Chapter 9

Gene Drive Strategies for Population Replacement

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INTRODUCTION

After 3.8 billion years of research and development, Nature has provided inspiration for a plethora of human design problems. During the Renaissance, Leonardo da Vinci designed a flying machine inspired by the anatomy of birds. Today, Nature's evolutionary solutions are informing the design of solar panels from photosynthesis, and digital displays using the light-refracting properties of butterfly wings. Nature's intricate structures and processes may also help in the fight against mosquito-borne diseases. Gene drive—the process whereby natural mechanisms for spreading genes into populations are used to drive desirable genes into populations (e.g., genes conferring refractoriness to malaria or dengue fever in mosquitoes)— is another example of Nature's processes being applied for the benefit of humanity. Gene drive systems may either spread from low initial frequencies or display threshold properties such that they are likely to spread if released above a certain frequency in the population and are otherwise likely to be eliminated.

Population replacement, in this context, refers to the process whereby a population of disease-transmitting mosquitoes is replaced with a population of disease-refractory ones. Several approaches are being explored to engineer mosquitoes unable to transmit human diseases, and there have been a number of notable successes. For example, Isaacs et al. have engineered *Anopheles stephensi* mosquitoes expressing single-chain antibodies that prevent *Plasmodium falciparum* malaria parasites from developing in the mosquito, thus preventing onward transmission of the parasite [1]. Gene drive systems are expected to be instrumental in spreading disease-refractory genes into wild mosquito populations, given the wide geographical areas that these species inhabit and the expectation that refractory genes will be associated with

at least modest fitness costs [2]. Gene drive systems are also being considered to implement population suppression strategies whereby genes conferring a fitness load or gender bias are instead driven into the vector population, thereby reducing disease transmission.

Early Inspiration

Initial suggestions for spreading desirable genes into insect pest populations date back to the early 1940s and involved the proposition of translocations [3,4] and transposable elements (TEs) [5], inspired from natural systems. Translocations are rearrangements of parts between nonhomologous chromosomes. If insects homozygous for a translocation are introduced into a population at high frequency, they are predicted to spread to fixation [6], and if the translocation is linked to a disease-refractory gene, it is predicted to consequently be driven into the population as well. Initial field trials with translocations were unsuccessful in demonstrating spread [7]; but this is likely a result of those translocations being generated using X-rays, which often induce high fitness costs.

The suggestion of using TEs to drive disease-refractory genes into mosquito populations was largely inspired by the observation that a TE known as the *P* element spread through most of the global *Drosophila melanogaster* population within the span of a few decades following natural acquisition from *Drosophila willistoni* [8]. TEs are able to spread through a population due to mechanisms that enable them to increase their copy number within a host genome and hence to be inherited more frequently in subsequent generations. As a result, they are able to spread into a population from very low initial frequencies even if they incur a fitness cost to their host [9]. It was hoped that the *P* element invasion of *Drosophila* could be repeated in disease-transmitting mosquito species using a TE attached to a disease-refractory gene; however, early laboratory work on TEs in mosquito vector species has failed to identify elements with high remobilization rates following integration into mosquito lines [10].

Promising New Systems

Two of the most promising gene drive systems at present also involve technologies inspired by Nature—the use of homing endonuclease genes (HEGs) observed to spread in fungi, plants, and bacteria [11], and a selfish genetic element known as *Medea* observed to spread in *Tribolium* beetles [12,13]. A synthetic *Medea* element has been developed in *Drosophila* that works by the hypothesis that *Medea* encodes both a maternally expressed toxin and a zygotically expressed antidote [14]. This combination results in the death of wild-type offspring of *Medea*-bearing mothers, thus favoring the *Medea* allele in subsequent generations and mimicking the behavior of the natural element

in *Tribolium*. *Medea* was the first synthetic gene drive system to be developed and has a number of desirable design features; however, significant work is still ongoing to develop a *Medea* element in a mosquito disease vector.

   Recently, there has been much excitement around HEGs as, while *Medea* was first engineered in *Drosophila*, a naturally occurring HEG has been shown to spread in a laboratory population of *Anopheles gambiae*, the main African malaria vector, containing an engineered target sequence for the HEG [15]. HEGs spread by expressing an endonuclease that creates a double-stranded break at specific target sequences lacking the HEG. Homologous DNA repair then copies the HEG to the cut chromosome, increasing its representation in subsequent generations. Similar to the aforementioned gene drive systems, HEGs are being considered to drive disease-refractory genes into mosquito populations; however, a number of additional strategies for their application are also being considered, which aim to suppress rather than replace mosquito populations [11], and progress has been made toward these ends as well [16].

Design Criteria

As the technology for developing gene drive systems for population replacement develops on a number of fronts, it is useful to consider design criteria for assessing the safety and efficacy of the various approaches. An excellent review by Braig and Yan [17] proposes several biological properties that an ideal gene drive system should or must have:

1. The gene drive system must be effective. That is, it must be strong enough to compensate for any loss in host fitness due to the presence of both itself and its transgenic load (manifest as a reduction in host fertility, life span, or competitiveness). It must be able to spread to very high frequency in a population on a timescale relevant to disease control (i.e., a few years) and must be unimpeded by wild-type vectors immigrating into the target area.

2. The gene drive system must be able to carry with it several large genes and associated regulatory elements. At the very least, a disease-refractory and marker gene will be needed along with regulatory elements; but multiple disease-refractory genes are preferable in order to slow the rate at which the pathogen evolves resistance to each of them.

3. Features should be included to minimize the rate at which linkage is lost between the drive system and disease-refractory genes, as even rare recombination events could be significant for wide-scale spread over a long time period.

4. It should be possible to use the gene drive system to introduce waves of refractory genes over time to counteract the effects of evolution

of pathogen resistance, mutational inactivation of the refractory gene, or loss of linkage between the refractory gene and drive system.

5. The gene drive system should be easily adapted to multiple vector species. Human malaria, for instance, is transmitted by approximately 50 species of mosquitoes belonging to the genus *Anopheles*. In sub-Saharan Africa, the most important transmitters are *An. gambiae*, *Anopheles coluzzii*, *Anopheles arabiensis*, and *Anopheles funestus*, ideally all of which should be rendered refractory in a population replacement strategy.

   Additional features of an ideal gene drive system were proposed by James to address ecological, epidemiological, and social issues, including safety [2]. Safety is a broad criterion that should be assessed through risk assessment in which potential hazards are identified along with their corresponding magnitudes and likelihoods. This provides a framework for managing the most significant risks and for the overall safety of the system to be scored. However, prior to a comprehensive risk assessment, a few general safety criteria for gene drive systems can be imagined.

6. The behavior of the gene drive system in the target species should be stable and predictable, thus minimizing the likelihood of unpredictable side effects in target species.

7. A mechanism should be available to prevent horizontal transfer of the gene drive system and/or refractory gene to nontarget species, thus minimizing the wider ecological impact of the release.

8. The gene drive system and refractory gene should not cause undesirable effects for human health, for instance, by selecting for increased virulence in the pathogen population. The gene drive system should also include a mechanism for removing the refractory gene from the population in the event of any adverse effect.

9. The gene drive system must be consistent with the social and regulatory requirements of the affected communities. For instance, public attitude surveys in Mali [18] highlight the importance of confined field trials prior to a wide-scale release, which could be achieved through the initial use of gene drive systems with high release thresholds followed by subsequent releases with more invasive systems.

10. The gene drive system should be cost-effective, as budgets for disease control are limited and a number of alternative interventions are available. The initial development of gene drive systems is expensive; but ongoing investment can be minimized by designing systems that are resilient to evolutionary degradation.

Cost-effectiveness is an important consideration, as it is not only relevant to the choice of gene drive system, but to whether gene drive should be used at all. In a recent modeling study, Okamoto et al. demonstrated the economic feasibility of releasing large numbers of insects carrying a dengue-refractory gene

without a gene drive system in order to reduce the dengue transmission potential of *Aedes aegypti* mosquitoes in Iquitos, Peru [19]. Wide-scale control of *Anopheles* malaria vectors in sub-Saharan Africa is less likely amenable to the mass release strategy; however, it is essential to assess this in terms of efficacy, safety, and cost-effectiveness prior to implementation.

