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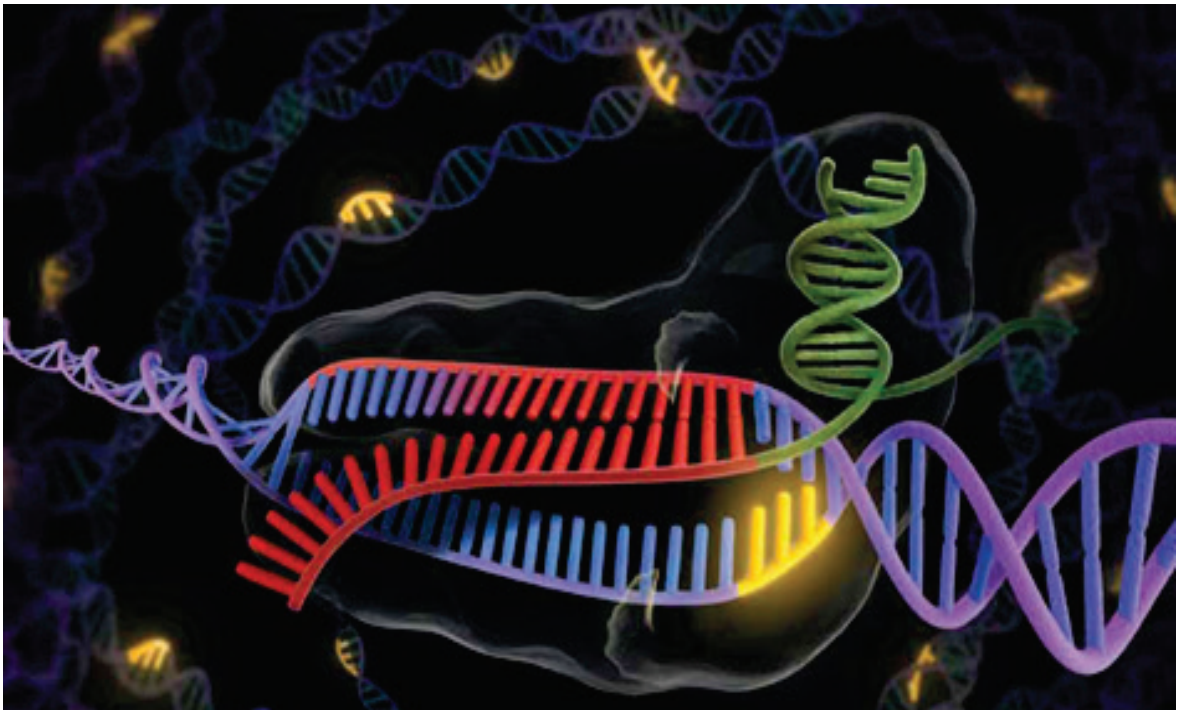
Invasive Species Management on Military Lands

Clustered Regularly Interspaced Short Palindromic Repeat/ CRISPR-associated protein 9 (CRISPR/Cas9)-based Gene Drives

Ping Gong

June 2017



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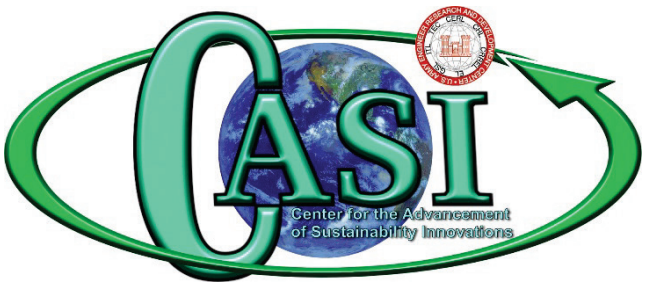

Final report

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Washington, DC 20314-1000

Under Project 611102AH68, "CRISPR/Cas9-based Gene Drives for Invasive Species Management on Military Lands"

Abstract

Applications of genetic engineering-based technologies to the control of invasive species are emerging as an important area of innovation. As a mechanism to spread the desired genes and associated traits into the target population, gene drive is recognized as a promising genetic biocontrol strategy to combat invasive species. Unlike existing technologies that depend on the cumbersome custom-making of new proteins for each DNA target, the CRISPR system uses RNA as its DNA-homing mechanism, which makes it low cost, high efficiency, easy to implement, and dramatically shortens the design-build-test cycle for gene drive development. The CRISPR/Cas9 technology can alter multiple loci at the target gene that prevent mutations from blocking the spread of the drive. It can also be used to develop multiple types of gene drives such as precision, immunization, and reversal drives to precisely target a specific subpopulation, protect a population from any future gene drive "invasions", and overwrite previously released drives in case of unanticipated effects, respectively. Poised to become a self-sustaining, highly efficient, environmentally benign, and cost-effective alternative for invasive species control, CRISPR/Cas9-mediated gene drives can sweep a "deleterious" gene through a population exponentially faster than the normal Mendelian inheritance, and thus suppress or eradicate the target invasive species.

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Preface

This study was conducted for the Headquarters, U.S. Army Corps of Engineers (HQ, USACE) under project 611102AH68, "CRISPR/Cas9-based Gene Drives for Invasive Species Management on Military Lands." The technical monitor was HQ, USACE.

This study was performed under the Center for the Advancement of Sustainability Innovations (CASI) Program for the U.S. Army Engineer Research and Development Center (ERDC) in coordination with the office of the Assistant Secretary of the Army Installations, Energy, and Environment (ASA(IE&E)) and the U.S. Army Installation Management Command (IMCOM). The Director of CASI was Franklin H. Holcomb.

The work was performed by the Environmental Processes Branch (EPP) of the Environmental Processes and Engineering Division (EP), ERDC Environmental Laboratory (ERDC-EL). At the time of publication, Dr. Brandon Lafferty was Chief of EPP; Warren Lorentz was Chief of EP; and Dr. Patrick N. Deliman was the Technical Director for the Civil Works Program. The Deputy Director of ERDC-EL was Dr. Jack Davis, and the Director was Dr. Beth Fleming.

COL Bryan S. Green was Commander of ERDC, and Dr. David W. Pittman was the Director.

1 Introduction

1.1 Background

The spread of invasive species is recognized as one of the major factors contributing to ecosystem change and instability throughout the world. Executive Order 13112 (National Archives and Records Administration (NARA) 1999) defines an invasive species as "an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health." The National Invasive Species Council (NISC) and the Invasive Species Advisory Committee (ISAC) further clarified and defined the term as "plants, animals, or pathogens that are non-native (or alien) to the ecosystem under consideration and that cause or are likely to cause economic or environmental harm or harm to human, animal, or plant health" (Beck et al. 2008).

Alien invasive species are a leading threat to the nation's rich biodiversity, as well as to national security, the economy, and human health (Weldy 2008). For instance, non-native species invasions have been shown to modify ecosystem processes (e.g., nutrient cycling, fire frequency, hydrologic cycles, sediment deposition, and erosion) (Kelly 2002). In addition, they have been shown to cause vector-borne infectious diseases (WHO 2015) such as West Nile virus (CDC 2015), suppression of native species (Pimentel et al. 2005), loss of biodiversity (Stein et al. 2000), and decreased values of recreational properties (Beck et al. 2008; Weldy 2008). In practice, a species is regarded as invasive if it has been introduced by human action to a location, area, or region where it did not previously occur naturally (i.e., is not a native species), becomes capable of establishing a breeding population in the new location without further intervention by humans, and becomes a pest in the new location, directly threatening agriculture or the local biodiversity.

In 2007, the Invasive Species Specialist Group (ISSG) of the International Union for Conservation of Nature's (IUCN) Species Survival Commission (SSC) surveyed records of harmful or potentially harmful species in 16 countries and identified 1,453 species with records of invasiveness and 14,121 taxa of potentially invasive species (ISSG 2007). In 2011, the Invasive Species Profile System of the U.S. Army Corps of Engineers (USACE) inventoried 205 aquatic and wetlands animal species, 309 aquatic and

wetlands plant species, 369 terrestrial animal species, and 1,429 terrestrial plant species (http://corpslakes.usace.army.mil/employees/invasive/isps/index.cfm). The lists of invasive species are growing and many databases have been built to curate information about invasive species on regional, national, continental, or global scales (see www.invasivespeciesinfo.gov/resources/databases.shtml). For instance, the Global Invasive Species Database (GISD) profiles invasive alien species that threaten native biodiversity and covers all taxonomic groups from microorganisms to animals and plants (www.issg.org/database/welcome/).

1.2 Invasive species management and control methods

The subject of invasive species management (e.g., integrated pest management) is very extensive and covers planning and prioritizing threats, inventory and survey, monitoring, control methods, prevention, and restoration or revegatation. The greatest challenge for invasive species management is the development of cost-effective, sustainable, and environmentally benign methods for invasive species control. Control and management efforts are usually site- and species-specific. A wide variety of control methods exists, including biological, chemical, and physical technologies (Tu et al. 2001). For instance, biopesticides derived from such natural materials as animals, plants, bacteria, and certain minerals are often used in biological control (biocontrol) of invasive species. A good example is *Pseudomonas fluorescens* strain CL145A, a biopesticide for the control of zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) (Molloy et al. 2013). Chemical control is the application of conventional chemical-based insecticides and herbicides, whereas examples of physical control are mechanical removal (e.g., mowing) and burning of invasive weeds. The focus of this Special Report is on the technologies developed and scientific progress made in one particular type of biocontrol methods, genetic engineering-based bio-control, or genetic control of invasive species.

1.3 Problem statement

Alien invasive species cause nearly $138 billion worth of loss and damages in the United States each year (Pimental et al. 2005), while worldwide the cost estimates are close to a trillion dollars (Pimental 2002). Invasive species management costs the U.S. Army millions of dollars by interfering with training exercises, posing health problems, and diverting funding from other projects (Shearer et al. 2013). Numerous military installations

across the country and overseas have employed a variety of chemical, physical, and biological methods to combat invasive species. These conventional methods have limited success and are inadequate because they are generally effective only on small-space scales or short time frames (Thresher 2007). Repeated treatments are often required due to the recurrence of invasive species infestation, for instance:

- Mechanical methods for weed control are particularly useful if the population is relatively small. Although this method can specifically target weed species and thereby minimize harm to other plants, mechanical removal can be labor intensive and impractical for large infestations (Hodgins et al. 2009).
- Insecticides alone are insufficient for chemical control of mosquito populations since reduced susceptibility and even resistance is being observed more and more frequently. There is also increased concern about the toxic effects of insecticides on non-target (even beneficial) insect populations, humans, and the environment (Bourtzis et al. 2014).
- In natural populations of alien species, insect herbivores can be introduced to control the spread of their invasive hosts. Biocontrol has met with considerable success in some species (Myers and Bazely 2003), but poses some additional concerns including the potential for the control agent to switch hosts from the introduced plant to a native species (Louda et al. 1997).

