Cas9 and gRNAs to create a highly modular nuclease that can be programmed to target essentially any gene at multiple positions immediately suggested them as tools that could bypass the problems noted above (73). Shortly thereafter, germline homing into *yellow* (*y*), a nonessential gene involved in body color determination, was demonstrated in *Drosophila* (78). This was, in turn, rapidly followed by other work in mosquitoes and *Drosophila* reporting germline homing of varying rates using complete elements designed to spread in wild-type populations (3, 80, 90–92, 108, 140, 146), or using split elements in which gRNAs and a dominant marker inserted at the homing locus are introduced into (and therefore can spread only within) a genetic background in which Cas9 (located elsewhere) is expressed in the germline (40, 44–46, 105, 106, 112, 116). Most (45, 113), but not all (91, 156), germline promoters currently in use result in high levels of maternal carryover of active Cas9–gRNA complexes from the maternal germline into the embryo. This can lead to homing into the embryonic germline of animals that lack the Cas9–gRNA transgene (40, 87, 105). Unfortunately, maternal carryover–dependent cleavage of the paternal genome in the zygote and cells of the developing embryo is also often repaired using error-prone NHEJ. This creates resistant alleles in the germline at high frequency (40, 44–46, 80, 90, 105, 106, 112, 116), as well as LOF alleles in somatic cells that can result in fitness costs to heterozygotes (e.g., 90, 108, 140).

Population modification with HEGs requires that several challenges be overcome. First, resistant alleles that retain gene function—naturally occurring or created by NHEJ—must be avoided. For example, their creation blocked drive in two studies in mosquitoes designed to drive a cargo gene conferring disease refractoriness into a population using homing (80, 146; although for recent successes, see 3, 47). The use of promoters that do not result in maternal carryover (91, 156), targeting of highly conserved essential genes (108, 156), and multiplexing of gRNAs and sites targeted (44, 49, 140) provide potential solutions, as does targeting an essential gene with a construct that contains a recoded rescuing fragment (3, 47, 73, 138). In addition, while rates of homing using Cas9-based cleavage are generally very high in *Anopheles* mosquitoes in both the male and female germlines (3, 80, 90, 108), rates in *Drosophila* and *Aedes aegypti* are often lower and more variable (44, 46, 106, 112, 140). The basis for these differences is important to understand, since it undoubtedly has genetic and cell-biological elements (46, 91), which makes it subject to natural selection in ways that could suppress the homing frequency below that needed to overcome fitness costs associated with carrying the HEG. Finally, it remains to be seen what the cargo loss and

mutation rates are in drive experiments in which the cargo resides within the HEG. We think that homing for modification will be most robust when carried out in a context in which the critical components to be driven into the population do not themselves move with the HEG, which then only has one job, to home into and disrupt an essential gene at high frequency.

Outcomes can be regulated in several ways. A small molecule can be used to activate Cas9 (116a), providing a possible point of control during homing. Alteration or reversal of modifications brought about through homing can, in principle, be achieved through a variety of means, although the dynamics can be complicated and result in failure in ways that depend on the system, fitness costs, and migration of wild types (38, 64, 73, 79, 82, 149, 153, 163, 175). However, none of these strategies return the population to the pretransgenic state except by a decrease in frequency, through natural selection, of remaining transgenes that can no longer drive. This process could be relatively rapid or very slow, depending on whether remaining fitness costs are dominant or recessive, respectively, and on their size. Species specificity with HEGs walks a fine line. The necessity of targeting very highly conserved sequences to prevent resistance allele formation, coupled with the ability to spread rapidly from low frequency, may create some opportunities for spread to populations that are only partly reproductively isolated from the target species (144). Spread from a target species into a subspecies may be desirable if members of the latter are also pests, disease vectors, or beneficial insects within which the cargo can perform a comparable (and desirable) function; this spread may not be desirable if members of the subspecies carry out other important functions that might be altered by the drive element, or if drive in them could serve as a bridge to invasion of other non-target species.