In this chapter, we review a range of gene drive systems being considered to drive disease-refractory genes into mosquito vector populations. We divide gene drive systems into two broad categories: (i) those that spread by causing a double-stranded break at a specific target sequence and insert themselves at this location through DNA repair (e.g., HEGs) and (ii) those that use combinations of toxins and antidotes, active at different life stages, to favor their own inheritance (e.g., *Medea*). We also review modern approaches to developing translocations as form of gene drive, which do not fit into either category. Systems using symbiotic or commensal microorganisms to mediate gene drive are covered in another chapter (e.g., *Wolbachia*). For each system, we review the biological mechanisms involved, the system's current stage of development, and its alignment with the abovementioned design criteria.

GENE DRIVE SYSTEMS THAT SPREAD VIA TARGET SITE CLEAVAGE AND REPAIR

We begin by reviewing gene drive systems that manipulate inheritance in their favor by causing a double-stranded break at one or more specific target sites in the host's genome and utilize the host's homologous DNA repair mechanism to increase their genomic copy number. Gene drive systems of this type include TEs, HEGs, and a number of recently proposed HEG analogs, such as zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeats (CRISPRs).

Transposable Elements

TEs are genomic components capable of changing their position and sometimes replicating within a genome. Consequently, they show widespread prevalence throughout the genomes of many taxa, with various families of TEs accounting for $\sim$90% of the Salamander genome, 50% of the *Ae. aegypti* genome, and 45% of the human genome. There are various classes of TEs, and those being considered for population replacement in mosquitoes belong to class 2. Class 2 elements contain both repeat sequences that mark their boundaries and their own transposase gene that catalyzes transposition. They move via a cut-and-paste mechanism [20], whereby transposition results in excision of the TE via two double-stranded breaks, leaving behind a gap where they have been excised. In some cases, this gap is filled by

homologous gap repair from a chromatid also having the TE. The excised TE is then inserted at another genomic location, resulting in their genomic copy number being increased by one. In a second replication mechanism, some TEs transpose during the S phase of the cell cycle. If a recently replicated element transposes to an unreplicated region of the genome, it will be replicated a second time, resulting in a net gain of one element in the genome.

Current Status. The widespread distribution of TEs in Nature together with observations of the rapid spread of the *P* element in *Drosophila* [8] inspired initial hopes that class 2 TEs could be inserted, along with disease-refractory and marker genes, into transgenic lines of *Ae. aegypti* (the main vector of dengue fever) and *Anopheles* vectors of malaria. Class 2 TEs lacking their transposase gene are often used as vectors for introducing novel genes into mosquitoes; hence, integration into mosquito lines is relatively straightforward. More problematic, however, has been the remobilization of TEs containing their own transposase gene once they have been integrated. An excellent review by O'Brochta et al. describes results from experiments in which four class 2 TEs—*Hermes*, *Mos1*, *Minos*, and *piggyback*—were used to create transgenic lines of *Ae. aegypti* [10]. In all cases, remobilization was shown to be highly inefficient. More recently, attempts were made to improve the post-integration mobility of *Hermes* in *Ae. aegypti* using an additional construct to express a transposase gene under the control of a testis-specific promoter [21]; however, remobilization was still only observed in less than 1% of the transgenic lines.

Design Criteria. The observed remobilization of natural TEs suggests that remobilization of introduced elements should also be possible; however, the regulation of TE mobility is complex, and it may require much experimentation to find TEs compatible with mosquito vectors. This work is likely not cost-effective, as TEs fail to satisfy most of the design criteria outlined earlier, and have been superseded by more recently proposed systems like HEGs and *Medea*. Of particular note, it is unlikely that TEs will be able to carry large inserts containing disease-refractory genes as transposition events are known to be imprecise and prone to DNA loss. Furthermore, a study on the *Himar1 mariner* element suggests that transposition rates decline substantially with increasing insert size [22], suggesting that elements which have lost their transgenic load will outspread those which have not [23]. Finally, the large numbers of target sites that TEs have undermine their predictability and stability in target species, and their wide species host range highlights the risk of horizontal gene transfer and spread in nontarget species.

Homing Endonuclease Genes

HEGs are highly efficient selfish genetic elements that spread by expressing an endonuclease that recognizes and cleaves a highly specific target sequence of 14−40 base pairs usually only present at a single site in the host

genome [24]. As the HEG is positioned directly opposite its target site, actually within its own recognition sequence, it induces a double-stranded break only in chromosomes lacking the HEG. The HEG is effectively copied to the target site, in a process referred to as "homing," when the cell's repair machinery uses the HEG-bearing chromosome as a template for homology-directed repair. When homing occurs in the germ line of the host organism, a HEG can be transmitted to progeny at a higher than Mendelian inheritance ratios, enabling its spread through a population (Figure 9.1A).

On the basis of observations of homing activity in a number of nonmetazoan organisms including yeast, fungi, algae, and plants, Burt proposed that HEGs could be used as a gene drive system for population replacement in mosquito disease vectors; however, he also proposed and favored their use as a population suppression system [11]. Burt proposed a suite of HEG-based
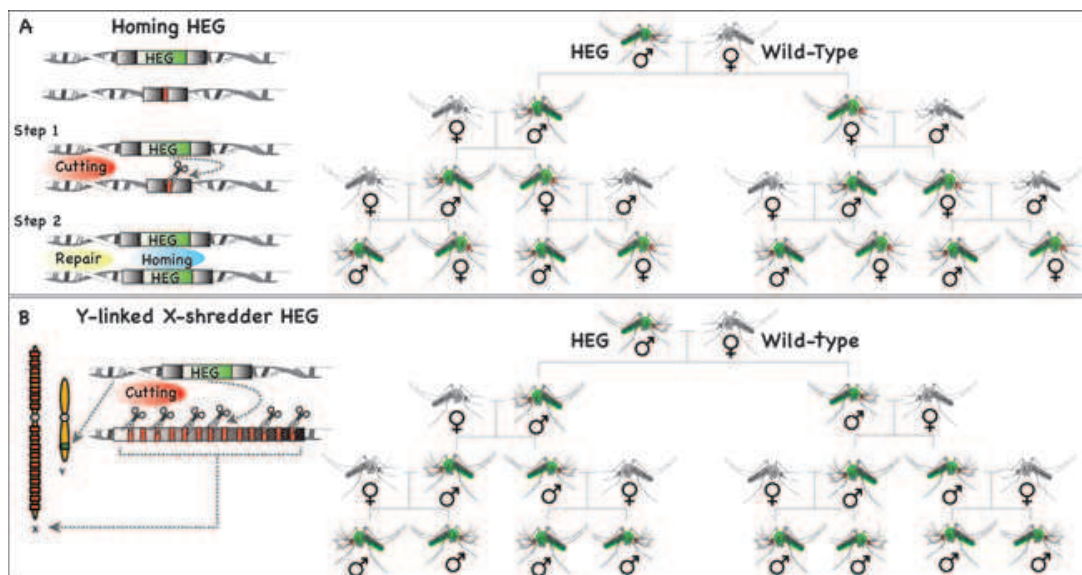

FIGURE 9.1 Preferential inheritance of homing-based gene drive systems. (A) Left panel: A homing HEG (green) encodes an endonuclease that recognizes and cleaves a specific target sequence (red) on a wild-type chromosome (step 1). Once the target site is cleaved, the cell repairs the chromosomal break through homologous recombination using the HEG-bearing chromosome as a template (step 2). This two-step process results in the HEG effectively being copied to the wild-type chromosome in a process referred to as "homing," thereby converting a HEG heterozygote into a HEG homozygote. Right panel: When a HEG-bearing male (green mosquito) is released into the wild and mates with a wild-type female (gray mosquito), the majority of their progeny inherit the HEG, and over time the HEG invades entire populations. (B) Left panel: For HEG-based population suppression, an X-shredder HEG is positioned on the Y chromosome (Y-linked X-shredder HEG). This HEG encodes an endonuclease that recognizes and cleaves chromosomal sequences that are repeated exclusively on the X-chromosome of the mosquito. When expressed during spermatogenesis, X-bearing spermatids are disrupted by the HEG, resulting in the majority of functional sperm being Y-bearing and containing the HEG. Right panel: When a Y-linked X-shredder HEG-bearing male (green mosquito) is released into the wild and mates with a wild-type female (gray mosquito), all resulting progenies are HEG-bearing males. Over time, this is predicted to induce an all-male population crash and potentially eventual extinction of the vector species.

strategies for genetic control of mosquito vectors—two involving population replacement and three involving population suppression:

1. First, the HEG could be linked to a disease-refractory gene and engineered to target a gene-sparse region of a chromosome (so as to reduce impacts on mosquito host fitness), thus carrying the disease-refractory gene with it as it spreads into the population.