Hence, there has been considerable incentive and increasing demand to develop innovative technologies for cost-effective, sustainable, and environmentally benign alternatives of invasive species control (Hodgins et al. 2009).

1.4 Objectives

This Special Report surveys genetic engineering-based technologies developed for invasive species control, describes the current status of technical development in this area, and provides recommendations for technical solutions, research and development (R&D) needs, and future direction. In particular, it focuses on a recently emerged powerful and innovative technology called CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated protein 9) (Pennisi 2013). Two proof-of-concept studies are presented to demonstrate how the CRISPR/Cas9 technology can be employed to develop cost-effective, self-sustaining, and

environmentally benign gene drives for efficient control and eradication of invasive pests. Biosafety, ecological risks, regulations, public perceptions, and limitations surrounding the development and release of CRISPR/Cas9-based gene drives are also discussed.

2 Review of Genetics-based Technologies for Bio-control of Invasive Species

During the early 1960s, entomologists speculated that genetics-based technologies could be a powerful means of controlling pest populations based on the observation that the meiotic drive, acting as a genetic sex ratio distorter through a mutant Y chromosome, had apparently driven some insect populations to extinction (Hamilton 1967). However, practical development of such technologies laid fallow. Recently, advances in genetic engineering technologies in combination with the lack of other options to effectively control established pest populations led to renewed interest in this idea (Thomas et al. 2000; Thresher et al. 2014). Meanwhile, a number of *in silico* studies have modeled the potential of these genetics-based methods that have been proposed for pest (e.g., Bax and Thresher 2009; Teem et al. 2014; Team and Gutierrez 2014; Gentile et al. 2015) and weed (Hodgins et al. 2009) control, incorporating varying degrees of ecological reality.

Genetics-based biocontrol (referred to as genetic control henceforth) refers to the intentional, environmental release of genetically manipulated organisms that are designed to disrupt the survival or reproduction of a targeted invasive species (Kapuscinski and Sharpe 2014). It depends on the dissemination of heritable factors that reduce pest damage or suppress or eradicate the population of an invasive species (Alphey et al. 2013; Alphey 2014; Gilna et al. 2014; Thresher et al. 2014). In general, it involves manipulations of chromosomes of a target species in order to skew sex ratios of the target species, recombinant DNA techniques to insert a deleterious gene construct into the target species' genome in order to disrupt the organism's life cycle, or a combination of both techniques (Kapuscinski and Sharpe 2014).

Genetic control strategies share the following key features (Alphey 2014):

1. They involve the rational and intentional manipulation (modification and/or introduction) of a heritable element into the target population, so that the "modified pest becomes a biocontrol agent against its unmodified brethren" (Gilna et al. 2014).

2. All genetic strategies exploit the mate-seeking behavior of the modified insect to provide a control agent that self-disperses and actively seeks wild pest insects.
3. They are area-wide methods, in contrast to vaccines, drugs, and bednets, which are directed at individual humans (though widespread use may have additional community effects). As a result, those in the program area are equally protected irrespective of wealth, social status, or education.
4. Because they are mating-based, genetic methods are also extremely species-specific. This minimizes off-target effects, but where multiple vector species are present, multiple control tools may be required.

This dependence on mating and vertical transmission distinguishes genetic control strategies from other biocontrol methods such as the use of predators, parasitoids, or infectious microbial agents. Instead, the control agent is a version of the pest itself with a heritable modification that alters its properties in a desirable way (Alphey et al. 2013).

Genetic control strategies can be classified in several ways. Thresher et al. (2014) divided those for invasive fish control into self-propagating, chromosomal, and recombinant approaches. The self-propagating approach uses a genetically modified agent (usually a virus) to spread a recombinant lethal or sterilizing construct, e.g., immune-contraception (Hardy et al. 2006). The chromosomal approach includes chromosomal manipulation resembling Trojan Y (Gutierrez and Teem 2006) and sterility by triploidy (Benfey 2011), whereas the recombinant approach includes "sterile feral" (e.g., the recombinant analogue of triploidy) (Thresher et al. 2009) and autocidal (Gould and Schliekelman 2004) technologies. The strengths and weaknesses of these approaches are summarized in Table 1.

Table 1. Strengths and weaknesses of different genetic options for invasive species control.

Genetic options	Chromosomal	Recombinant
Self-propagating	NA	Strength Potentially very high efficiency Potentially applicable to a wide range of vertebrates Low maintenance costs after release Weakness High developmental costs Species-specificity uncertain (particularly for viral agents)

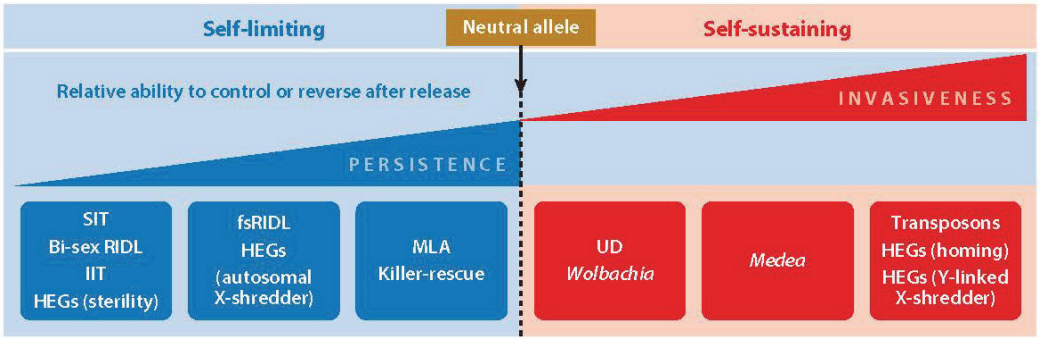
Genetic options	Chromosomal	Recombinant
		Public acceptability is likely to be low Legislative/policy impediments in some jurisdictions Introduction to native range may result in unintended extinction
Sterile male/ female release	Strength Technology available (triploidy) for fish and amphibians; applicability to other vertebrate taxa is unlikely Species-specific outside of naturally occurring hybridization cases (e.g., carp-goldfish) Public acceptability high Few legislative/policy impediments Low cost Weakness Only males participate in matings High stocking rates required Reduced growth rate in some taxa, potentially resulting in low competitiveness and/or survival	Strength Prototype available for fish Potentially applicable to wide range of vertebrates Both sexes fertile and sterile Species-specific Potential recombinant options for increasing efficacy Weakness Developmental work still required Moderately high cost to produce and maintain brood lines High stocking rates required Public acceptability uncertain Legislative/policy impediments in some jurisdictions
Autocidal	Strength Technology (Trojan Y) available in part (YY males); viability of YY females uncertain and likely to differ between species Applicable to fish and amphibians; applicability to other vertebrates unknown Applicable to live-bearing fish Species-specific outside of naturally occurring hybridization cases (e.g., carp-goldfish) Publicly acceptable Few legislative/policy impediments Low cost Efficacy is unknown, but potentially moderate Weakness Sex ratio manipulation only Carrier fitness and competitiveness unknown Effects of autosomal sex modifying genes Effects of environmental temperature on sex ratios unknown Developmental work required, although basic technology for chromosome and gender manipulations available	Strength Questionable efficacy, but potentially moderate to high, depending on copy number Potential recombinant options for increasing efficacy Potentially applicable to wide range of vertebrates Alternative and complementary genetic strategies can be targeted at vulnerable life history stages/traits Alternative and complementary genetic strategies can be applied sequentially or simultaneously to increase efficacy Weakness High developmental costs Developmental work still required Carrier fitness and competitiveness unknown and likely to vary depending on construct Species-specificity is likely to vary depending on construct Public acceptability is uncertain Legislative/policy impediments in some jurisdictions

Genetic control strategies can also be categorized according to the intended outcome, or the expected dynamics (persistence) of the genetic element in the target population (Alphey et al. 2013; Alphey 2014; Burt 2014). In

outcome-based classification, individuals in the target population are either suppressed or replaced. Similar to the goal of insecticide-based programs, the suppression strategies reduce the number of competent individuals in the target population or area (Alphey 2014). In contrast, replacement strategies do not actually replace; instead, they spread a novel trait, such as reduced ability to transmit a pathogen, into the target population through breeding of released genetically modified individuals with wild individuals, thereby changing the phenotype of some or all individuals in that population – those that carry the new genetic element (Alphey et al. 2013).

In terms of persistence (and invasiveness), some genetic constructs are self-limiting, having an inherent tendency to decline in frequency and disappear from the population. Repeated releases are necessary to maintain these constructs in the target population, and relatively large (i.e., inundative) releases will usually be necessary to have a significant epidemiological effect. Release of sterile males for population suppression is a clear example. By contrast, other constructs are meant to be self-sustaining, with an inherent tendency to increase in frequency in the target population over multiple generations and maintain themselves at a high frequency. Releases need occur only once or a few times and can often contain relatively fewer mosquitoes (i.e., inoculative releases). The relative persistence or invasiveness of various genetic control systems is shown in Figure 1.

Figure 1. Self-limiting and self-sustaining genetic systems underdominance (Alphey 2014).



Self-sustaining element: a genetic element expected or designed to persist indefinitely and perhaps to increase in frequency and/or invade other populations or species; Self-limiting element: a genetic element or modification that disappears from the target population over time, e.g., through natural selection. Abbreviations: RIDL, release of insects carrying a dominant lethal genetic system; fsRIDL, female-specific RIDL; HEG, homing endonuclease gene; IIT, incompatible insect technique; MLA, multi-locus assortment; SIT, sterile insect technique; UD, underdominance.