4. HIGH-THRESHOLD GENE DRIVE FOR POPULATION MODIFICATION

4.1. Underdominance

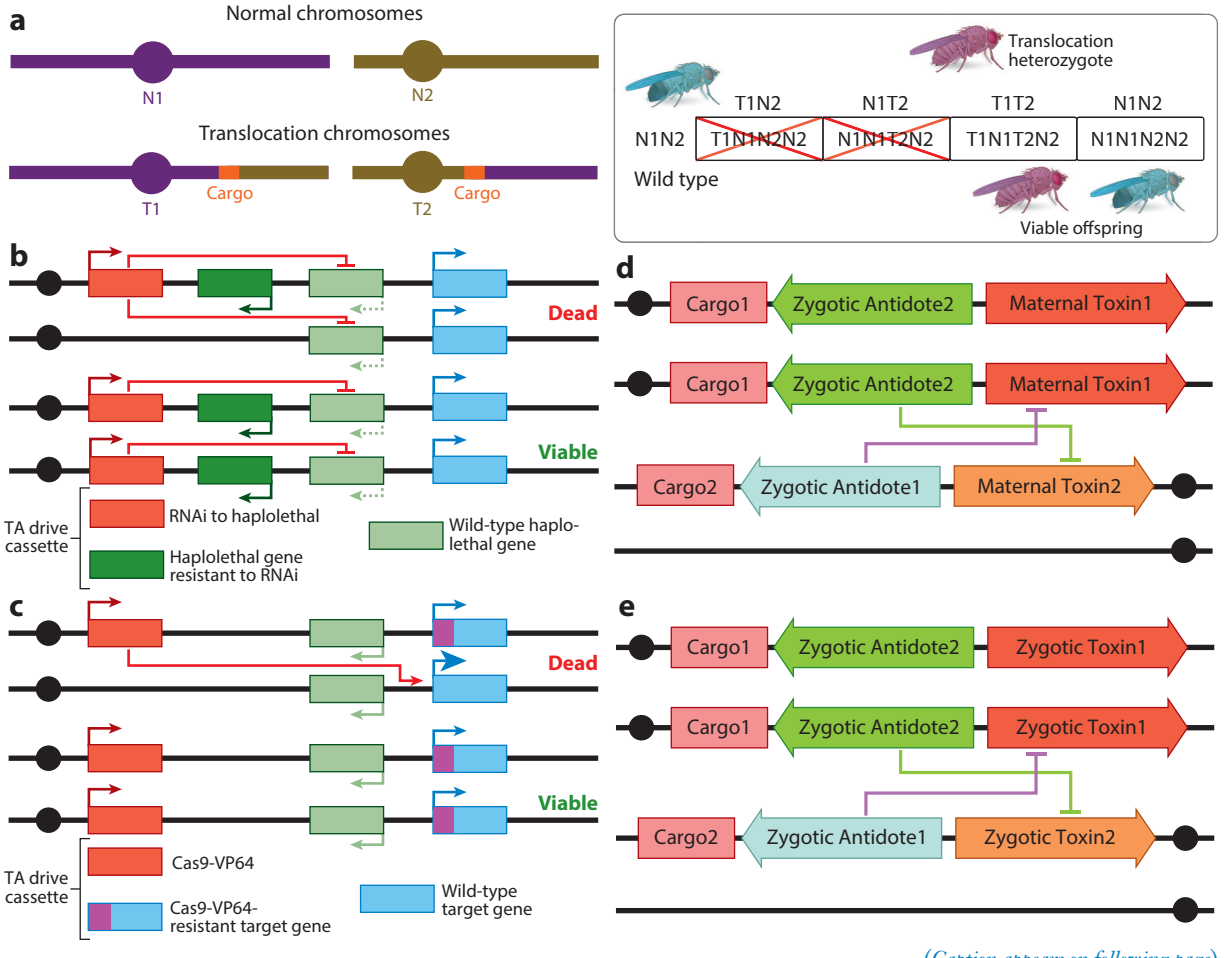
High-threshold gene drive mechanisms all use the phenomenon of underdominance. In its simplest form, underdominance occurs when the fitness of heterozygotes at a specific locus is lower than that of either homozygous genotype. Such a system has no stable internal equilibrium, and selection drives it to one of the two homozygous states. Thus, if one allele or chromosome type carries a transgene, and its frequency is above some critical threshold, then it spreads until the wild-type genotype is eliminated. Conversely, if it falls below the critical threshold, then it is lost in favor of wild type.

4.2. Translocations

Chromosome rearrangements such as inversions, compound chromosomes, and reciprocal translocations show underdominant behavior that can result in drive (for reviews, see 86, 170). Translocations have been a primary focus of research and were the first gene drive vehicle proposed (55). A reciprocal chromosome translocation results in the mutual exchange of DNA between two non-homologous chromosomes. Provided that the translocation breakpoints do not alter the activity of nearby genes, translocation heterozygotes and homozygotes are phenotypically normal. However, translocation heterozygotes are semisterile, producing approximately 50% inviable offspring (**Figure 4*a***). This occurs because meiosis in a translocation heterozygote produces gametes with a full or aneuploid gene complement in roughly equal proportions. Progeny that inherit the aneuploid gene set die unless they inherit a complementary aneuploid set from the other parent. In consequence, if the frequency of the translocation in the population is low, then its elimination is the result. However, if its frequency is high—and the fitness of translocation

homozygotes is also high—then the translocation can spread to allele fixation at the expense of wild type, eliminating the latter from the population. A translocation with no fitness cost has a threshold introduction frequency of 50%. On the basis of these observations, if a gene beneficial to humans or other species could be linked to the translocation breakpoint (**Figure 4*a***), then this behavior of translocations could be used to spread the gene to high frequency (55). Efforts to bring about population modification using translocations created in the lab have generally been unsuccessful (for reviews, see 11, 86, 148), probably due to fitness costs associated with their production by X-rays, which create background mutations and/or breakpoint locations in sequences important for fitness.