2. In a related population replacement approach, the HEG could be engineered to target an endogenous gene involved in the development or transmission of the pathogen, thus reducing vector competence as it spreads [25]. This approach has the benefit that it does not involve an effector gene and hence is more resilient to evolutionary degradation; however, it does require a gene to be identified, the disruption of which would block pathogen transmission, and for a HEG to be engineered to target this, which is quite arduous.

3. In terms of population suppression, a HEG could be engineered that targets a native mosquito gene required in at least one copy for either mosquito survival or fertility. If a HEG of this type is active in the mosquito germ line, then it will increase in frequency in the population, inducing a genetic fitness load on the population as it spreads. This could lead to either population suppression or an eventual population crash.

4. An alternative to the homing-based applications of HEGs is to rely entirely on their target site cleavage activity. In the first of these approaches, known as the "autosomal X-shredder" strategy, a HEG can be designed to specifically cleave the X chromosome at multiple locations, effectively destroying it. If an X-shredder HEG is expressed during male meiosis, it will result in destruction of X-bearing male sperm. If females mate with males having the X-shredder, most viable sperm will be Y-bearing and hence most of the progeny will be male. This strategy will reduce the reproductive potential of the population; but it requires regular releases since the X-shredding gene is associated with a fitness cost and will only persist in the population for a few generations.

5. Finally, Burt proposed a "Y-linked X-shredder" strategy whereby, if the X-shredder HEG is located on the Y chromosome, then it will be driven into the population along with the transgenic Y chromosome as it induces an increasingly male gender bias. This approach would mimic naturally existing meiotic drive systems that bias sex ratios, although it could potentially induce a much larger gender bias than those observed in Nature [26−28], causing a cascade of male-only population crashes that could potentially lead to species extinction (Figure 9.1B).

Current Status. An encouraging result for homing-based HEG strategies has been the engineering of a naturally occurring HEG, I-*Sce*I, which has been shown to cleave in *Ae. aegypti* [29] and spread in laboratory populations of both *D. melanogaster* and *An. gambiae* containing an engineered

target sequence for the HEG [15,30,31]. These results are encouraging because they show that, although HEGs have not been discovered in any metazoan species to date, there is nothing intrinsic about metazoan biology that prevents HEGs from homing. Furthermore, the fact that this was achieved in *An. gambiae*, the most important African malaria vector, is hopeful for its application to disease control. For the population replacement strategy to work in the wild, a HEG must be engineered or identified which has a target sequence in the wild mosquito genome. Engineering HEGs to recognize and cleave new target sequences has proven difficult thus far [32−34], and future research should focus on the development of novel approaches to circumvent these difficulties.

Population suppression strategies that rely solely on the target site cleave activity of HEGs have shown remarkable progress in recent years. A HEG originally discovered in the slime mold *Physarum polycephalum*, I-*Ppo*1 [35], was integrated into the *An. gambiae* genome and shown to recognize and cleave a conserved DNA sequence, repeated hundreds of times and located exclusively on the X chromosome cluster of ribosomal DNA genes in *An. gambiae* [36]. This cleavage activity is highly applicable to both the autosomal and Y-linked X-shredder strategies of HEG-driven population suppression and has also provided a novel genetic approach to the sterile insect technique for *An. gambiae*. The expression of I-*Ppo*1 during spermatogenesis in *An. gambiae* resulted in cleavage of the paternal X chromosome in differentiating spermatozoa, which was expected to result in a male bias among progeny. However, it turned out that the I-*Ppo*1 from mature sperm cells was carried over into the zygote, thus shredding the zygotic X chromosomes as well and rendering the transgenic males completely sterile [37]. It was later shown that transgenic mosquitoes engineered with *I-Ppo*1 could induce high levels on sterility in large cage populations, confirming the suitability of this technology for use in sterile insect population suppression programs [38]. This could be a useful first application of HEG technology in the wild given the self-limiting nature of sterile insect releases.

For X-shredder strategies to work, I-*Ppo*1 would need to be destabilized in order to minimize its carryover into the zygote by mature sperm. To this end, recent work by Galizi et al. has succeeded in expressing destabilized autosomal versions of I-*Ppo*1, which result in efficient shredding of the paternal X chromosome and are restricted to male meiosis [16]. Consequently, males carrying this construct are fully fertile and some insertions produce >95% male offspring bias. Males inheriting the autosomal I-*Ppo*1 gene also produce a male bias in their progeny, showing that the gender-biasing effect of autosomal X-shredders will remain in the population for several generations; however, continued releases would be required, as the X-shredder gene is not favored through inheritance when located on an autosome and is expected to be eliminated due to fitness costs. Nevertheless, for repeated releases, population suppression is expected, which would be

more efficient than the previously mentioned sterile male releases and would also be self-limiting, albeit over a longer period. Autosomal X-shredders could therefore be an appropriate second application of HEG technology.

The only remaining steps in order to realize the Y-linked X-shredder strategy are to dock the destabilized I-*Ppo*1 HEG onto the *An. gambiae* Y chromosome and ensure that it is expressed during spermatogenesis. To this end, recent progress has been made in developing a Y chromosome docking line in *An. gambiae* [39]. Future work will focus on docking the HEG onto the Y chromosome and ensuring it can be expressed and function as anticipated.

Design Criteria. HEG-based strategies for genetic control of vector-borne diseases are extremely promising given the remarkable progress made recently, most notably in the malaria vector *An. gambiae*. HEGs are highly effective as a gene drive system, capable of spreading for low initial frequencies to high frequency on a short timescale. They are also relatively short sequences targeting very precise regions of the genome, suggesting both stability and a low rate of corruption due to evolutionary degradation. Species-specific regulatory sequences can be included to limit their horizontal transfer to nontarget species, and furthermore, a strategy has been proposed to reverse the spread of a deleterious HEG through the release of HEG-resistant alleles in the event of unforeseen consequences [11]. Additionally, a wide range of HEG strategies are available displaying different levels of confineability, allowing them to be used at all stages of a phased release and to be tailored to the social and regulatory requirements of affected communities.

Target site cleavage strategies show more promise than those reliant on homing activity as they sidestep many of the abovementioned design criteria and are independent of disease-refractory genes. Target site mutagenesis and gap repair through nonhomologous end joining can both result in disruption of the HEG cleavage site, rendering certain individuals immune to the HEG and preventing the HEG from spreading through an entire population. For strategies in which a HEG disrupts a gene required for mosquito survival or fertility, HEG-resistant mutants will be favored in a population once they emerge. Furthermore, there is a possibility of losing the disease-refractory gene either through mutagenesis or during homology-directed repair—a concern that becomes more serious for larger inserts, and would render a population replacement strategy futile. The Y-linked X-shredder strategy is less vulnerable to target site mutagenesis as it targets so many loci on the X chromosome at once. It is, however, dependent on germ line gene expression on the *An. gambiae* Y chromosome although this could potentially be achieved through the use of insulator sequences.

TALENs and ZFNs

TALENs and ZFNs have been proposed as alternative platforms for engineering homing-based gene drive systems [40]—that is, systems that spread

by cleaving a specific target sequence and then using the cell's repair machinery to copy themselves to the target site. The benefit of TALENs and ZFNs over HEGs is that they can be easily engineered to target desired DNA sequences due to the modular nature of their DNA-binding domains. TALENs are derived from naturally occurring proteins that are secreted by the pathogenic bacteria *Xanthomonas* spp. to alter gene expression in host plant cells [41,42]. These proteins contain arrays of highly conserved, repetitive DNA-binding domains, each recognizing only a single base pair, with specificity being determined by repeat-variable di-residues [43,44]. The relationship between these repeats and DNA recognition can be exploited to design TALENs that target virtually any desired DNA sequence. For ZFNs, DNA-binding specificity can be similarly manipulated, being determined by an array of finger modules that can be generated either by selection using large combinatorial libraries, or by rational design [45].

For both TALENs and ZFNs, DNA-binding modules can be combined with several types of domains, including transcriptional activators, nucleases, and recombinases, allowing for a comprehensive range of genetic modifications [46]. In terms of cleavage activity, a wide range of tailored recognition sequences can be cleaved efficiently as TALENs and ZFNs are fusion proteins consisting of a nonspecific fok1 nuclease linked to a DNA-binding motif [47,48]. The TALEN or ZFN may then be copied to the cleaved target side by homology-directed repair, and hence used as a gene drive system for driving disease-refractory genes into mosquito populations.