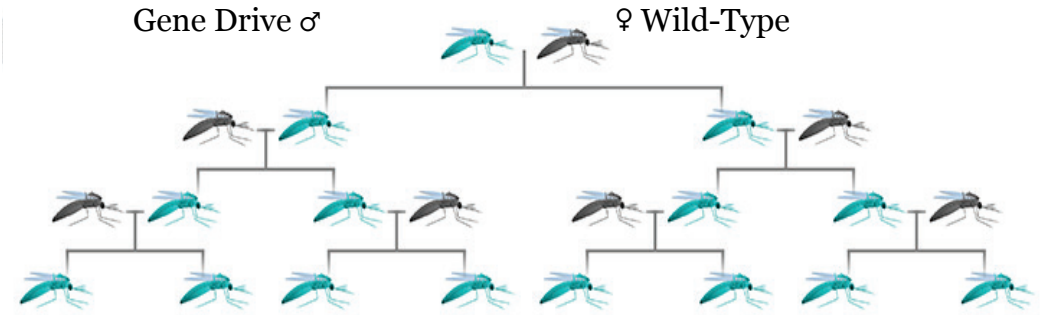
3 Gene Drive and Homing Endonuclease Genes (HEGs)

A gene drive refers to a mechanism that spreads the desired gene and its effect (e.g., sterility or inability to transmit a pathogen) into the target population in a timeframe that is appropriate for disease control or population eradication (Benedict et al. 2008). As the practice of "stimulating biased inheritance of particular genes to alter entire populations" (Pennisi 2014), gene drive systems utilize selfish genetic elements to drive a trait through the target population, where the trait in question may be deleterious, e.g., sterility, or relatively benign, like resistance to parasite or virus carriage (Chan et al. 2011). Selfish genes are DNA elements that increase their rate of genetic transmission at the expense of other genes in the genome and can therefore quickly spread within a population (Simino et al. 2014). For example, an organism carrying one copy of an altered gene (e.g., male determination gene) normally passes it on to 50% of offspring. A gene drive can ensure that nearly all offspring inherit the altered gene, causing it to rapidly spread through the population - even if it is mildly deleterious to each individual's chance of reproducing (Figure 2). The advantages of gene drive strategies over other genetic controls are apparent, they allow for a smaller release population since the frequency of the selfish element is expected to grow in the population. In addition, the selfish element could potentially have persistent negative impacts on the target population. Finally, selfish elements' ability to spread allows effects that are distant from the site of release, a characteristic that is very useful when the target area is inaccessible owing to infrastructure or human conflict (Chan et al. 2011).

Homing endonuclease genes (HEGs) are a collection of selfish DNA elements encoding proteins (endonucleases) that recognize and cleave specific DNA sequences of ~20–30 nucleotides (Alphey 2014). These sequences are called HEG recognition loci. HEGs catalyze the hydrolysis of genomic DNA within the cells that synthesize them, but do so at very few, or even singular, locations. Repair of the hydrolyzed DNA by the host cell frequently results in the gene encoding the homing endonuclease having been copied into the cleavage site, hence the term "homing" to describe the movement of these genes. The coding sequences of HEGs are located in the middle of their own recognition sequence at the same locus on the ho-

mologous chromosome, thus preventing the HEG-bearing chromosome from being cleaved.

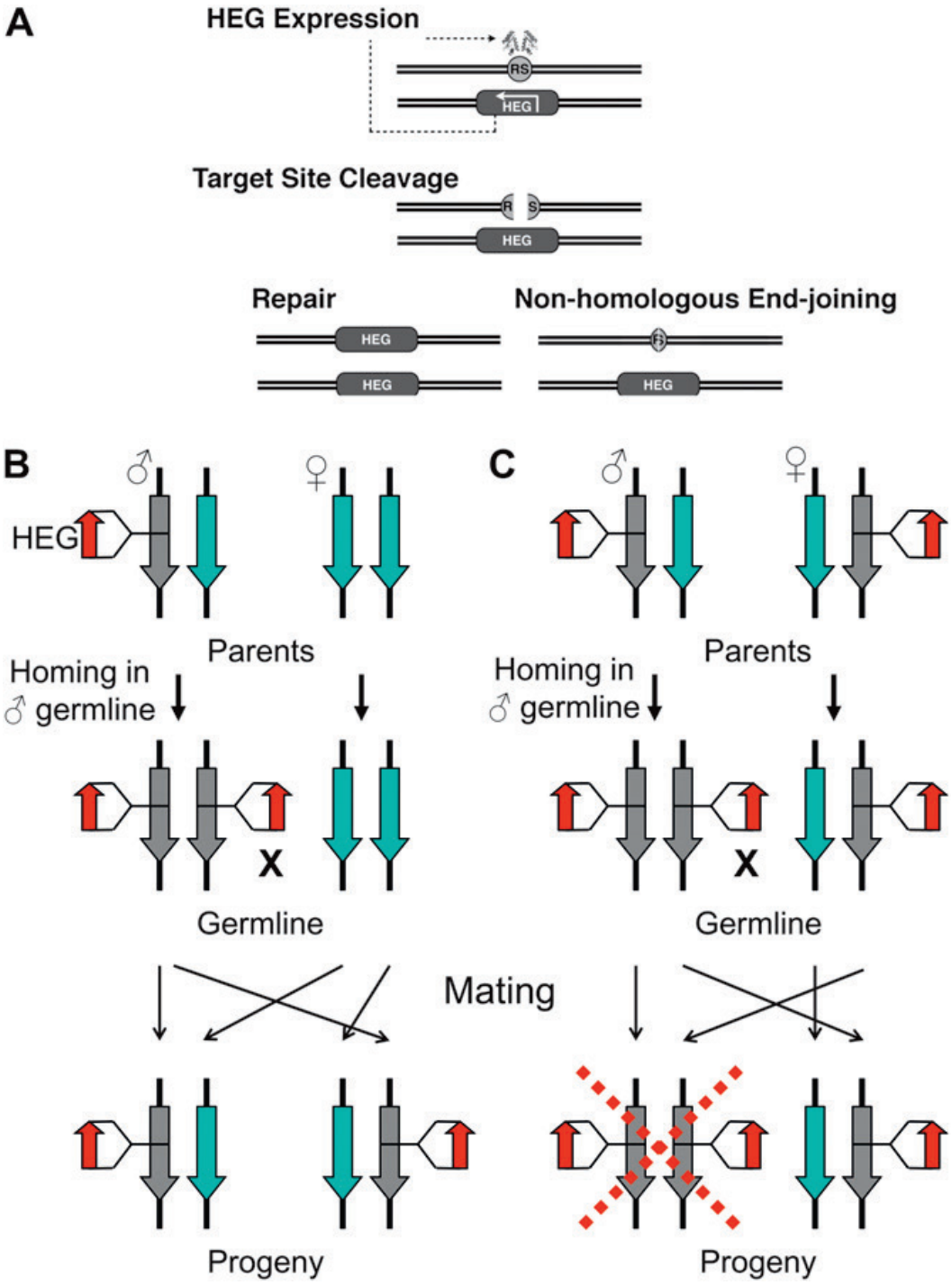
Figure 2. Hypothetic illustration of a male-dominant gene drive quickly spreading through the entire target population via mating after a number of generations (Adapted from Esvelt et al. 2014).



In an animal trans-heterozygous for an HEG-containing chromosome, transcription and translation of the HEG in the germline produce an active nuclease (Figure 3A). The enzyme makes a double-strand break (DSB) at its recognition sequence (RS) on the target. The DSB can be repaired by the desired homologous recombination route, converting the target chromosome to an HEG-containing donor, or by an undesirable nonhomologous end-joining event.

Figure 3B illustrates a population control strategy by considering an HEG transgene inserted into and disrupting a target gene that it homes with 100% efficiency in the male germline. It is further assumed that the target gene is recessive lethal because it plays an essential somatic role, but is not required for fertility. When a male trans-heterozygous for the transgene is mated to a wild-type female, the HEG homes onto its homologous chromosome in the germline. The animal remains viable as somatic tissues remain heterozygous for the HEG insertion; however, all gametes from the male now bear the HEG insertion. As a consequence, all progeny from this mating will be heterozygous for the HEG insertion, thereby increasing the frequency of this insertion within the population. In the event of two heterozygotes mating (Figure 3C), all sperm from the male carry the HEG insertion, while only half of the oocytes do. Half of the progeny will be homozygous for the HEG insertion-disrupted gene and therefore inviable while the remaining progeny are heterozygous for the HEG insertion and available to further propagate the HEG insertion.

Figure 3. Conceptual illustration of an HEG strategy (Chan et al. 2011).



HEGs can thereby transmit horizontally within a host population, increasing their allele frequency at greater than Mendelian rates. It has long been proposed that the elegant mechanisms by which such naturally occurring selfish genetic elements as HEGs spread at the expense of their hosts have enormous potential and can be harnessed to build effective and self-

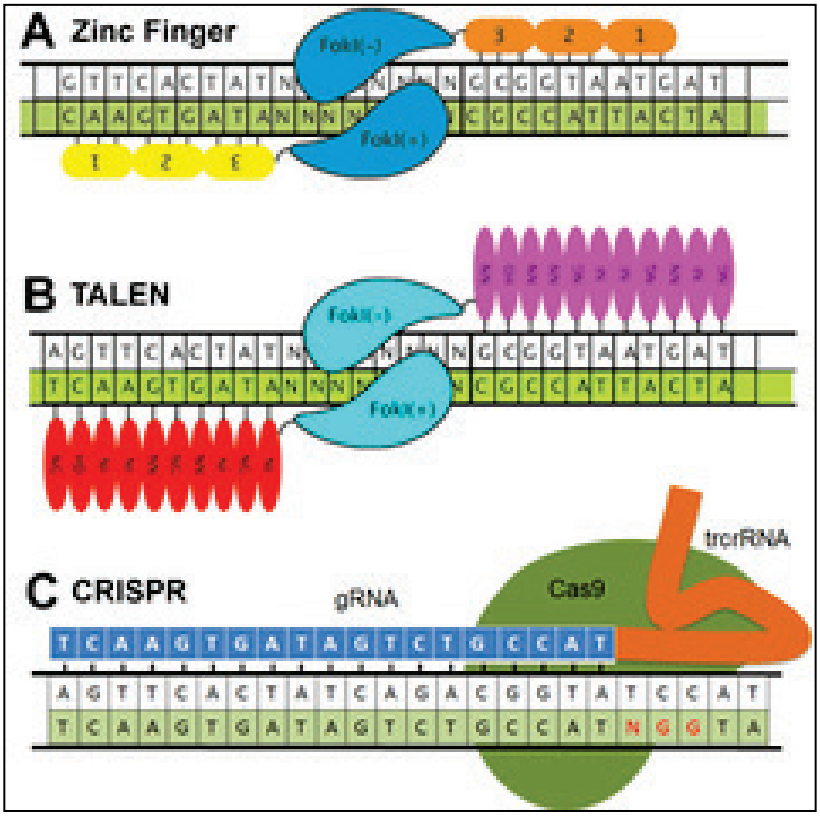
sustaining gene drive systems for the control of invasive species (Burt 2003; Sinkins and Gould 2006).