Buchman and colleagues (29) avoided these problems by using a site-specific nuclease to create targeted breaks within transgene cassettes at known gene-poor locations on two different chromosomes, followed by homologous recombination between these cassettes. Several translocations generated using this approach spread when introduced into a wild-type laboratory population at high frequency (>50%) but were eliminated when introduced at lower frequencies (29). Related approaches to generating translocations using Cas9 and gRNAs to create defined translocation breakpoints should be possible.



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Underdominance drive methods. (*a*) Translocations. (*Left*) Two wild-type non-homologous chromosomes are shown, along with reciprocal translocation-bearing counterparts. Cargo genes are located at the translocation breakpoints. (*Right*) The genotypes and phenotypes (dead and alive) of a cross between a wild-type individual (N1N2) and a translocation heterozygote (N1T1N2T2) are shown. Translocation heterozygotes produce four gamete genotypes, in roughly equal frequency. Live progeny of both sexes carry balanced sets of the two chromosome types, while dead progeny carry unbalanced sets. (*b*) Single-locus underdominance with reproductive isolation based on RNAi of a haplolethal gene. The toxin in the TA cassette consists of a gene (*red rectangle*) that drives the expression of RNAs that bring about RNAi, which silences (*dotted lines* and *arrow*) the expression of a haplolethal gene (*light green rectangle*). The antidote is a recoded version of the haplolethal gene resistant to RNAi (*dark green rectangle*). Importantly, however, the antidote is only able to rescue viability when present in two copies—in homozygotes. The drive cassette (RNAi to a haplolethal gene and a tightly linked version of the haplolethal resistant to RNAi) is illustrated as being on the same chromosome as the target haplolethal gene, but this is not essential. When individuals carry only one copy of the drive cassette, they die because the reduced expression from the endogenous haplolethal gene (*dotted line* and *arrow*; compare with same gene in panel *c*) is not compensated for by the single copy of the RNAi-resistant version (*top*). In contrast, those who carry two copies of the recoded haplolethal gene (*bottom*) survive. (*c*) Single-locus underdominance with reproductive isolation based on overexpression of an endogenous gene. The toxin in the TA cassette consists of gRNAs and the transcriptional activator Cas9-VP64. Together, these bind to the promoter region of an endogenous gene (*blue rectangle*) and promote lethal levels of gene overexpression (*larger arrow*). The antidote consists of both copies of the endogenous gene, each with promoter mutations (*pink rectangle*) that prevent Cas9-VP64 from binding and driving gene overexpression. Heterozygous individuals carrying one copy of the TA cassette die because overexpression from a single copy of the wild-type target locus is sufficient to cause death (*top*). Those carrying two copies of the recoded version (and thus no copies of the wild-type version) survive. Note that what is essential is not the presence of two copies (the gene targeted for overexpression is haplosufficient), but rather the absence of wild-type copies. Note also that the toxin and antidote (the components of the TA cassette) will often not be tightly linked in this system—Cas9, gRNAs, and cargo are tightly linked, while the target locus is typically located elsewhere. (*d*) Two-locus *UD*^{Mel}*/Double Medea*. Each of two non-homologous chromosomes carries an unmatched maternal toxin–zygotic antidote pair. For the example shown (when the individual is a mother), two different toxins are provided maternally to all offspring. Each antidote acts in the zygote to rescue survival from the cognate maternal toxin. The only surviving progeny of such a mother are those who inherit both chromosome types, from one or both parents. One copy of an antidote is sufficient to rescue survival when mothers carry two copies of the cognate toxin. (*e*) Two-locus zygotic underdominance. Each of two non-homologous chromosomes carries an unmatched zygotic toxin–zygotic antidote pair. Other conditions are as in panel *d*.

The high introduction threshold makes a translocation a confinable gene drive system (124). However, because translocation heterozygotes are viable, they come with the cost that the flow of wild types from neighboring regions can keep the equilibrium frequency of transgene-bearing individuals below 100% in the target area, while allowing some level of transgenics in nontarget areas (29, 124). Modeling also suggests that, in spatially distributed populations, underdominant alleles with high thresholds such as translocations (and others discussed below) are unlikely to spread well from a point source into a larger target area, and the actual outcome will depend on the distribution of wild-type and transgene-bearing population densities moving both inward and outward at borders (13, 14, 48, 62, 151). Together, these observations suggest that translocations and other high-threshold drive systems are likely to be most useful in target areas circumscribed by significant barriers to migration and situations where the target area can be more or less painted in its entirety with transgenics to guarantee spread into all habitats. While the introduction percentages required represent a large fraction of the population, and constitute a large number of individuals if the area to be covered is large, population modification is plausible in some contexts, since the numbers involved are still substantially lower than those used in earlier nontransgenic insect population suppression programs (68).