Current Status and Design Criteria. Both TALENs and ZFNs rely upon homing activity and thus, for the purposes of population replacement and control, are functionally similar to HEGs. Given this similarity, the range of replacement and suppression strategies outlined earlier is also applicable to these systems and many of the design issues are similar too. For example, TALENs and ZFNs are also expected to spread from low initial frequencies, species-specificity can be incorporated through the addition of regulatory elements, and a deleterious TALEN or ZFN can be removed from a population through the release of TALEN- or ZFN-resistant alleles. However, there are some important differences. In terms of cost-efficiency, both TALENs and ZFNs are easier to engineer to target specific DNA sequences, and consequently, they could be straightforwardly adapted to multiple vector species, which is particularly important for malaria control. However, concerns arise regarding their stability, as their repetitive nature makes them more prone to mutation and evolutionary degradation. Recent progress toward developing both TALEN- and ZFN-based gene drive systems in *D. melanogaster* have successfully demonstrated DNA-binding specificity, cleavage, and homing through homology-directed DNA repair; however, mutational inactivation led to a decline in effectiveness over just a short period of time [40]. Thus, if TALENs or ZFNs are to be useful as gene drive systems in the future, their stability issues must first be overcome.

Clustered, Regularly Interspaced, Short Palindromic Repeats

CRISPR is another promising system proposed, although not yet demonstrated, as an alternative platform for homing-based gene drive. The system is based on an adaptive immune process in bacteria whereby sequences derived from invading bacteriophages or plasmids are integrated into the bacterial CRISPR locus. This essentially provides bacterial cells with the ability to "remember" and protect themselves against previously encountered viral genomes and invasive, mobile genetic elements [49]. To perform nuclease activities, CRISPR systems use an array of CRISPR RNAs (crRNAs) derived from exogenous DNA targets (e.g., viral genomes), noncoding transactivating RNAs, and a cluster of CRISPR-associated (Cas) genes. Three types of CRISPR systems have been discovered, with type II CRISPR systems being best characterized. These consist of a Cas9 nuclease and a crRNA array encoding guide RNAs and auxiliary transactivating crRNAs to mediate target site cleavage [50]. As for the homing-based systems described earlier, if the double-stranded break is repaired by homology-directed repair, the CRISPR system may be copied to the cleaved target site and hence used as a gene drive system for population replacement similar to HEGs. If the target site cleavage activity is directed toward the X chromosome, then the population suppression strategies initially described for HEGs could also be realized.

Current Status. Recent encouragement for CRISPR-based gene drive has been provided by proof-of-principle studies showing that the type II CRISPR system from *Streptococcus pyogenes* can be modified to target endogenous genes in bacteria [51] and human cell lines [52,53]. It has subsequently been shown that CRISPR can be used to alter genes in a range of other species including insects such as *D. melanogaster* [54,55] and mosquitoes. Straightforwardly, utilizing this system in other organisms requires only two components—the Cas9 nuclease and guide RNAs [52,56]. DNA-binding specificity is determined by the first 20 nucleotides of the guide RNA as these designate the DNA target side that Cas9 will be guided to according to Watson−Crick DNA−RNA base pairing rules. The only restriction for the target site selection is that it must lie directly upstream of a protospacer adjacent motif sequence that matches the canonical form 5′-NGG. Aside from that, it is possible that the CRISPR system can be engineered to target and cleave essentially any genomic location, with subsequent homing and gene drive occurring via homology-directed repair, however this remains to be demonstrated.

Design Criteria. CRISPR-based gene drive has yet to be implemented; however, its mechanisms imply that the approach is achievable. In terms of design criteria, the system is very similar to TALENs and ZFNs—it is expected to spread from low initial frequencies, species-specificity can be incorporated through regulatory elements, and a deleterious CRISPR can be removed through release of CRISPR-resistant alleles. The system is active

in a range of species and target sites are even easier to engineer than for TALENs, suggesting the system would be easily adapted to multiple vector species. Another advantage of the CRISPR system is that it can be used to target multiple sequences in a single experiment [57], increasing its potential efficacy and decreasing the rate at which target site mutagenesis could slow its spread. A major concern, however, is that the CRISPR system itself may be degraded. The CRISPR system is quite large, consisting of promoters, the Cas9 gene, guide RNAs and, depending on the strategy being implemented, multiple disease-refractory genes and associated regulatory elements. A system this size is prone to mutation and errors introduced during homing, including potential loss of function of disease-refractory genes. These considerations may lead to population suppression strategies being favored for CRISPR-based drive systems; however, this would place selection pressure on mutant CRISPR alleles having lost their function and so the evolutionary stability of the CRISPR system will need to be explored and optimized if it is to provide a cost-effective alternative to the relatively stable yet difficult-to-engineer X-shredding HEGs.

TOXIN−ANTIDOTE GENE DRIVE SYSTEMS

We now move on to gene drive systems that use combinations of toxins and antidotes, active at different life stages, to favor their own inheritance [58]. Gene drive systems of this type include *Medea*, engineered forms of underdominance such as UD^{MEL}, self-limiting systems such as killer-rescue, and other toxin−antidote possibilities such as *Semele*, *Medusa*, and inverse *Medea*.

Medea

The story of *Medea* has origins in both Greek mythology and beetle biology. In Greek mythology, Medea was the wife of the hero Jason, to whom she had two children. Her marriage to Jason was hard-earned, transpiring only after she enabled him to plough a field with fire-breathing oxen, among other achievements; but despite this, he left her when the king of Corinth offered him his daughter. As a form of revenge, Medea killed their two children. From a biological perspective, such infanticide would make Medea an unfit mother; but if the trait is genetic and children that inherit it also have the ability to defend themselves, then mathematical models show that it actually has a selective advantage and, if present at modest levels in a population, is expected to become present among all individuals within a matter of generations [59,60]. This is simply because children who are able to defend themselves against a murderous parent are more fit than those who cannot.

The Greek analogy sounds bizarre; but genes displaying these properties do actually exist in Nature and have been discovered and characterized in

various regions of the world [12,61,62]. The first such element to be identified was in the flour beetle *Tribolium castaneum* [12] and was given the name *Medea* after both the character from Greek mythology, and as an acronym for "maternal-effect dominant embryonic arrest." By crossing individuals from geographically isolated locations, it was found that *Medea*-bearing males gave rise to both wild-type and *Medea*-bearing offspring; but that *Medea*-bearing females only gave rise to *Medea*-bearing offspring. It appeared that *Medea*-bearing mothers were selectively killing non-*Medea*-bearing offspring; or alternatively that they were trying to kill all offspring and the *Medea*-bearing offspring were able to defend themselves.

The genetic factors involved in this behavior remain obscure; but the dynamics suggest a model in which *Medea* consists of two tightly linked genes—a maternally expressed toxin gene, the product of which causes all eggs to become unviable and a zygotically expressed antidote gene, the product of which rescues *Medea*-bearing eggs from the effects of the toxin [12,63]. In *Tribolium*, *Medea* dynamics are attributed to an insertion of a composite Tc1 transposon inserted between two genes both having maternal and zygotic components [13]. Remarkably, this system was reverse-engineered using entirely synthetic components in laboratory populations of *D. melanogaster* and was shown to rapidly drive population replacement [14,64]. These synthetic elements were constructed using two unique, tightly linked components—a maternal toxin consisting of maternally deposited microRNA designed to target an essential embryonic gene; and a zygotic antidote consisting of a tightly linked, zygotically expressed, microRNA-resistant version of the embryonic essential gene. The combination of these components results in the death of wild-type offspring of *Medea*-bearing mothers, thus favoring the *Medea* allele in subsequent generations and mimicking the behavior of the natural element in *Tribolium* (Figure 9.2A).

Current Status. *Medea* was the first synthetic gene drive system to be developed, in this case in *D. melanogaster* [14]. Given that the synthetic *Medea* elements were constructed using rationally designed synthetic components and well-understood, conserved molecular and genetic mechanisms, it should be possible to engineer *Medea* elements in a range of other insects including mosquitoes. The *Medea* drive strategy is particularly well-suited to driving disease-refractory genes into mosquito populations, and hence the development of several efficient refractory genes for each disease of interest is encouraged.