The HEG-based gene drive approach has shown promise in initial laboratory evaluations with both the fruit fly, *Drosophila melanogaster* (Chan et al. 2011, 2013a) and the malaria vector, *Anopheles gambiae* (Windbichler et al. 2008, 2011; Galizi et al. 2014). Considerable efforts have been directed towards deploying the strategy for the control of mosquito populations. In models of HEG drive systems, it is strategically advantageous to target female-specific genes required for fertility (Deredec et al. 2008, 2011); A panel of putative *Anopheles gambiae* female germline-specific genes was identified using bioinformatics approaches (Baker et al. 2011). Screening this panel against a library of known target sites for naturally-occurring LAGLIDADG homing endonucleases (LHE) yielded a number of hits that were subsequently ranked by predicted ease of creating redesigned HEGs (Taylor et al. 2012). For instance, it has been demonstrated that a synthetic sex distortion system based on the homing endonuclease I-Ppol is able to selectively cleave ribosomal gene sequence of *Anopheles gambiae* that are located exclusively on the mosquito's X chromosome (Windbichler et al. 2008, 2011), and that shredding of the paternal X chromosome (X-shredder) during spermatogenesis leads to fully fertile mosquito strains that produce >95% male offspring (Galizi et al. 2014).

There are two main requirements for the success of the engineered HEG strategy. First, homing must occur in a metazoan. To date, all examples of homing have been observed in unicellular organisms and no genes resembling HEGs have been found in any metazoan genome (e.g., see Burt and Trivers 2005). Second, to effectively initiate the HEG strategy, an HEG must be precisely inserted at the cleavage site within its host target gene, for example through repair of the cleavage from an ectopic template containing the HEG insert at a non-homologous site (Chan et al. 2011). Although many genetic engineering studies have demonstrated successful site-specific cutting and homing of HEGs in several metazoan hosts (e.g., Chan et al. 2011, 2013a; Windbichler et al. 2008, 2011), routinely redirecting or redesigning the DNA target specificity of an HEG remains a major hurdle. A wide variety of methods including computational design, structure-based engineering, and selection or directed evolution has been attempted for this purpose (Stoddard 2011; Takeuchi et al. 2014; Thyme et al. 2014). Currently, there are six known HEG families with the following conserved structural motifs: LAGLIDADG, GIY-YIG, His-Cys box, H-N-H,

PD-(D/E)xK, and Vsr-like (Belfort and Roberts 1997). A bioinformatic analysis found hundreds of recognizable LHE genes within microbial genomic sequence databases (Takeuchi et al. 2011; Taylor et al. 2012). Until now, only one LHE (i.e., I-CreI) (Gao et al. 2010; Munoz et al. 2011) and one His-Cys box homing endonuclease (i.e., I-PpoI) (Galizi et al. 2014) had been successfully engineered and used for the targeted modification of actual physiological coding sequences. However, several additional LHEs (including I-MsoI, I-SceI, I-AniI and I-PpoI) had been redesigned in a more limited manner (Stoddard 2011) or had produced a reduced homing frequency (e.g., I-OnuI, Chan et al. 2013b). These results, despite early hopes, have proven that it is difficult to redesign homing endonucleases with customized specificity, i.e., engineer HEGs to cleave new target sequences of interest (Esvelt et al. 2014).

Figure 4. Schemes of ZFN, TALEN, and CRISPR-Cas9 technologies that allow the introduction of double stranded breaks at specific sequences.



To alleviate the difficulties in customized specificity of HEGs, efforts have been made to engineer other modular endonuclease (Sakuma and Woltjen 2014), such as transcription activator-like effector nucleases (TALENs) (Miller et al. 2011) and zinc-finger nucleases (ZFNs) (Urnov et al. 2010) into site-specific synthetic selfish elements to cut new target sequences.

Recently, new versions of the fruit fly gene drive using modular ZFNs or TALENs were constructed in place of the homing endonuclease (Simoni et al. 2014). They both act as a dimer in which each monomer binds on complementary DNA strands and the FoKI nuclease directs its activity to the spacer between the recognition sequences generating a 5'-overhang (Figure 4A and 4B). ZFN technology utilizes a restriction endonuclease FokI as the DNA-cleavage domain and binds DNA by engineered Cys_2His_2 zinc fingers. Specific zinc fingers recognize different nucleotide triplets and dimerize the FokI nuclease. The activated nuclease introduces a double stranded break between the two distinct zinc finger binding sites (Figure 4A). TALENs are artificial restriction enzymes generated by fusing a transcription activator-like effector (TALE) domain derived from the *Xanthomonas spp*. to a FokI domain. By modifying the amino acid repeats in the TALEs, one can customize TALEN systems to specifically bind target DNA and induce cleavage by the nuclease between the two distinct TALE array-binding sites. Individual TALEN modules bind to single nucleotide and 15 to 19 modules are assembled together to form a functional nuclease (Figure 4B). While initially successful at cutting and homing in the fruit fly, both ZFN and TALEN declined in effectiveness over time due to the evolutionary instability of the modular repeats inherent to those proteins (Simoni et al. 2014; Esvelt et al. 2014).

Early attempts described above demonstrate that it is possible to build synthetic gene drives, and they emphasize the importance of cutting any desired gene and remaining stable during copying (Esvelt et al. 2014). Until now, a gene drive capable of spreading efficiently through a wild population had not been developed, mainly owing to the difficulty of programming drives to cut desired sequences at high efficiency (Oye et al. 2014). The recent discovery of the RNA-guided CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR associated) nuclease system represents a promising solution. Unlike ZFN and TALEN technologies, both of which depend on custom-making new proteins for each DNA target, the CRISPR/Cas systems use RNA (i.e., guide RNA (gRNA)) as its DNA-homing mechanism (Figure 4C). These gRNAs are much easier to make than proteins used in ZFNs and TALENs.

4 CRISPR-Cas9-mediated Gene Drives as a Novel Genetic Control Approach

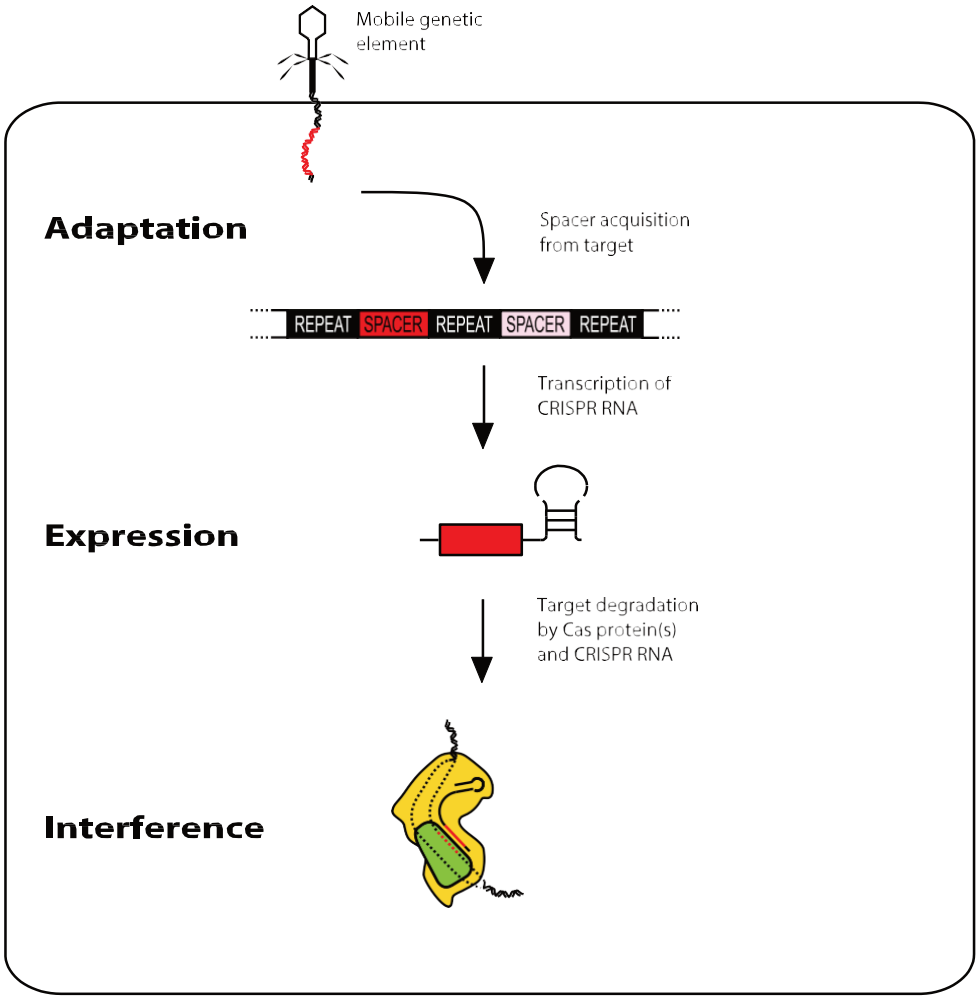
The following sections provide a brief overview of CRISPR-Cas9-mediated gene drives, detail the mechanistic reasons that RNA-guided gene drives are likely to be effective in many species, and outlines probable capabilities and limitations. Furthermore, novel gene drive architectures are proposed that may substantially improve the control over gene drives and their effects; possible applications are discussed; guidelines for the safe development and evaluation of this promising, but, as yet, unrealized technology are suggested; and risk governance and regulatory issues intended specifically for policymakers are discussed.

4.1 The biological mechanism of CRISPR/Cas-mediated adaptive defense

CRISPR/Cas systems have evolved in bacteria and archaea as an elegant adaptive defense mechanism to provide acquired resistance to invading viruses and plasmids (Horvath and Barrangou 2010; Terns and Terns 2011; Wiedenheft et al. 2012). As suggested by its name, the CRISPR is an array of short repeated sequences separated by spacers with unique sequences. CRISPRs function together with CRISPR-associated (cas) genes that typically flack CRISPR loci in the genome, and the entire pathway is consequently referred to as CRISPR-Cas (Sternberg and Doudna 2015). The CRISPR-Cas mediated defense process can be divided into three stages (Figure 5).