4.3. Single-Locus Zygotic Underdominance

In the first proposed form of single-locus underdominance, each of a pair of homologous chromosomes carries, at the same position, a toxin and an antidote that rescues death from the toxin on the other homologous chromosome (57). When toxins and antidotes are expressed in the zygote,

the only individuals that survive are those that carry both constructs, since, while they express both toxins, they also express both antidotes. Thus, in this system, only 50% of progeny of a cross between transheterozygotes are viable. This behavior gives rise to a very high introduction threshold in which transgenics must account for approximately 67% of the population. In addition, the system is intrinsically evolutionarily unstable, since mutations in either toxin, which are selected for strongly in each generation, will result in rapid loss of drive. Such a system has yet to be implemented.

Several alternative versions of single-locus underdominance are much more evolutionarily robust. In the first, the toxin is RNAi to a haploinsufficient or haplolethal gene (a gene whose presence in two functional copies is needed for high fitness or survival, often encoding a ribosomal protein). This is tightly linked to an antidote encoding an RNAi-resistant version of the haploinsufficient gene. Heterozygotes for this TA construct are unfit (or dead), since both endogenous copies of the haploinsufficient gene are silenced, and they express only one copy's worth of the RNAi-resistant version. In contrast, homozygotes are fit, since they carry two copies of the RNAi-resistant version (**Figure 4*b***). In consequence, the drive chromosome spreads to allele fixation, at which point selection against mutation of the toxin cassette of the drive chromosome (in the absence of other fitness effects associated with its expression) ceases to occur. Such a drive mechanism has a minimal introduction frequency of 50%. Interestingly, the system can also be thought of as a form of reproductive isolation (speciation). Matings between homozygotes (within a species) give rise to fit progeny, while matings with wild types (between two species) give rise to unfit or dead progeny. This single-locus system has been implemented in *Drosophila*, in a proof-of-principal format, and shows high-threshold gene drive (147).

A different approach to achieving the same goals—reproductive isolation and high-threshold gene drive with a 50% introduction threshold—was first implemented in yeast (129). In this approach, expression of a cleavage-dead version of Cas9 linked to a transcriptional activator is used to drive lethal levels of gene overexpression from a wild-type target locus. Those homozygous for this construct do not die because they carry only recoded versions of the target locus (located elsewhere) in which Cas9 no longer binds, and thus Cas9-dependent overexpression does not happen. However, when matings between these individuals and wild types take place, all progeny die due to lethal overexpression from the wild-type target locus (**Figure 4*c***). Multiple implementations of this system have recently been described in *Drosophila* (128), although achieving success required extensive tweaking of constructs to achieve the desired behavior; Reference 167 provides further discussion of challenges to implementation of this strategy.

Because the above systems (when they result in heterozygote lethality) reproductively isolate the drive population from the wild population—throughout the entire genome—introgression of drive and endogenous alleles between the two populations cannot not occur in the wild. Crosses between homozygotes produce fit progeny, but all heterozygotes die, preventing any possibility of introgression throughout the genome. Thus, if such an engineered strain is introduced into the wild (and it is fit and introduced at a high frequency), then the complete gene and allele composition of the wild population or species would literally end up being swapped for that of the engineered strain. This may have utility if one goal of drive (in addition to carrying a cargo transgene) is to simplify the wild population in terms of its genetic diversity and perhaps also bring about introgression of unlinked loci from the engineered strain that confer desirable traits such as insecticide sensitivity. However, it also seems generally unlikely that engineered strains released into the wild will be as fit as wild type (and thus they may not spread), since they lack alleles from the target population that confer adaptation to that environment. As part of the development of these interesting forms of underdominance, it will be important to develop ways of introgressing the engineered drive alleles into the genetic background of the target population before full

reproductive isolation is brought about. Versions in which heterozygotes have reduced fitness, but are not dead, could achieve this goal. However, systems in which some heterozygotes survive create opportunities for unlinked suppressor mutations to accumulate (because they would allow more heterozygotes to survive), which could slow or prevent drive. For both kinds of single-locus underdominance, modeling has identified conditions under which drive can succeed (7, 8, 48, 57, 107, 110, 147). The important factors are similar to those for translocations, with the exception that the frequency of transgenes in neighboring populations, and wild types in modified populations, are greatly reduced due to the death of heterozygotes (124).

4.4. Two-Locus Zygotic Underdominance

A two-locus version of zygotic underdominance utilizes two independently segregating chromosomes, with each chromosome carrying a toxin and an antidote that rescues death from the toxin on the other non-homologous chromosome (57). When one copy of an antidote is sufficient to rescue death in the presence of two copies of the toxin (as is commonly desired, and illustrated in **Figure 4*e***), this system gives rise to many more surviving genotypes than does the single-locus version. This results in a much lower introduction threshold frequency for an element with no other fitness costs. In consequence, the threshold migration rate needed to drive in neighboring populations is also lower (48, 71, 124). Double homozygotes are viable, and thus, as the system spreads, selection against the toxin is decreased because most remaining genotypes are viable, thereby prolonging functional lifetime in the wild. Two-locus zygotic underdominance has not yet been implemented but is interesting because it combines important aspects of confinability, reversibility (through dilution with wild types), evolutionary stability, and the ability to introgress alleles from the local population and carry out male-only double homozygote releases (because one copy of each construct is sufficient for rescue). Several variants of this system are possible (70), and modeling has begun to explore the conditions under which two-locus underdominance could be useful (48, 63, 70, 71, 99, 100, 107, 120).