Design Criteria. In many ways, *Medea* is the ideal system for replacement of wild mosquito populations with disease-refractory varieties. Solutions are available for all of the design criteria outlined earlier, and *Medea* has an advantage over homing-based strategies for population replacement since it is stably integrated into the host chromosome, thus not affected by the substantial risk of loss during homology-directed repair. If introduced at modest population frequencies, *Medea* can spread and rapidly
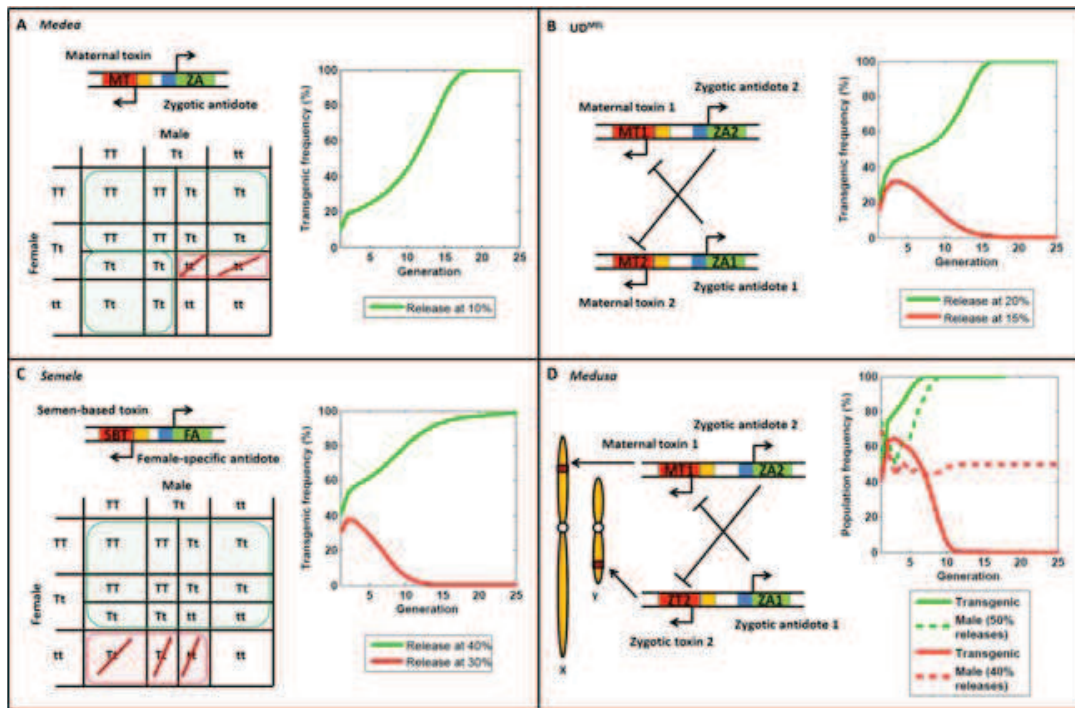
FIGURE 9.2 Dynamics of toxin−antidote-based gene drive systems. (A) *Medea* elements distort the offspring ratio in their favor through the action of a maternally expressed toxin (MT) and a zygotically expressed antidote (ZA). This results in the death of wild-type offspring of heterozygous mothers and enables the *Medea* element to spread into a population from very low initial frequencies. Dynamics here are shown for a *Medea* element with no fitness cost, released at 10% in the population. Transgenic frequency refers to any individual carrying at least one copy of the element. (B) UD^{MEL} (maternal-effect lethal underdominance) is a toxin−antidote-based underdominant system consisting of two constructs, each of which possesses a maternally expressed toxin (MT1 and MT2) whose activity is manifest during progeny embryogenesis and a zygotic antidote (ZA1 and ZA2) capable of neutralizing the maternal toxin expressed by the opposite construct. This results in heterozygous females being sterile if mated to wild-type individuals, thus leading to the characteristic bistable dynamics of underdominant systems. Dynamics here are shown for UD^{MEL} constructs at independently assorting loci having no fitness costs. If released at a population frequency of 20%, the system spreads to fixation in the population; but if released at 15%, the system is eliminated. (C) *Semele* elements distort the offspring ratio in their favor through the action of a semen-based toxin (SBT) and a female-specific antidote (FA). This results in unviable crosses between transgenic males and wild-type females and favors transgenic individuals provided the *Semele* element is present at population frequencies exceeding ∼36% (above this frequency, the selective advantage of the antidote exceeds the selective disadvantage of the toxin). Dynamics here are shown for a *Semele* element with no fitness cost. If released at a population frequency of 40%, the element spreads to fixation in the population; but if released at 30%, the system is eliminated. (D) *Medusa* is a two-construct, sex chromosome-linked drive system capable of inducing confineable and reversible population suppression. The system consists of four components—a maternally expressed, X-linked toxin (MT1) causes suppression of the female population and selects for the transgene-bearing Y since only transgenic male offspring have the corresponding Y-linked zygotically expressed antidote (ZA1). A zygotically expressed, Y-linked toxin (ZT2) and a zygotically expressed, X-linked antidote (ZA2) then selects for the transgene-bearing X when the transgene-bearing Y is present, creating a balanced lethal system. When present above a certain threshold frequency, *Medusa* spreads while creating a strong male gender bias leading to population suppression. Dynamics here are shown for *Medusa* constructs having no fitness costs. For two consecutive male-only releases at a population frequency of 50%, the population becomes entirely male as the system spreads to fixation in the population; but for two consecutive male-only releases at a population frequency of 40%, the system is eliminated.

replace a population, even in the presence of modest fitness costs [60]; however, *Medea* is unlikely to spread following a small-scale accidental release because its driving ability is low at low population frequencies [18].

Tight linkage between the toxin, antidote, and refractory genes by placing the toxin and refractory genes within an intron of the antidote gene can improve system stability and reduce the rate of loss of the refractory gene through recombination. However, in the event that the *Medea* element or refractory gene become unlinked, mutated, or rendered ineffective through parasite evolution, second-generation *Medea* elements can be generated that utilize toxin−antidote combinations distinct from those of the first-generation elements [14], making it possible to carry out multiple cycles of population replacement. This strategy can also be used to remove refractory genes from populations in the event of adverse effects. As the functional components of *Medea* are developed in mosquito species, it will become more cost-efficient to develop these elements and to adapt them to multiple vector species.

Toxin−Antidote-Based Underdominance

Underdominant systems display the property that heterozygotes, or their progeny, have lower fitness than either homozygote [65]. In the simplest case of a single biallelic locus for which matings between opposite homozygotes are sterile, whichever allele is more frequent in the population will tend to spread to fixation. Underdominant systems therefore display features similar to that of a bistable switch at the population level—if the system is present above a critical threshold frequency, it will tend to spread to fixation, while if it is present below the threshold, it will tend to be eliminated in favor of the alternative allele or chromosome. A variety of toxin−antidote systems have been proposed to achieve these underdominant dynamics and the critical threshold frequency depends on the system and fitness cost.

A range of underdominant systems is available in Nature, including chromosomal alternations such as inversions, translocations, and compound chromosomes [3,4]. We will return to translocations in the Translocation section; but will concentrate here on novel forms of underdominance that are in principle straightforward to engineer using combinations of toxins and antidotes. Toxin−antidote approaches to underdominance were originally proposed by Davis et al., who suggested an elegant system having two transgenic constructs, each of which possesses a gene whose expression induces lethality and a gene that suppresses the expression or activity of the gene inducing lethality carried by the other construct [66]. The constructs can either be inserted at the same locus on a pair of homologous chromosomes or at different loci on nonhomologous chromosomes. These systems display underdominant properties because individuals carrying neither or both constructs are viable; but a proportion of their offspring—those carrying just one of the

constructs—are unviable. The critical threshold for the two-locus system is ∼27%, above which it is predicted to spread to fixation, and for the single-locus system is ∼67% [66].

Current Status. Attempts to engineer the underdominance system proposed by Davis et al. have thus far been unsuccessful [66]; however, a related novel underdominant system known as maternal-effect lethal underdominance (UD^MEL) has recently been engineered in *D. melanogaster* and demonstrated to replace wild-type laboratory populations in a threshold-dependent manner [67,68]. In the UD^MEL system, there are two transgenic constructs, each of which possesses a maternally expressed toxin gene whose activity is manifest during progeny embryogenesis and a zygotic antidote gene capable of neutralizing the maternal toxin expressed by the opposite construct. From the crosses produced by this system (Figure S1 of Akbari et al. [67]), it can be seen that heterozygous females are sterile if mated to wild-type individuals, while populations of transgenic homozygotes are fully viable, as are wild-type populations. This leads to the characteristic bistable dynamics of underdominant systems. As per the system proposed by Davis et al., the UD^MEL constructs can be inserted at the same locus or on a pair of homologous chromosomes [66]. The critical threshold for the two-locus system is ∼19% and for the single-locus system is ∼64%, assuming no fitness costs [67], and threshold-dependent drive has been demonstrated in the laboratory for both cases (Figure 9.2B).