The first stage, adaptation or acquisition, leads to insertion of new spacers in the CRISPR locus. In the second stage, expression, the system prepares for action by expressing the cas genes and transcribing the CRISPR into a long precursor CRISPR RNA (pre-crRNA). The pre-crRNA is subsequently processed into mature crRNA by Cas proteins and accessory factors. In the third and final stage, interference, target nucleic acid is recognized and destroyed by the combined action of crRNA and Cas proteins (Rath et al. 2015). The spacers (termed protospacers) are often derived from nucleic acid of viruses and plasmids, an observation that gave rise to the idea that CRISPRs are part of an anti-virus system (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005).

Figure 5. The key steps of CRISPR-Cas immunity (Rath et al. 2015).

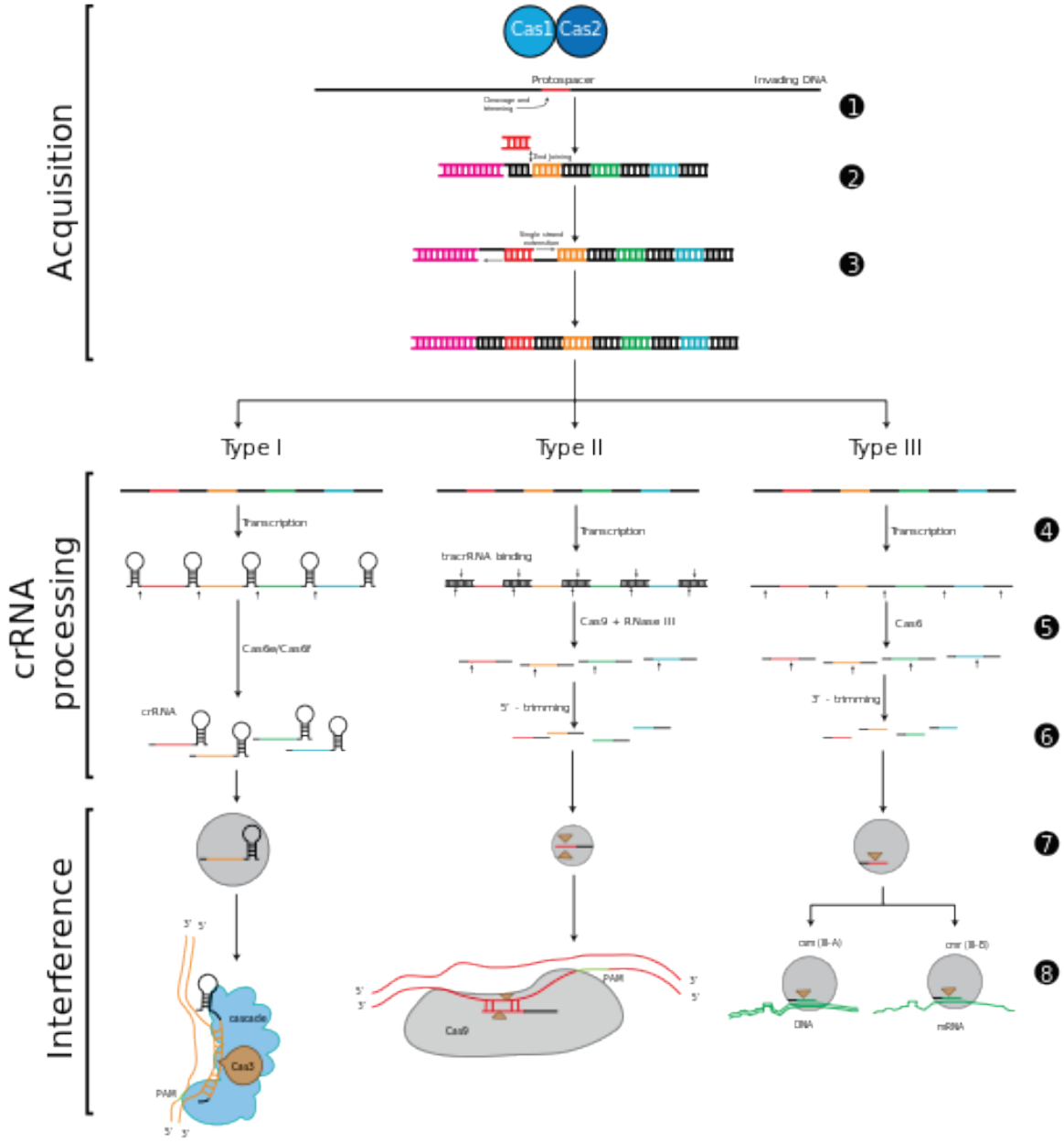


Adaptation: insertion of new spacers into the CRISPR locus. Expression: transcription of the CRISPR locus and processing of CRISPR RNA. Interference: detection and degradation of mobile genetic elements by CRISPR RNA and Cas protein(s).

So far, three distinct bacterial CRISPR systems have been identified and termed Type I, II, and III. The main steps of their pathways are as follows (Figure 6): (1) Acquisition begins by recognition of invading DNA by Cas1 and Cas2 and cleavage of a protospacer. (2) The protospacer is ligated to the direct repeat adjacent to the leader sequence, and (3) a single strand extension repairs the CRISPR and duplicates the direct repeat. The crRNA processing and interference stages occur differently in each of the three major CRISPR systems. (4) The primary CRISPR transcript is cleaved by cas genes to produce crRNAs. (5) In Type I systems, Cas6e/Cas6f cleave at the junction of single-stranded (ss) and double-stranded (ds) RNA formed by hairpin loops in the direct repeat. Type II systems use a trans-activating (tracr) RNA to form dsRNA, which is cleaved by Cas9 and RNase III. Type III systems use a Cas6 homolog that does not require hairpin loops in the

direct repeat for cleavage. (6) In Type II and III systems, secondary trimming is performed at either the 5' or 3' end to produce mature crRNAs. (7) Mature crRNAs associate with Cas proteins to form interference complexes. (8) In Type I and II systems, base-pairing between the crRNA and a short (3-5 bp) DNA sequence adjacent to protospacers termed Protospacer Adjacent Motif (PAM) causes degradation of invading DNA. Type III systems do not require a PAM for successful degradation.

Figure 6. The three major types of CRISPR-mediated adaptive immunity (Wikipedia 2015).



The classification of CRISPR-Cas immune systems is based primarily on cas gene phylogeny (Makarova et al. 2011). Although many mechanistic features are widely conserved, significant differences exist. For example, Type I and III systems require only crRNA for targeting, while Type II systems use both crRNA and tracrRNA (Deltcheva et al. 2011). In addition, the protein composition of crRNA-Cas targeting complexes is highly variable with complexes in Type I and III systems, typically comprising greater than eight subunits (Brouns et al. 2008; Hale et al. 2009). In contrast, Type II systems require only a single polypeptide: Cas9 (Sapranauskas et al. 2011). The Type II system is the basis for the current genome engineering technology, often referred to as the CRISPR-Cas9 technology.

4.2 The CRISPR-Cas9 technology: a brief description

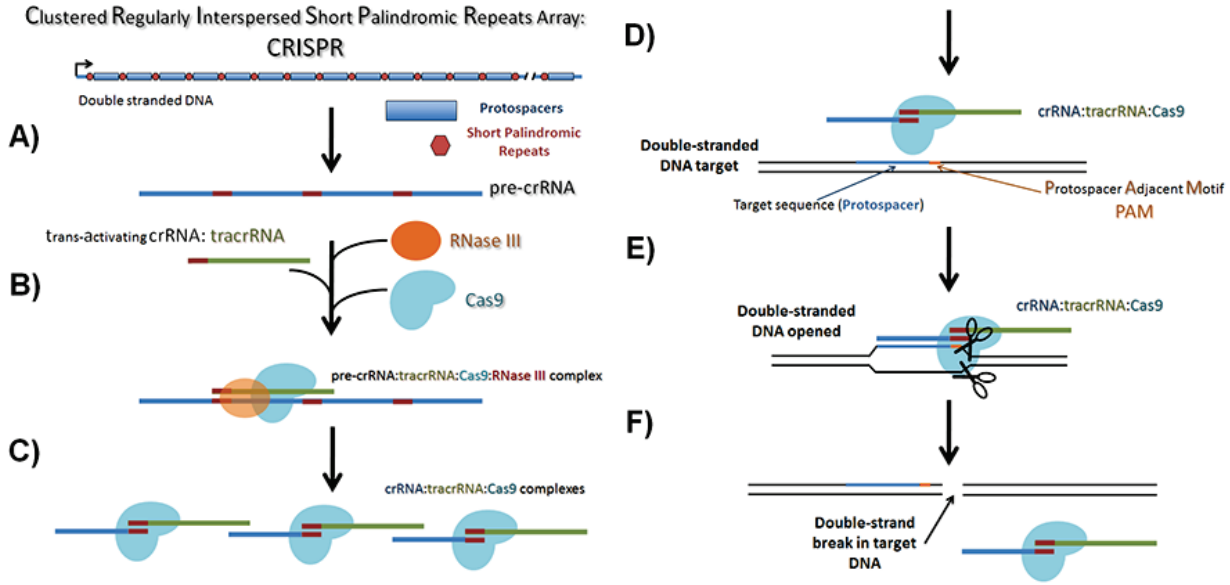
As shown in Figure 7, the crRNA targeting sequences are transcribed from DNA sequences known as protospacers. Protospacers are clustered in the bacterial genome in a group called a CRISPR array. The protospacers are short sequences (~20bp) of known foreign DNA separated by a short palindromic repeat and kept like a record against future encounters. To create the CRISPR targeting RNA (crRNA), the array is transcribed and the RNA is processed to separate the individual recognition sequences between the repeats. The processing of the CRISPR array transcript (pre-crRNA) into individual crRNAs is dependent on the presence of a trans-activating crRNA (tracrRNA) that has sequence complementary to the palindromic repeat. When the tracrRNA hybridizes to the short palindromic repeat, it triggers processing by the bacterial double-stranded RNA-specific ribonuclease, RNase III. At that point, any crRNA and the tracrRNA can both bind to the Cas9 nuclease, which then becomes activated and specific to the DNA sequence complimentary to the crRNA.

Cas9 functions naturally with a dual-guide RNA composed of crRNA and tracrRNA (Jinek et al. 2012); the 5' end of the crRNA base pairs with target DNA, whereas the 3' end forms a double-stranded stem with the tracrRNA to facilitate Cas9 recruitment. Because DNA targets are recognized via RNA-DNA base pairing, changing the sequence of the guide RNA easily alters DNA specificity (Sternberg and Doudna 2015).