4.5. *UD^{Mel}/Double Medea*

One form of purely synthetic single- and two-locus underdominance has been implemented in wild-type *Drosophila*. This system, known as underdominance maternal-effect lethal (*UD^{Mel}*), or *Double Medea*, uses two *Medea* toxin–antidote pairs, with each antidote located on the same chromosome and tightly linked to the toxin for the other *Medea* (4). The *Medea* toxin brings about maternal-effect killing (**Figure 4*d***). Therefore, when only one of the mismatched pairs is present in a female, the only progeny that survive are those who inherit the other mismatched toxin–antidote pair from the father, as this includes the correct antidote. When females are transheterozygous for both constructs, progeny must inherit both constructs to survive. The frequency of both rescuing events is frequency dependent, creating an underdominant situation. *UD^{Mel}/Double Medea* has been implemented in single-locus and two-locus formats and spreads to transgene fixation in laboratory populations of wild-type *Drosophila* (4). In contrast to the other synthetic toxin–antidote combination noted above, in which it is difficult to generate the unfit heterozygotes needed to create fit homozygotes, this is relatively straightforward for *UD^{Mel}/Double Medea*, since the transgenes can be introduced into the male germline, which shows normal Mendelian transmission. In idealized deterministic populations, the single-locus system has a minimal introduction frequency of 78% of the population. For a two-locus system with genes on an autosome and the X chromosome, this drops to 37%, and for a two-locus autosomal system, it drops to an even lower 24%. Single-locus *UD^{mel}/Double Medea* is thus highly confinable, while two-locus versions can be confined for low but not high migration rates. Reversibility can, in

principle, be achieved for each of the above underdominant systems through dilution with wild types (e.g., 4, 29, 147, 151), although this is much easier for single-locus systems. For discussion of other proposed high-threshold gene drive systems for population modification, we refer the reader to References 39, 84, 102, 123, 125, and 127.

5. SELF-SUSTAINING POPULATION SUPPRESSION

Several methods have been suggested for bringing about population suppression using gene drive (5, 32, 42, 86, 126, 143, 154, 169), some of which are high threshold. In this section, we focus on two low-threshold mechanisms for which much progress has been made, introduction of a fitness cost through homing and creation of all-male populations.

5.1. Driving a Fitness Cost into a Population Using Homing

When homing rates are high and an HEG targets a recessive gene required for viability or fertility, population suppression or even population extinction can result. This can happen when heterozygotes are fit and the rate of spread of the HEG outpaces the fitness cost incurred by the creation of HEG homozygotes, thereby driving the population toward the homozygous, unfit state (32). In the case where there is some fitness cost to being heterozygous for the HEG, or homing rates are lower, an internal equilibrium may be reached in which the rate of drive is balanced by the fitness costs to carriers. This can still result in a greatly reduced mean fitness of the population (18, 59, 60, 83). Under the ideal conditions for suppression, homing occurs after the time when the gene is required in the germline (if it is required in the germline), thus maintaining germline fitness in heterozygotes in which homing is occurring; homing occurs in both sexes (maximizing the homing frequency); and the target is a gene required for female fertility or viability (which allows fit homozygous males to continue transmitting the HEG). Other variables can also be important (6, 59, 60, 83).

The first attempts to bring about population suppression involved HEG-based targeting of genes expressed in somatic follicle cells of the mosquito ovary that are essential for eggshell formation. High levels of homing were observed over multiple generations (90), but subsequent analysis of the most promising of these HEGs over more generations uncovered the formation of cleavage-insensitive but functional alleles of the target gene. These increased over time and blocked drive (92). Drive followed by the creation of resistant alleles (albeit with spread resulting in extinction in several cages) was also recently reported for a HEG targeting a different gene, *kynurenine hydroxylase-white*, which is required for pigment formation in the eye and detoxification of the female-specific blood meal in *Anopheles stephensi* (146). Drive and resistant allele formation was also observed in experiments targeting *Drosophila transformer*, required for female sex determination (106). Mutiplexing of gRNAs provides one strategy to reduce the frequency of resistant alleles (44, 49, 73, 122, 140). Targeting sequences that cannot easily mutate to a cleavage-resistant but functional sequence provides another.