Design Criteria. Toxin−antidote-based underdominant systems such as UD^MEL are an excellent option during the testing phase of population replacement, or whenever a confined release is desired. The threshold nature of these systems has three advantages in these scenarios. First, they are unlikely to spread following an accidental released because escapees will inevitably be present at subthreshold levels and be eliminated from the environment [18]. Second, they are expected to be confineable to isolated release sites because transgenic insects released at superthreshold frequencies are expected to spread transgenes locally while they remain at subthreshold levels at nearby locations. And third, releases are reversible as transgenes can be eliminated by diluting them to subthreshold frequencies through a sustained release of wild-type insects.

It should be noted that the confineability of these systems, although likely, is not guaranteed.

In theory, chance events could lead to underdominant systems gaining a foothold and spreading in structured populations, presumably beginning from a single individual; however, this is more likely to occur on an evolutionary timescale than on a human timescale. Underdominant systems may be better-suited to *An. gambiae* because it disperses quickly over the range of a single village [69,70], reducing the chance of its spread being confined to smaller subpopulations. The small-scale population structure of *Ae. aegypti*, however, may prevent its village-wide spread in natural populations of these vectors.

Otherwise, similarly to *Medea*, solutions are available for all of the design criteria outlined earlier. As the functional components are developed and identified in mosquitoes—microRNAs, maternal and early-zygote-specific promoters and essential genes—these systems will be highly useful for confined population replacement of vector species such as *An. gambiae*.

Killer-Rescue

Killer-rescue is an intriguingly simple two-locus gene drive system proposed by Gould et al. for both its ease of engineering and its ability to spread into a population in a time-limited way [71]. Both these qualities are desirable in the early stages of a population replacement program. The system consists of two alleles at unlinked loci—one that encodes a toxin (a killer allele) and another that confers immunity to the toxin (a rescue allele), which could be tightly linked to a gene for disease refractoriness. A release of individuals homozygous for both alleles results in temporary drive as the alleles segregate and the presence of the killer allele in the population confers a benefit to those also carrying the rescue allele. In an alternative configuration, a second killer allele can be included at an independently assorting locus to enhance the selective benefit of the rescue allele. However, regardless of the conformation, the killer allele soon declines in frequency due to its inherent fitness cost and, as it does, the selective benefit of the rescue allele is lost. As this happens, if the rescue allele or disease-refractory gene confers a fitness cost to the host, then it will gradually be eliminated from the population as well over a timeframe determined by the magnitude of its fitness cost—a higher fitness cost leading to it being eliminated more quickly.

Design Criteria. As mentioned earlier, the killer-rescue system is intriguing for its ability to spread in a time-limited manner, thus reducing risks, as appropriate during field trials of transgenic mosquitoes carrying disease-refractory genes. The system is also spatially limited, as it only has a window of time in which to disperse to neighboring populations, and will spread to much lower levels in these populations than at the population of release [72]. Similar to underdominant systems, it will not persist following an accidental release, and its elimination from a population can be accelerated through large-scale releases of wild-type insects. Also, similar to other toxin−antidote systems, solutions are available for all of the design criteria outlined earlier.

Some consideration should go into the fitness cost of the rescue allele and refractory gene, as a high fitness cost will lead to rapid elimination, but the maximum frequency of the disease-refractory allele in the population will be compromised; while small fitness costs will allow the system to spread to very high maximum frequencies, but it may take several years for the system to be eliminated from the population entirely. Further

complicating this, fitness costs are exceedingly difficult to quantify in the field. The bistable nature of underdominant systems therefore makes them more controllable in terms of confinement and reversibility; however, the major benefit of the killer-rescue system is its ease of engineering. Molecular tools are already available to engineer the system in a variety of mosquito species, allowing the system to be implemented with relative ease in a range of disease vectors.

Other Confineable Toxin−Antidote Systems

As *Medea*, killer-rescue, and the various forms of engineered underdominance highlight, there are many ways in which toxins and antidotes can be used to favor the inheritance of one allele over another. For example, even if we limit ourselves to single-locus systems like *Medea*, either the toxin or antidote gene could be placed under the control of a paternal, maternal, or zygote-specific promoter, function through a recessive or dominant mechanism, and be located on a sex chromosome or autosome [73]. The possibilities multiply if we also consider multilocus systems. A few additional toxin−antidote systems displaying unique population dynamics are *Semele*, inverse *Medea*, and *Medusa*, all of which are also confineable to partially isolated populations.

Semele. *Semele* is a single-locus system consisting of a toxin gene expressed in the semen of transgenic males that either kills or renders infertile wild-type females and an antidote gene expressed in females that protects them against the effects of the toxin [74]. The name is an acronym for "semen-based lethality" and, like *Medea*, also has Greek origins. In Greek mythology, Semele was a mortal female who attracted the attention of Zeus while slaughtering a bull at his altar (Zeus, at this point, was flying overhead disguised as an eagle). Zeus became infatuated with Semele and impregnated her, but Semele died after witnessing his godliness because she was not herself a god. The story parallels the biology of the *Semele* construct, in which wild-type females die (or become infertile) upon mating with transgenic males.

Semele has several interesting population dynamic properties. If only males carrying the *Semele* allele are released into a wild population, they are expected to suppress the population size when released in large numbers. This happens because all of the wild females that mate with the *Semele* males are susceptible to their toxic semen. If both males and females carrying the *Semele* allele are released, the system displays bistable dynamics with a threshold frequency of $\sim 36\%$ in the absence of fitness costs [74]. Above the threshold, the selective advantage of the female antidote outweighs the reproductive disadvantage conferred by the toxic semen and the system spreads into the population. In combination, this means that an initial release of *Semele* males could be used to suppress a population

preceding a superthreshold release of males and females, thus reducing the release size required to exceed the critical population frequency (Figure 9.2C).

Inverse Medea. Inverse *Medea* is another single-locus system capable of achieving confined population replacement [75]. The system consists of a zygotic toxin and maternal antidote—essentially the same components as the *Medea* system with the promoters switched. This has the effect of rendering heterozygous offspring of wild-type mothers unviable and leads to bistable dynamics in which the system spreads when it represents a majority of the alleles in a population, and is otherwise eliminated. While similar dynamic properties are displayed by other underdominant toxin−antidote systems, the benefit of inverse *Medea* is its ease of engineering once the components to generate *Medea* elements in mosquito vectors have been identified. Several approaches to engineering these elements are available—for example, the toxin could be a microRNA that silences expression of a gene whose activity is required for early embryo development, and the antidote could be a maternally expressed RNA that restores the necessary activity to the zygote and is resistant to silencing.

Medusa. *Medusa* is a two-construct, sex chromosome-linked drive system capable of inducing confineable and reversible population suppression [76]. The system consists of four components—two at a locus on the X chromosome and two at a locus on the Y chromosome. The combination of a maternally expressed, X-linked toxin and a zygotically expressed, Y-linked antidote causes suppression of the female population and selects for the transgene-bearing Y since only transgenic male offspring of *Medusa*-bearing females are protected from the effects of the toxin. At the same time, the combination of a zygotically expressed, Y-linked toxin and a zygotically expressed, X-linked antidote selects for the transgene-bearing X when the transgene-bearing Y is present. Together, this creates a balanced lethal system that, when present above a certain threshold frequency, spreads while creating a strong male gender bias, hence causing population suppression (Figure 9.2D). Characteristic of all drive systems with thresholds, releases of *Medusa* mosquitoes are confineable and reversible, making the system an ideal tool for confined population suppression. This could be particularly useful in the lead-up to releases of invasive population suppression systems such as Y-linked X-shredder HEGs [76].

The name *Medusa* is an acronym for "sex chromosome-associated *Medea* underdominance," as its components are identical to those of *Medea* and engineered underdominance. The name also has origins in Greek mythology, where *Medusa* is a beautiful yet terrifying woman who caused onlookers to be turned to stone (toxin) but was ultimately beheaded by Perseus who distracted himself with Athena's mirrored shield (antidote). Simple population dynamic models show that an all-male release of *Medusa* males, carried out over six generations, is expected to induce a population crash within 12

generations for modest release sizes [76]. Reinvasion of wild-type insects can result in a population rebound; however, this can be prevented through regular releases of modest numbers of *Medusa* males.

Design Criteria. The vast range of possible toxin−antidote combinations highlights the versatility of this approach to engineering gene drive systems. *Semele* is an excellent option for confined population replacement due to its ability to suppress a vector population prior to replacement, inverse *Medea* is an excellent underdominant system that is easy to engineer once the components of the *Medea* system have been identified in mosquito vectors, and *Medusa* is an ideal system for confined population suppression in preparation for invasive X-shredder strategies [76]. Other toxin−antidote systems are imaginable and may be favored depending on the components first identified in molecular work on vector species [73]. As toxin−antidote systems, the design criteria outlined earlier are generally satisfied, and as largely confineable systems, the systems highlighted here are excellent options during the testing phase of population replacement, or whenever a confined release is desired.