Efficient targeting also requires the presence of a short sequence motif (PAM) proximal to the DNA target sequence (Mojica et al. 2009). By specifically selecting spacers at PAM sites during acquisition (Yosef et al. 2012), CRISPR-Cas9 immune systems can discriminate between self and

non-self sequences during interference via PAM recognition. Targets found in foreign DNA contain a PAM and are targeted, whereas matching targets in the CRISPR locus itself, from which the crRNA is transcribed, do not contain a PAM and are avoided. Cas9 from *Streptococcus pyogenes*, which has been the focus of most studies to date, recognizes a 5'-NGG-3' PAM sequence (Jinek et al. 2012; Mojica et al. 2009).

Figure 7. An overview of the endogenous Type II bacterial CRISPR/Cas9 system (Addgene 2015).



Within a bacterial genome (Figure 7), a CRISPR array contains many unique protospacer sequences that have homology to various foreign DNA (e.g. viral DNA). Protospacers are separated by a short palindromic repeat sequence. (A) The CRISPR array is transcribed to make the pre-CRISPR RNA (pre-crRNA). (B) The pre-crRNA is processed into individual crRNAs by a special trans-activating crRNA (tracrRNA) with homology to the short palindromic repeat. The tracrRNA helps recruit the RNAse III and Cas9 enzymes, which together separate the individual crRNAs. (C) The tracrRNA and Cas9 nuclease form a complex with each individual, unique crRNA. (D) Each crRNA:tracrRNA:Cas9 complex seeks out the DNA sequence complimentary to the crRNA. In the Type II CRISPR system, a potential target sequence is only valid if it contains a special Protospacer Adjacent Motif (PAM) directly after where the crRNA would bind. (E) After the complex binds, the Cas9 separates the double stranded DNA target and cleaves both strands after the PAM. (F) The crRNA:tracrRNA:Cas9 complex unbinds after the double strand break.

While Cas9 shares molecular capabilities with other CRISPR-Cas systems, its compositional simplicity has been paramount to its successful application. Not only does it encompass only a single polypeptide, but remarkably, it retains full activity with a chimeric single-guide RNA (sgRNA), generated by connecting the 3' end of the crRNA to the 5' end of the tracrRNA (Jinek et al. 2012). sgRNA-programmed Cas9 was shown to be as effective as Cas9 programmed with separate tracrRNA and crRNA in guiding targeted gene alterations.

4.3 Applications of the CRISPR-Cas9 technology

As a technological breakthrough in genetic engineering (especially targeted genome engineering), the CRISPR-Cas9 system requires only the redesign of the crRNA to change target specificity. This contrasts with other genome editing tools, including ZFNs and TALENs, where redesign of the protein-DNA interface is required. Several recent reviews have described a wide variety of applications of CRISPR-Cas9 in basic biology, translational medicine, synthetic biology, biotechnology, and population ecology (Hsu et al. 2014; Jiang and Marraffini 2015; Rath et al. 2015; Sternberg and Doudna 2015). The rapid growth in these applications has also been greatly accelerated through a combination of open-source distributors such as Addgene, as well as a number of online user forums and computational tools of sgRNA design that minimize off-target effects such as http://www.genome-engineering.org, http://www.rgenome.net/cas-offinder, and http://www.egenome.org.

Generally speaking, the CRISPR-Cas9 technology holds great promises in enabling a broad range of applications from basic biology to biotechnology and medicine (see Figure 8 clockwise from top). Causal genetic mutations or epigenetic variants associated with altered biological function or disease phenotypes can now be rapidly and efficiently recapitulated in animal or cellular models (Animal models, Genetic variation). Manipulating biological circuits could also facilitate the generation of useful synthetic materials, such as algae-derived, silica-based diatoms for oral drug delivery (Materials). Additionally, precise genetic engineering of important agricultural crops could confer resistance to environmental deprivation or pathogenic infection, improving food security while avoiding the introduction of foreign DNA (Food). Sustainable and cost-effective biofuels are attractive sources for renewable energy, which could be achieved by creating efficient metabolic pathways for ethanol production in algae or corn (Fuel). Direct *in vivo* correction of genetic or epigenetic defects in somatic tissue

would be permanent genetic solutions that address the root cause of genetically encoded disorders (Gene surgery). Finally, engineering cells to optimize high yield generation of drug precursors in bacterial factories could significantly reduce the cost and accessibility of useful therapeutics (Drug development). More details about these applications are described below (Figure 9).

Figure 8. Potential applications of the CRISPR-Cas9-based genome engineering technology (Hsu et al. 2014).

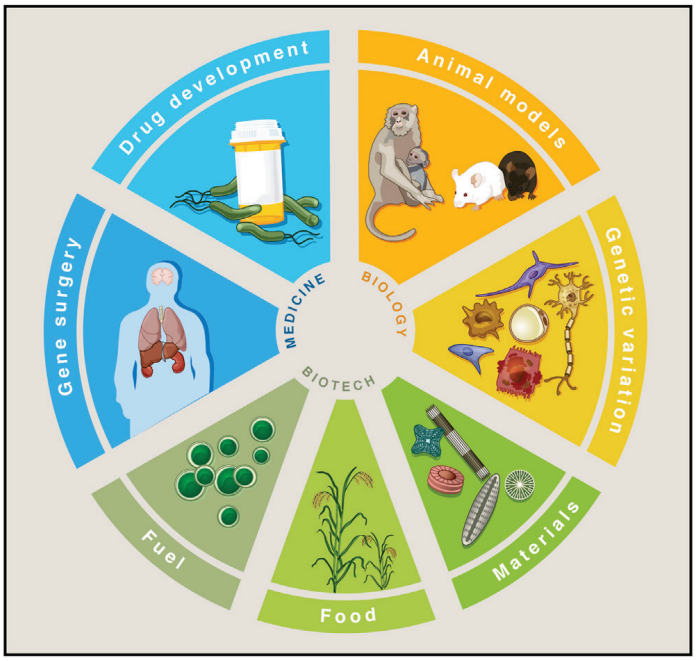
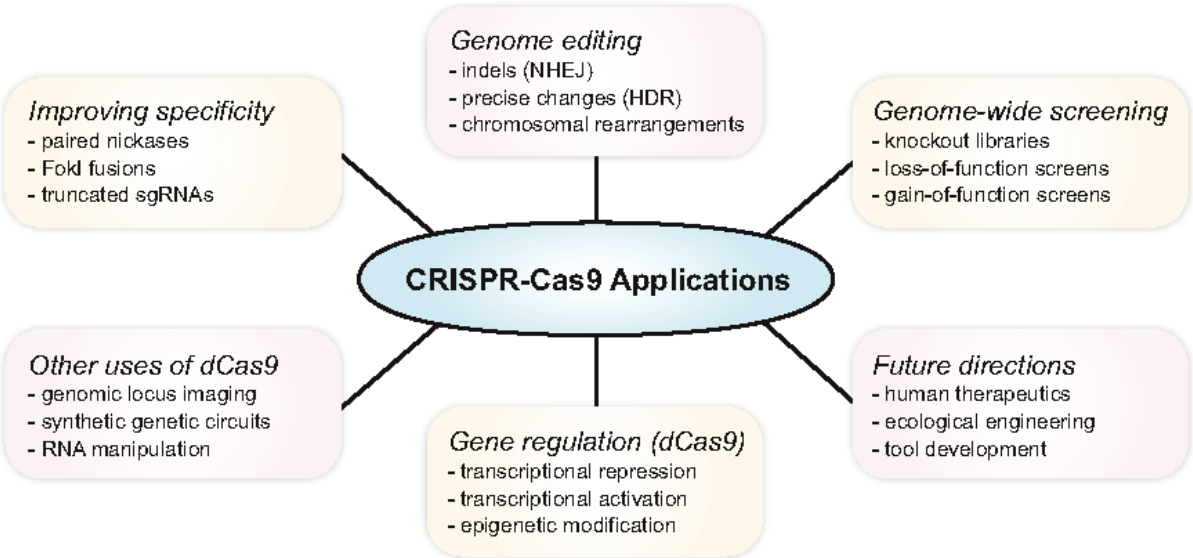


Figure 9. Current and future applications of the CRISPR-Cas9 technology (Sternberg and Doudna 2015).



4.3.1 Genome editing

Targeted genome editing and gene regulation mediated by Cas9 are easy to program, scale, and multiplex, allowing researchers to decipher the causal link between genetic and phenotypic variation. It has been widely adopted as a genome editing tool (i.e., delete, insert, activate, and suppress target genes) and has already been successfully used to target genes of interest in virtually any organism throughout the tree of life (Terns and Terns 2014). In some of those species, precision genetic alteration has never been possible before. Several research groups have now taken advantage of this technology to introduce single point mutations (deletions or insertions) in a particular target gene, via a single gRNA (Nishimasu et al. 2014). Using a pair of gRNA-directed Cas9 nucleases instead makes it possible to induce large deletions or genomic rearrangements, such as inversions or translocations (Gratz et al. 2013).

4.3.2 Genome-wide screening

CRISPR-Cas9 enables rapid genome-scale interrogation of gene function by generating large gRNA libraries for genomic screening (Mali et al. 2013). Using lentiviral sgRNA libraries and catalytically active Cas9, loss-of-function gene knockout screens have been performed in both human and mouse cells (Koike-Yusa et al. 2014; Shalem et al. 2014; Wang et al. 2014a; Zhou et al. 2014). Deep sequencing of the sgRNA pool after either positive or negative selection revealed genes essential for cell viability, as well as genes involved in resistance to specific small-molecule drugs. While focused libraries will prove useful for targeted screens in which the candidate genes are selected by the researcher, genome-wide libraries that query all protein-coding genes will have a greater likelihood of discovering novel hits that were not previously identified (Koike-Yusa et al. 2014; Shalem et al. 2014).

4.3.3 Gene regulation via dCas9

A recent exciting development is that the wild-type Cas9 nuclease can be converted into a generic RNA-guided homing device by deactivating the two conserved nuclease domains (HNH and RuvC) via point mutations. This results in catalytically inactive Cas9 (dCas9). By fusing dCas9 with a transcriptional activation domain or a repressor, targeted transcriptional regulation (i.e., activation or repression) can be achieved (Cheng et al. 2013; Farzadfard et al. 2013; Gilbert et al. 2013; Mali et al. 2013). By add-

ing multiple activating domains, strong induction can be achieved (Konermann et al. 2015). dCas9 can also be used to probe and manipulate the genome in other ways that ultimately rely on specific nucleic acid targeting. For example, microscopic visualization of specific genome loci can be imaged in live cells using dCas9-GFP fusions (Chen et al. 2013); dCas9 fusions to effector domains that install epigenetic markers may also enable specific perturbation of epigenetic regulation (Hu et al. 2014).