Kyrou and colleagues (108) took this latter approach, focusing on a highly conserved sequence that they identified within an intron of the gene *doublesex* (*dsx*) in the mosquito *A. gambiae*. Male and female proteins resulting from sex-specific splicing of *dsx* are required for sex determination, probably in all insects (164), and loss of the female-specific exon adjoining this conserved intron results in the conversion of female *A. gambiae* mosquitoes to a sterile intersex phenotype (108). A HEG designed to target the conserved intron sequence (disrupting female- but not male-specific splicing) resulted in a high frequency of homing, thereby driving the population toward a state dominated by homozygous genetic females that are intersex and sterile and males that are homozygous transgenic and fertile. Importantly, while NHEJ occurred and created uncleavable

alleles, these alleles did not increase in frequency during the drive experiment, indicating that they were not under positive selection and are likely just LOF alleles for the female *dsx* isoform. This HEG continued to spread to fixation, ultimately resulting in the extinction of two laboratory cage populations (108). In other recent work in *A. gambiae*, homing occurred into the same position in *dsx* of a cargo consisting of a site-specific nuclease designed to shred the X chromosome during spermatogenesis (see below), resulting in a strong bias in HEG-bearing males toward Y-bearing mature sperm. The combined action of these two activities is predicted to bring about stronger and more evolutionarily robust suppression than targeting female fertility alone. This element also brought about rapid population elimination, starting from a satisfyingly low 2.5% allele introduction frequency, and resistant alleles were not observed (156). These remarkable results show that population elimination can be achieved with a synthetic HEG in a major vector of human disease in a way that is robust to (at least some) sequence variation.

These positive results notwithstanding, genetic diversity and spatial structure will still provide challenges to HEG-based population suppression. First, extragenic suppressors (sequence variants at other loci) that decrease homing rates may exist. These, coupled with maternal-effect killing (or other fitness costs) in heterozygotes due to the use of germline promoters that have some level of carryover of active Cas9–gRNA complexes into the zygote (thereby creating somatic LOF mutations that compromise heterozygote fitness), can result in the creation of a HEG internal equilibrium frequency that maintains the population at some level (18, 59, 60). Second, inbreeding is favored when a drive with a fitness cost is introduced into a population (30), and it can work to remove an element that carries a fitness cost (30, 31, 65, 176). Finally, spatial structure (particularly when combined with inbreeding) can also hinder suppression, creating local areas in which extinction and loss of the HEG is followed by repopulation with wild types (18, 31, 41, 139). The significance of these variables will depend on the species, environment, and degree of inbreeding depression. That said, partial solutions involve tuning Cas9 such that its expression is limited to the germline (e.g., 91, 156), seeding the target area with more transgene-bearing individuals in a uniform distribution (as compared with a point source), and continuing this activity as the HEG spreads, so as to ensure seeding of all vector habitats and bring about suppression before selection for suppressor mutations has a chance to take hold. Deliberate introduction of resistant alleles (32) or other transgenes that disrupt Cas9 function (149, 153, 171) (thereby reducing the rate of homing) can be used to terminate a population-suppression program, although these do not necessarily return the population to the pretransgenic state, except through natural selection, which could be quite slow for a recessive locus.

5.2. Spatially Constrained Homing-Based Modification and Suppression

Homing can also be combined with other techniques to bring about local population modification or suppression. In the tethered homing approach, a higher-threshold gene drive method such as single- or two-locus underdominance, translocations, or *ClvR* (in cases where *ClvR* has a threshold due to fitness costs) is used to drive a source of Cas9 (and any other payload desired) into a local population. In a second step, or in parallel if they are introduced at low frequency, gRNAs located within a target gene (along with a cargo if desired) are introduced. Homing, using the now-common source of Cas9, drives the gRNA-bearing construct into the population, bringing about modification and/or suppression if the target is an essential gene (61). This approach seems particularly powerful when the goal is to bring about localized population suppression, since strains carrying multiple independent gRNA cassettes (each located within an essential gene and carrying nothing else) could be deployed simultaneously, in a single strain, into populations fixed for Cas9. This creates redundancy in terms of suppression, while only permitting suppression in Cas9-bearing individuals or their progeny. Tethered homing also has the important feature

that it decouples the fitness cost of the cargo from that of the component undergoing threshold-dependent drive. This makes it possible for tethered homing to drive in (in a localized manner) cargo with much larger fitness costs than is possible with other high-threshold drive mechanisms, in which the cargo and drive element are located together and thus share the costs of drive into the population.

In a somewhat related idea, which may be particularly useful in testing scenarios and on islands or in other isolated regions, homing of a complete HEG is made to occur into alleles that are fixed within the local population (locally fixed alleles, or private alleles) but that are polymorphic in other locations in which the insect is present (159). Such alleles may be naturally occurring, they could be engineered into essential genes through homologous recombination and the insects introduced into an otherwise empty habitat (e.g., for testing purposes, an island that could support mosquitoes but that does not currently do so), or they could be driven into an existing population using drive mechanisms such as *ClvR* that spread a novel essential gene sequence while eliminating wild-type copies but that are also very weak drivers when rare. The essential point is that HEG spread can occur locally into these private alleles, but if rare HEG-bearing individuals escape and mate with wild counterparts on a mainland, then gene drive by single- or two-locus underdominance, translocations, or *ClvR*-type systems will be insignificant, and the HEG will immediately encounter polymorphisms in target sites that prevent its further spread to high frequency.