TRANSLOCATIONS

As the first gene drive system to be proposed [4], translocations have since undergone a lull in interest following the observation that radiation-generated translocations failed to spread in the field, likely due to high fitness costs induced by X-rays [7]. However, recent developments in molecular biology permit the creation of translocations without relying upon radiation suggesting that, after several decades of inactivity, the application of this gene drive system could be revisited. Translocations result from the mutual exchange of chromosomal segments between nonhomologous chromosomes. Translocation heterozygotes are usually partially sterile, while translocation homozygotes are usually fully fertile. This effect is manifest during meiosis when nearly half of the gametes from a translocation heterozygote have a duplication of one chromosomal segment and a deficiency of another. The haploid gametes are functional, but when they fuse with native gametes following fertilization, the resulting zygotes are inviable. This produces the bistable dynamics described for other underdominant systems.

Current Status. Curtis proposed that if a translocation strain was developed that had a disease-refractory gene tightly linked to the translocation break point, disease-resistance would spread into that population as the translocation fixes [4]. To test this hypothesis, mosquito strains with chromosomal translocations were developed using X-ray mutagenesis; however, the low fitness associated with these strains and the difficulty of bringing disease-refractory genotypes into appropriate genetic backgrounds inhibited these approaches from further development. It is now possible to generate translocations at almost any genomic location without irradiation as a result

of progress in genome sequencing and synthetic biology [77,78]. This will reduce the fitness costs associated with translocations and will allow disease-refractory genes to be more easily linked to translocation break points, making them a feasible, future gene drive system for confined population replacement.

Design Criteria. As an underdominant system displaying bistable dynamics, translocations provide another option for confined population replacement. As modern molecular techniques are yet to be applied to the development of this system, its agreement with several of the design criteria mentioned earlier are yet to be determined, and its attractiveness as a local gene drive system will depend on its ease of engineering and satisfaction of these criteria in comparison to toxin−antidote-based underdominant systems. Toxin−antidote-based systems may be preferable for phased releases as their components are more similar to invasive *Medea* elements that could be used for subsequent wide-scale population replacement. That said there is a theoretical expectation that translocations are an effective gene drive system for local population replacement [4] and that the loss of disease-refractory genes will be minimized by inserting them at translocation break points. As a bistable system, translocations could be eliminated from a population through mass release of wild-type insects and would satisfy social and regulatory requirements when confinement is desired.

CONCLUSION

In 2006, Sinkins and Gould published an excellent review of gene drive systems for insect disease vectors which today provides a testament to how quickly the field has progressed in less than a decade [79]. As the authors state, "ultimately, the drive system that becomes most widely used might be one that is entirely novel and not described here." Interestingly, the majority of the drive systems described in this chapter—TALENs, CRISPRs, UD^{MEL}, killer-rescue, *Semele*, inverse *Medea*, and *Medusa*— were not mentioned in the Sinkins and Gould review as they are have only been recently published.

Of the systems that were mentioned by Sinkins and Gould, progress has been rapid. In mentioning *Medea*, for instance, the authors stated that "a molecular understanding of its function could lead to the development of artificial *Medea*-like constructs"—something that was achieved the following year [14] and is now one of the most promising approaches for population replacement. Regarding HEGs, the authors stated that, "Unfortunately, HEGs have only been reported in fungi, plants, bacteria and bacteriophages . . . the potential for developing an HEG-based functional system in insects is unknown." The following year, a HEG isolated from a species of slime mold demonstrated cleavage activity in *An. gambiae* [36], and a few years later, another naturally occurring HEG was shown to spread in

laboratory populations of both *D. melanogaster* and *An. gambiae* [15,30,31]. HEGs are now one of the most promising gene drive systems for inducing population suppression (Table 9.1). Research on using *Wolbachia* to control vector-borne diseases has been even more rapid, with large-scale field trials already having taken place in several countries including Australia and Vietnam [80]. This prompts the question of what the gene drive field will look like a decade from now?

Gene Drive for Any Situation

Sinkins and Gould also pointed out that "the various types of drive mechanisms should not be viewed as competing systems," adding that, "Different characteristics will be needed in different situations." Gene drive systems can lead to a number of outcomes in terms of population dynamics, and the optimal system in each case will depend upon the desired outcome. For driving disease-refractory genes into mosquito populations over a wide area, *Medea* seems to be a very promising system, as it is capable of spreading from low initial frequencies and is also stably integrated into the host chromosome. When population replacement is desired over a wide geographic area, stability in the face of evolutionary degradation is an important consideration, and *Medea* may be preferable to homing-based strategies incorporating disease-refractory genes because these are susceptible to DNA loss during homology-directed repair, which is expected to become increasingly significant over large spatial and temporal scales.

Systems with release thresholds are preferable when a confined release is desired because these systems are likely to be confineable to their population of release and to be reversible through releases of wild-type insects [72]. Toxin−antidote-based underdominant systems would be an obvious choice if the goal were to test the concept of population replacement prior to a release of toxin−antidote-based *Medea* elements. The bistable nature of these systems makes them particularly amenable to confinement; however, killer-rescue systems and a mass release of transgenic insects with disease-refractory genes [19,71] should also be considered, as these are significantly easier to engineer in a wide range of vector species and the spreading a disease-refractory gene into an isolated population will not always require gene drive.

For population suppression, Y-linked X-shredder HEGs are an ideal system, assuming the X-shredding HEG can be docked onto the Y chromosome and expressed during spermatogenesis. The major benefits of the X-shredder HEG are the generally small size of HEGs, making them less susceptible to evolutionary degradation, and the large number of loci cleaved on the X chromosome, making the strategy less susceptible to target site mutagenesis [11]. Autosomal X-shredders, as a self-limiting population suppression system acting through the same molecular

TABLE 9.1 Alignment of Potential Gene Drive Systems with Design Criteria Outlined in the Introduction[a]

Design Criteria	Target Site Cleavage-Based Gene Drive Systems				Toxin−Antidote-Based Gene Drive Systems		Engineered Translocations
	Engineered TEs	Engineered HEGs	ZFNs, TALENs	CRISPRs	*Medea*, UD[MEL]	Killer-rescue, *Semele*, *Medusa*	
Effectiveness of spread	Maybe [10] (not yet effective in vector species)	Yes [11] (very effective, first drive system shown to spread in a malaria vector)	Probably [40] (homing demonstrated, currently compromised by mutational inactivation)	Probably [51−57] (components identified, can target multiple sequences at once)	Probably [14,64,67] (observed to spread in laboratory *Drosophila* populations)	Theoretically [71,74,76] (not yet engineered, models predict spread)	Theoretically [4,77,78] (models predict spread, not yet engineered using modern components)
Ability to carry large effector genes	No [22] (transposition rate declines with increasing insert size)	Possibly [11] (could be lost during homology-directed repair)	Possibly [40] (could be lost during homology-directed repair)	Possibly (could be lost during homology-directed repair)	Yes [14,67] (stably integrated into host chromosome)	Yes [71,74,76] (stably integrated into host chromosome)	Yes [4] (stably integrated into host chromosome)
Tight linkage with effector genes	No [23] (transposition events prone to DNA loss)	No [11] (homology-directed repair prone to DNA loss)	No [40] (homology-directed repair prone to DNA loss)	No (homology-directed repair prone to DNA loss)	Yes [14,67] (place toxin and effector genes within intron of antidote gene)	Yes [71,74,76] (place effector gene within intron of antidote gene)	Yes [4] (very tight if effector gene linked to a translocation break point)
Waves of introductions	Maybe [10] (difficult-to-engineer multiple TEs)	Maybe [11] (difficult to engineer)	Yes [40] (easier to engineer than HEGs)	Yes (easier to engineer than HEGs)	Yes [14,67] (use distinct toxin−antidote combinations)	Yes [71,74,76] (use distinct toxin−antidote combinations)	Yes [4] (use threshold properties)