4.3.4 Transgenic models

Cas9-mediated genome editing has enabled accelerated generation of transgenic models and expanded biological research beyond traditional, genetically tractable animal model organisms (Sander and Joung 2014). For generation of cellular models, Cas9 can be easily introduced into the target cells using transient transfection of plasmids carrying Cas9 and the appropriately designed sgRNA (Hsu et al. 2014). For generation of transgenic animal models, Cas9 protein and transcribed sgRNA can be directly injected into fertilized zygotes to achieve heritable gene modification at one or multiple alleles in models such as rodents (Wang et al. 2013) and mosquitoes (Kistler et al. 2014).

4.3.5 Gene therapy

Cas9 holds great promise as a therapeutic strategy to treat human genetic diseases, as evidenced by the recent emergence of numerous companies dedicated to this cause (Ledford 2015; Sternberg and Doudna 2015). In proof-of-concept experiments, a disease-causing Fah mutation was successfully corrected in adult mice by hydrodynamic injection of a donor DNA template and plasmid DNA encoding Cas9 and sgRNA (Yin et al. 2014), and Duchenne muscular dystrophy was prevented by directly injecting Cas9 mRNA, sgRNA, and a donor DNA template into the mouse germline (Long et al. 2014). Significant hurdles exist before similar experiments can be performed on human patients, but successes in ZFN-based human clinical trials demonstrate the exciting potential of general approaches using programmable nucleases (Tebas et al. 2014).

4.3.6 Ecological engineering

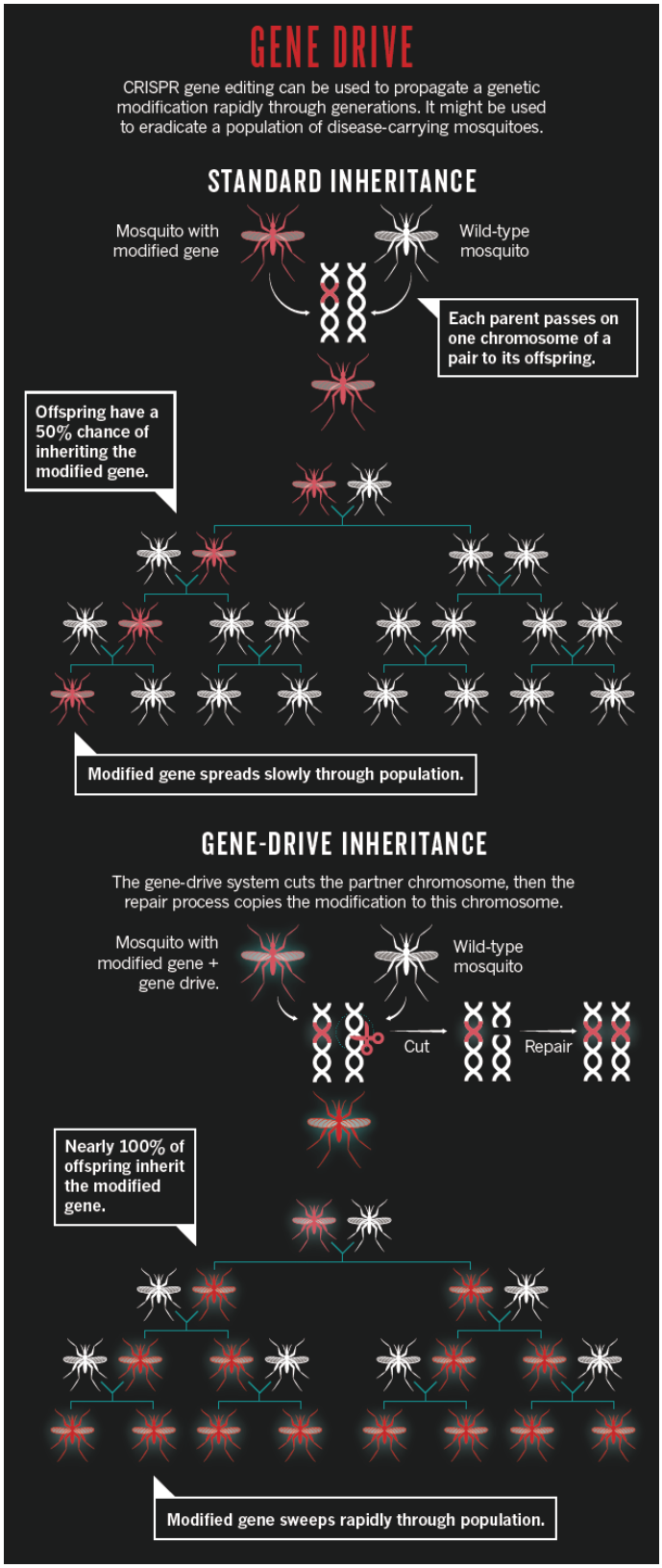
A number of recent studies have highlighted the ability of CRISPR-Cas9 to specifically alter ecological populations. Within microbial communities, Cas9 was packaged in bacteriophage and programmed to selectively kill

virulent bacteria by targeting virulence genes, while leaving other bacteria unaffected (Bikard et al. 2014; Citorik et al. 2014). In animal populations that undergo sexual reproduction, Cas9-based gene drives could be used to rapidly spread altered traits and control invasive species (Esvelt et al., 2014). Two recently published studies have demonstrated that RNA-guided gene drives mediated by CRISPR-Cas9 can efficiently bias inheritance in wild yeast (DiCarlo et al. 2015) and fruit flies (Gantz and Bier 2015). Finally, CRISPR-Cas9 can be used to genetically improve major staple crops such as bread wheat (Wang et al. 2014b). Many of these applications will require renewed attention to existing and future regulatory challenges (Oye et al. 2014; Voytas and Gao 2014).

4.4 Building RNA-guided gene drives using CRISPR-Cas9 for invasive species control

CRISPR-Cas9 relies on exactly the same copying mechanism as homing endonucleases used in gene drives, which can sweep an edited gene through a population exponentially faster than normal (Figure 10). Hence, it is naturally feasible to build gene drives using the CRISPR-Cas9 technology. These Cas9-mediated gene drives can be used for invasive species control, e.g., wiping out disease-carrying mosquitoes or ticks, eliminating invasive plants, or eradicating herbicide resistance in pigweed, which plagues some U.S. farmers (Esvelt et al. 2014; Ledford 2015). However, turning the concept into effective gene drives requires us to consider a number of molecular factors relevant to homing, which includes cutting, specificity, copying, and evolutionary robustness. The following provides a detailed technical analysis of the extent to which CRISPR-Cas9 can address each of these challenges (Esvelt et al. 2014; Figure 11).

Figure 10. The basic concept of CRISPR-Cas9-enabled gene drives for propagating a genetic modification rapidly through a target population (Ledford 2015).



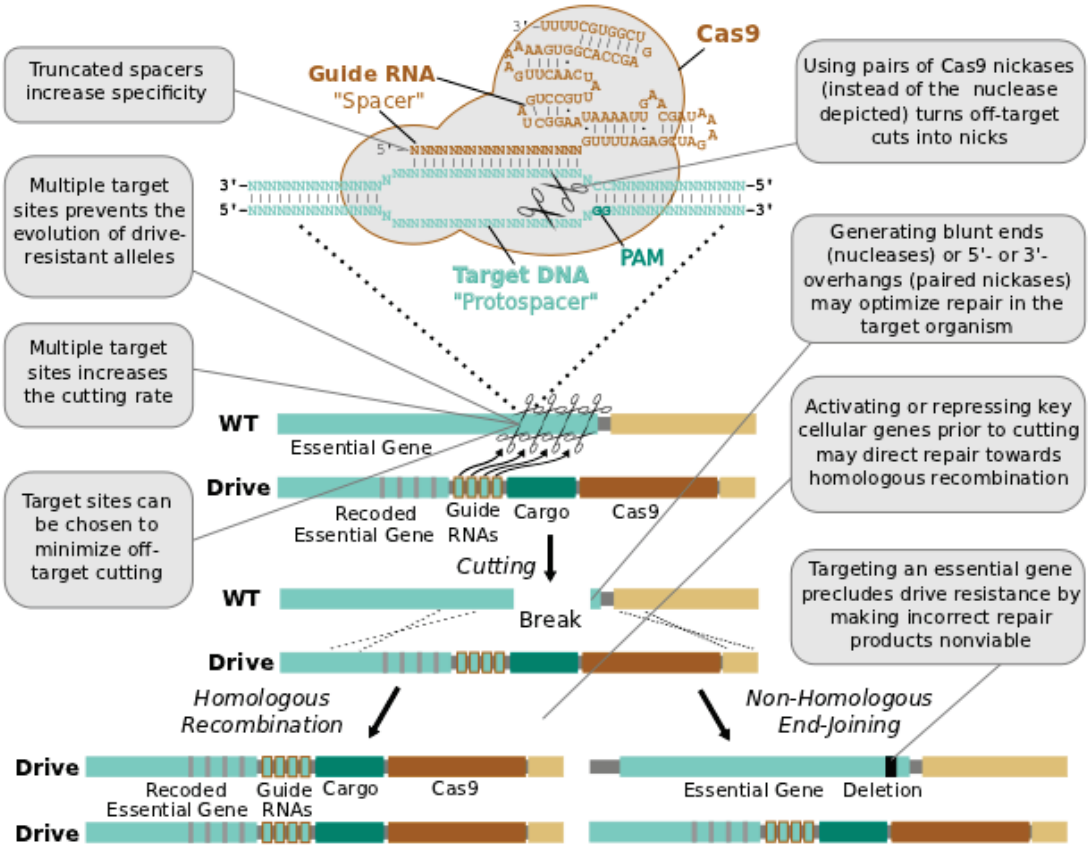
- Cutting efficiency: The first requirement for every endonuclease gene drive is to cut the target sequence. However, this is impractical for homing endonucleases and quite difficult for zinc-finger nucleases and TALENs, as each additional sequence requires a new nuclease protein to be engineered or evolved and then co-expressed. In contrast, the RNA-guided Cas9 nuclease can be readily directed to cleave additional sequences by expressing additional gRNAs. Using more than two gRNAs should further enhance cutting. The notable success of Cas9-based genome engineering in many different species, including studies that targeted every gene in the genome (Shalem et al. 2014; Wang et al. 2014a), demonstrates that most sequences can be efficiently targeted independent of species and cell type. Thus, RNA-guided gene drives should be capable of efficiently cutting any given gene.
- Cutting specificity: The second requirement is to avoid cutting non-targeted sequences. While several studies (e.g., Mali et al. 2013) have reported that Cas9 is prone to cutting off-target sequences that are closely related to the target, more recent developments and strategies designed to improve specificity (e.g., Guilinger et al. 2014; Tsai et al. 2014) have demonstrated that the off-target rate can be reduced to nearly undetectable levels. Notably, Cas9 does not appear to represent a noticeable fitness burden when expressed at a moderate level in fruit flies with or without gRNAs (Kondo and Ueda 2013). Organisms with larger genomes may require more careful target site selection due to the increased number of potential off-target sequences present.
- Homologous recombination (copying) rate: The third and most challenging requirement involves ensuring that the cut sequence is repaired using homologous recombination (HR) or homology-directed repair (HDR) to copy the drive rather than the competing non-homologous end-joining (NHEJ) pathway. HR rates are known to vary across cell types, developmental stages, species, and the phase of the cell cycle. Ideally, drives should be activated only in germline cells at developmental stages with a high rate of HR, but this may be challenging in many species. Targeting sites adjacent to both homology arms may also promote more efficient drive copying by biasing repair towards HR (Paix et al. 2014) and increasing the overall frequency of cutting (Kondo and Ueda 2013). The ability to regulate gene expression with Cas9 might be used to temporarily increase the rate of homologous recombination while the drive is active. For instance, the Cas9 nuclease involved in cutting might simultaneously repress genes involved in NHEJ (Gilbert et al. 2013), and therefore increase the frequency of