5.3. Creation of All-Male Populations

The number of females and their fecundity are the primary determinants of population size. Thus, strategies that use gene drive to skew the sex ratio toward males have the potential to bring about population suppression or elimination. In this section, we consider two strategies.

5.3.1. Homing of a male-determining locus. Sex determination in insects occurs through many different mechanisms. Male fertility, as distinct from phenotypic maleness, often requires the activity of multiple genes present on a Y-like chromosome. However, there are several important examples in which phenotypic maleness and male fertility are conferred by the presence of a single dominant allele of a male-determining locus. Medflies provide one example (133). Houseflies may provide another (155). In *A. aegypti*, the gene *Nix* is sufficient for maleness (88) and (assisted) male fertility (10), with the nearby gene *myo-sex* probably providing the missing factor needed for females transformed by *Nix* into males to be able to fly (and thus mate unassisted) (10). If high-frequency homing of a male-determining locus (along with any other needed loci, such as *myo-sex*) could be achieved in these species, then this could lead to population suppression. This represents a form of population modification, in which the trait being driven into the population is maleness. As such, success with this strategy is subject to the same considerations as those discussed above for homing of a cargo. An alternative, recently described strategy (132) involves linking a male determining locus with an autosomal X shredder (discussed below).

5.3.2. Y-drive. In species with heterogametic sex chromosomes (X and Y), there are many natural examples of what is known as male meiotic drive or segregation distortion, in which genetic elements located on one or the other sex chromosome result in a bias toward the production of X or Y gametes, and thus female or male progeny, respectively (for reviews, see 96, 114). The ability to coopt a naturally occurring segregation distorter system, or create a fully synthetic system, that induces a strong male bias (known as Y-drive) has long provoked interest as a potential way of bringing about population suppression or elimination through the creation of males that only

give rise to male progeny (54, 89, 118). Modeling has identified conditions under which population suppression and disease prevention using Y-drive might be expected to occur (15, 16, 41, 69).

The full molecular basis for naturally occurring sperm segregation distortion is not known in any system. However, observations of male-biased segregation distorters in several mosquito species indicated that drive is associated with breakage of the X chromosome during meiosis (136, 160). While it is unclear if breakage is a causal event, the possibility that it might be was instrumental in formulating the idea that Y-drive could result if a site-specific nuclease targeting one or more sequences on the X chromosome, and expressed at an appropriate stage during spermatogenesis, was located on the Y chromosome (32).

Recent work supports this hypothesis. Fortuitously, the I-PpoI homing endonuclease, derived from the slime mold *Physarum polycephalum*, cuts a conserved sequence within ribosomal rDNA repeats, which are located exclusively on the *A. gambiae* X chromosome (173). Expression of I-PpoI during spermatogenesis in *Anopheles* results in cleavage of the paternal X chromosome, and most embryos fertilized by these males are chromosomally male, indicating that cleavage is somehow resulting in the loss of functional X-bearing sperm. However, due to paternal carryover of I-PpoI into the zygote, the maternal X chromosome is also cleaved. As a result, all progeny die, presumably due to chromosome aneuploidy and/or insufficient rDNA expression (174). Importantly, destabilization of I-PpoI in the male germline largely eliminates cleavage in the zygote, resulting in males that give rise to >95% viable male progeny (75). This chromosome does not drive, since the construct is located on an autosome and therefore finds itself in females half the time, but these males are able to bring about population suppression when introduced repeatedly into population cages [and, as noted above, when carried into a population at super-Mendelian frequencies in association with a HEG (156)]. These results indicate that, if a similar transgene was located on the Y chromosome and expressed during the appropriate time during spermatogenesis, then Y-drive should occur.

Transgene insertions on the largely heterochromatic *Anopheles* and *Drosophila* Y chromosome that drive expression in somatic tissues have been identified (23, 27). The problem that remains to be overcome is to bring about male meiotic germline expression from such a transgene. This may not be a trivial task. Species with heterogametic sex chromosomes usually engage in a process known as meiotic sex chromosome inactivation, in which most or all transcription is suppressed on sex chromosomes (for a review, see 56). In part, this may be an evolutionary response designed to shut down sex-linked segregation distorters, which, if left unopposed, decrease population fitness (89). It is unclear how to avoid this process. Transcriptional profiling in *Anopheles* has identified rare sex chromosome–linked genes that may escape silencing (161). It is possible that regulatory elements from these genes could be used to support nuclease expression at the right time and place. Alternatively, it may be possible to take advantage of the fact that specific steps of spermatogenesis often utilize translational control elements that suppress translation of expressed transcripts until a later stage (26, 152), as a way to bring about nuclease expression at the appropriate time. Finally, it will be interesting to see if coupling of an X shredder with a male-determining locus can be used to avoid the problem of meiotic silencing (132).