Easily adapted to other species	No [10] (difficult to find TEs compatible with vector species)	No (difficult to engineer target site)	Maybe [40] (once components identified in species)	Maybe (once components identified in species)	Maybe [14,67] (once components identified in species)	Maybe [71,74,76] (once components identified in species)	Maybe [77,78] (once components identified in species)
Stability in target species	No [8] (large number of target sites undermine predictability)	Yes [11] (short sequences targeting precise genomic regions)	Moderate to low [40] (prone to mutation due to repetitive nature)	Moderate to low (more prone to mutation due to repetitive nature and large size)	Yes [14,67] (stably integrated into host chromosome)	Yes [71,74,76] (stably integrated into host chromosomes)	Yes [77,78] (very stable)
Minimal horizontal gene transfer	No [8] (wide species host range)	Yes [11] (include species-specific regulatory sequences)	Yes [11,40] (include species-specific regulatory sequences)	Yes [11] (include species-specific regulatory sequences)	Yes [14,67] (include species-specific regulatory sequences)	Yes [71,74,76] (include species-specific regulatory sequences)	Yes
Mechanism for removal	No	Yes [11] (design second HEG to target first HEG)	Yes [11,40] (design second ZFN or TALEN to target first ZFN or TALEN)	Yes [11] (design second CRISPR to target first CRISPR)	Yes [14,67] (use threshold properties or second-generation element to remove refractory gene)	Yes [71,74,76] (use threshold properties or dilution with wild-types)	Yes [4] (use threshold properties)
Social and regulatory requirements	No [79] (not confineable or reversible)	Yes [11] (wide range of strategies with different levels of confineability)	Yes [11,40] (wide range of strategies with different levels of confineability)	Yes [11] (wide range of strategies with different levels of confineability)	Yes [18,72] (confineable or unlikely to spread following small accidental release)	Yes [72] (confineable to partially isolated populations)	Yes [72] (confineable to partially isolated populations)

[a]In many cases, data supporting satisfaction of design criteria are preliminary. TEs, transposable elements; HEGs, homing endonuclease genes; ZFNs, zinc-finger nucleases; TALENs, transcription-activator-like effector nucleases; CRISPRs, clustered, regularly interspaced, short palindromic repeats; UD^{MEL}, maternal-effect lethal underdominance.

mechanism, are an obvious choice for testing this drive system prior to a wide-scale release. Similar approaches using ZFNs, TALENs, and CRISPRs should also be considered, especially considering their relative ease of engineering. However, the repetitive nature of ZFNs and TALENs and the large size of CRISPRs generally will make them more susceptible to mutation and evolutionary degradation (Figure 9.3).

Outstanding Issues and Future Outlook

In 1899, US patent officer Charles Duell famously stated that, "Everything that can be invented already has been invented." It would be just as foolish to say that all imaginable gene drive systems have already been imagined. The coming decades are bound to witness the emergence of a plethora of novel mechanisms for spreading desirable genes into insect populations, and it will be fascinating to see how these systems align with the design criteria mentioned earlier. Furthermore, of the systems for which development has already begun, it will be fascinating to see how their laboratory and field studies progress. Progress on toxin−antidote-based systems will be greatly facilitated by the development of their functional components—toxins, antidotes and regulatory elements—in mosquito vectors. It will also be interesting to see how modern approaches to translocations perform against toxin−antidote-based approaches to underdominance. Regarding homing-based systems, critical developments will be the engineering of HEGs for other vector species, the insertion and expression of X-shredders on the Y chromosome, and determining the resilience of alternative homing-based systems to evolutionary degradation.

As a technology capable of engineering or eliminating entire species, the development of gene drive systems carries with it both great promise and great responsibility. Issues are heightened by the ability of invasive systems to spread into neighboring communities and countries without their consent [81]. Comprehensive risk assessments that address ecological, epidemiological, and social issues are therefore essential, and such technology should only be used in the absence of significant risks. On the flip side, gene drive technology has the potential to make a profound impact on relieving the global vector-borne disease burden [2]. Considering malaria as an example, traditional interventions such as bed nets and antimalarial drugs require human compliance, which never truly exceeds ∼80% coverage, meaning that there is always a residual human population capable of sustaining transmission [82]. Replacement of disease-transmitting mosquitoes with disease-refractory ones has the unique benefit that it does not require human compliance, and can spread into areas where interventions are difficult to apply. This makes it one of the most promising components of future integrated strategies for the elimination of vector-borne diseases.
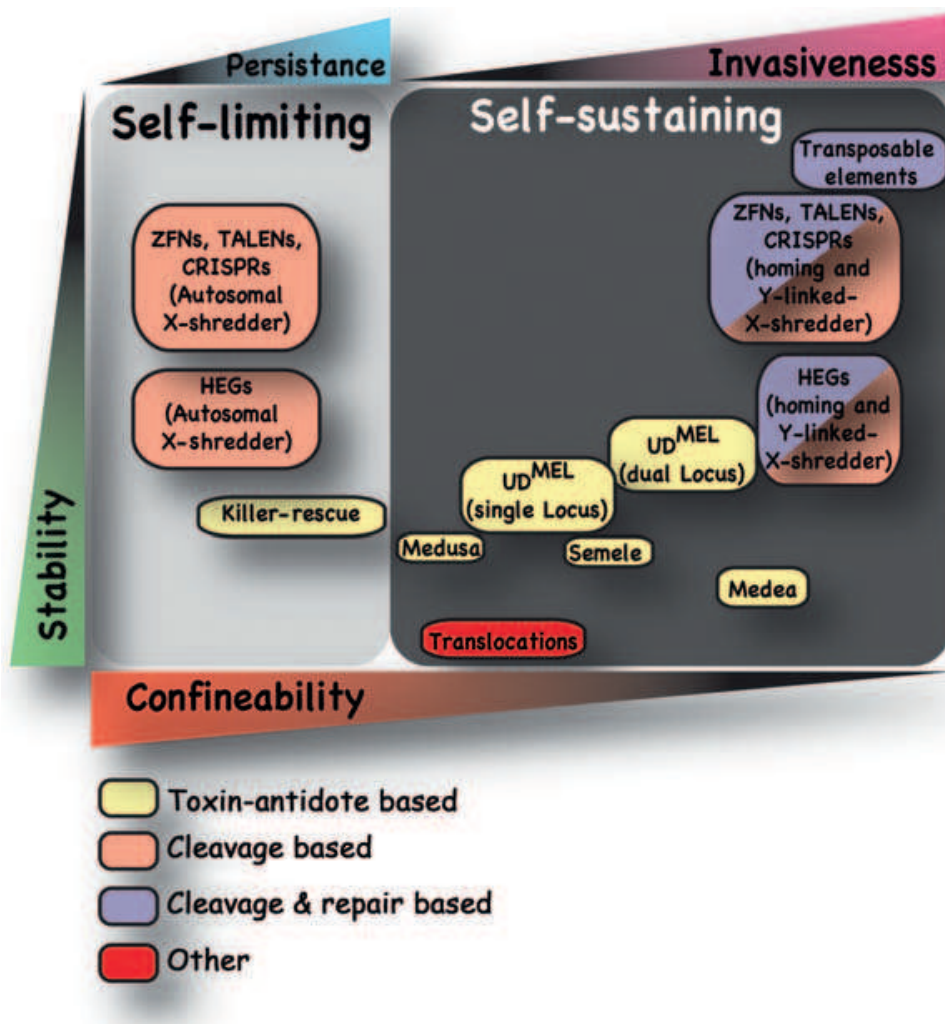
FIGURE 9.3 Confineability and stability of potential gene drive systems. The potential gene drive systems described in this chapter differ in multiple ways, including their confineability (the ability to limit their spatial spread following a release) and their stability (resilience against evolutionary degradation, predictable behavior in the host organism and infrequent spread into nontarget species). Here, we depict the potential gene drive systems in a two-dimensional graph according to these properties. Self-limiting systems eliminate themselves from a population as a result of their own dynamics and hence are highly confineable, although some persist in a population longer than others. Self-sustaining systems are capable of maintaining a high population frequency but are relatively confineable if they display threshold properties in terms of release frequency. Self-sustaining systems not displaying threshold dynamics can be highly invasive. Toxin−antidote-based systems (yellow) are relatively stable but have differing levels of confineability. Cleavage-based population replacement systems (purple) are relatively invasive whether they carry disease-refractory genes or induce a population fitness load. The process of homing also causes them to be relatively unstable due to errors introduced during gap repair. Cleavage-based population suppression systems (salmon) can be either invasive if located on the Y chromosome or self-limiting if located on an autosome. ZFNs, zinc-finger nucleases; TALENs, transcription-activator-like effector nucleases; CRISPRs, clustered, regularly inter-spaced, short palindromic repeats; HEGs, homing endonuclease genes; UD^{MEL}, maternal-effect lethal underdominance.

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