Many species of interest with XY sex chromosomes do not carry rDNA repeats exclusively on the X chromosome, and therefore cannot be targeted using a single site-specific nuclease such as I-PpoI. However, the X chromosomes of these species should contain many other unique or repeated sequences that are not shared with other chromosomes. Cas9 and gRNAs can target these for cleavage. The feasibility of such an approach is indicated by the fact that the Y-based segregation distortion seen in *Anopheles* with autosomal I-PpoI transgenes can be recapitulated using Cas9 and a gRNA targeted to the rDNA repeats cleaved by I-PpoI (76). With this observation in hand, strategies have been devised to identify X chromosome–specific sequences, particularly

repetitive ones, in other species (145). Recent evidence indicates that male-biased drive of autosomal elements targeting some of these sequences with Cas9 and gRNAs in *Drosophila* (74) and the medfly *Ceratitis capitata* (132) does result in some level of sex ratio distortion, although the level of distortion is generally less extreme than in *Anopheles*. The lower rates of sex ratio distortion observed in these insects provide a cautionary note, as they highlight the fact that segregation distortion following cleavage occurs in a biological context about which much remains unknown.

Y-drive is expected to work best when sperm is not limiting and females mate only once. In cases in which the goal is population suppression, there will be strong selection for suppressor mutations that restore the sex ratio to 1:1 (16, 89, 101, 119), as with endogenous segregation distorters (53, 104, 114). Suppressor mutations can arise in multiple ways (e.g., 16, 119), and modeling has begun to explore their effects (16). In the specific context of a site-specific nuclease, suppressors can occur as mutations of target sites that prevent cleavage while leaving target gene functions intact. They can also arise through the action of polymorphisms at other loci that prevent Cas9 and gRNA expression or cleavage. Finally, suppressor mutations may prevent the needed consequence of cleavage, the resorption of X-bearing sperm. If X-bearing sperm are functionally dead (unable to create viable progeny) but are still present and compete for fertilization with an equal number of Y-bearing sperm, then drive is suppressed, since Y-drive males then give rise to only as many male offspring as wild-type males (as with the autosomally located X-cleaving constructs).

For this last kind of suppressor, the issue is that the loss of X-bearing sperm following cleavage is in no way inevitable. The presence of DNA in sperm is not even required for successful fertilization in *Drosophila* (115), and chromosome breakage associated with X-irradiation of males for sterile male release programs does not prevent fertilization (68). It seems likely that the resorption of sperm carrying cleaved (damaged) DNA is the result of some form of endogenous quality control, tasked with eliminating defective sperm that could compete with functional sperm for fertilization. This process is undoubtedly under genetic control and thus subject to mutational inactivation. The critical empirical questions are whether loss of components in pathways that mediate removal of X-bearing sperm results in a significant cost to carriers and if populations are already polymorphic for suppressor alleles (16). These are questions that can be addressed in tests of current autosomal drive elements in genetically diverse populations of *Anopheles*.

SUMMARY POINTS

1. Population modification can be achieved in *Drosophila* using low- and high-threshold gene drive approaches: *Medea*, single-locus underdominance, *UD^{Mel}/Double Medea*, translocations, and *ClvR*. Versions of *ClvR*, which have been implemented in multiple formats, have many desirable features and should be easily portable to diverse species.
2. Homing-based methods for population modification in which the cargo moves with the HEG are under active development, and recent results are encouraging. Strategies in which the cargo does not home are most promising in terms of evolutionary stability but remain to be implemented.
3. Population elimination through homing in *Anopheles* mosquitoes can be achieved by targeting highly conserved sequences in the *dsx* gene that are required for female sex determination alone or in combination with an autosomal X-shredder. Similar approaches may be possible in other insect species.

4. Male-determining loci have been identified in some species and transgenes that bring about X shredding during spermatogenesis to create a Y chromosome bias in functional sperm work when placed on autosomes. It remains to be seen if similar transgenes can be expressed from the Y chromosome at the appropriate time so as to create males that only generate male progeny.

FUTURE ISSUES

1. Mechanisms by which the diverse genetics found in wild populations will work to silence drive mechanisms or subvert their function remain to be explored.
2. Modeling is beginning to provide hints as to which drive methods will work best in specific environments. However, these remain to be tested in facsimiles of real environments with genetically diverse populations.
3. When considering the regulatory path for use of a first-generation drive element, will next-generation elements with the ability to remove or modify first-generation elements need to already be in place and approved? Can an infinite regress of regulatory approvals be avoided, perhaps through the use of high-threshold methods that are reversible to the pretransgenic state through dilution with wild types and/or an insect-free state through the removal (on a small uninhabited island) of an essential food source?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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