HR if supplied with a shortened gRNA that directs it to bind and block transcription, but not cut (Bikard et la. 2013; Sternberg et al. 2014). Alternatively, an orthogonal and nuclease-null Cas9 protein (Esvelt et al. 2013) encoded within the drive cassette could repress NHEJ genes and activate HR genes before activating the Cas9 nuclease. Lastly, Cas9 might be used directly to recruit key HR-directing proteins to the cut sites, potentially biasing repair towards that pathway.

- Evolutionary stability: Even a perfectly efficient endonuclease gene drive is vulnerable to the evolution of drive resistance in the population. Whenever a cut is repaired using the NHEJ pathway, the result is typically a drive-resistant allele with insertions or deletions in the target sequence that prevent it from being cut by the endonuclease. Natural sequence polymorphisms in the population could also prevent cutting. These alleles will typically increase in abundance and eventually eliminate the drive because most drives - like most transgenes - are likely to slightly reduce the fitness of the organism. A second path to gene drive resistance would involve the target organism evolving a method of specifically inhibiting the drive endonuclease. The best defense against previously existing or recently evolved drive-resistant alleles is to target multiple sites. Because mutations in target sites are evolutionarily favored only when they survive confrontation with the gene drive, using many target sites can render it statistically improbable for any one allele to survive long enough to accumulate mutations at all of the sites, as long as cutting rates are high (Burt 2003). However, very large populations - such as those of some insects - might require unfeasibly large numbers of gRNAs to prevent resistance. In these cases, it may be necessary to release several successive gene drives, each targeting multiple sites, to overcome resistant alleles as they emerge. From an evolutionary perspective, the ability to preclude resistance by targeting multiple sites is the single greatest advantage of RNA-guided gene drives.
- Development time: The CRISPR-Cas9 technology has made it much easier and faster to make transgenic organisms with custom-designed gene drives. The design-build-test cycle for any new gene drives is limited only by the generation time of the organism. Nevertheless, many practical difficulties are likely to arise when constructing a particular gene drive in a given species (especially a non-model organism with limited genomic information). Early success is as unlikely as ever when engineering complex biological systems. With the parallel rapid advancement of next-generation sequencing technologies and bioinfor-

matics, it is predictable that the development time will become even shorter.

Figure 11. Technical advantages of RNA-guided gene drives mediated by Cas9 (Esvelt et al. 2014).



4.5 Proof-of-concept studies

Two recent papers published in early 2015 provided the proof-of-concept evidence for the feasibility and effectiveness of CRISPR-Cas9-based gene drives.

In one study, DiCarlo et al. (2015) constructed a Cas9-mediated gene drive that cut and disrupt the *ADE2* gene in haploid cells of the yeast *Saccharomyces cerevisiae*. Disruption in *ADE2* generated a red phenotype on adenine-limiting media due to the buildup of red pigments. When these red-colored, *ADE2*-deficient cells ($ADE2^{drv}$) mated to cream-colored, wild-type haploid strains ($ADE2^{+}$), an exclusively red diploid progeny ($ADE2^{drv/drv}$) was yielded (Figure 12B). In the absence of the target site or Cas9, normal 2:2 segregation was observed, demonstrating that the drive only functions in yeast populations encoding both Cas9 and gRNA (Figure 12C).

To verify that the *ADE2* alleles from wild-type parents were indeed lost, the authors sporulated the mated diploids and examined their resultant haploid progeny. Upon dissecting 18 cas9$^+$ diploids, they observed a perfect 4:0 ratio of red:cream haploids, confirming that all copies of the *ADE2* locus were disrupted (Figure 12D).

To determine whether *ADE2* disruptions in red diploids were the result of successful copying of the drive element via HR, researchers sequenced the 72 haploids derived from dissected cas9$^+$ diploids. All sequenced colonies contained intact drives without additional mutations, indicating that drive mobilization was efficient and occurred at high fidelity.

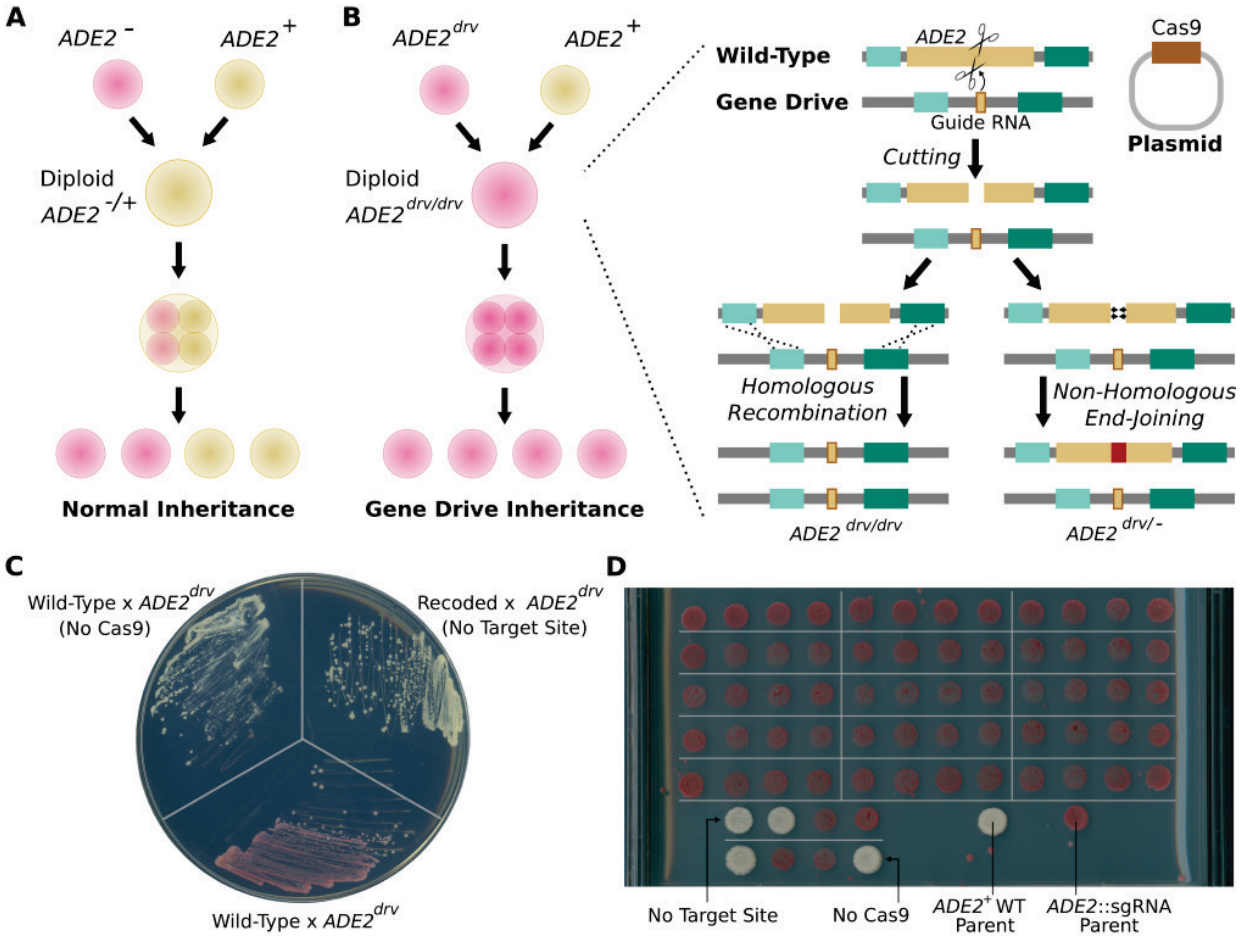
The authors further mated *ADE2* drive-containing haploids with a phylogenetically and phenotypically diverse group of six native haploid *S. cerevisiae* strains. The mean fraction of diploid chromosomes containing the *ADE2* gene drive was over 99% regardless of wild-type parent, attesting to the robustness of the drive in diverse backgrounds.

They also mated several haploid offspring of the first-round *ADE2* gene drive diploids to wild-type haploids containing the Cas9-expressing plasmid. All of the gene drive constructs biased inheritance at the same efficiency in the second generation as they did in the first, indicating a continued ability to spread through sexually reproducing populations over multiple generations.

This system (Figure 12) is self-contained because it consists of two physically separate genetic parts: an episomally encoded Cas9 gene and a drive element encoding the gRNA. (A) Mutations in *ADE2* generate a red phenotype on adenine-limiting media due to the buildup of red pigments. Mating a red mutant haploid to a wild-type haploid produced cream-colored diploids, which yielded 50% red and 50% cream-colored progeny upon sporulation. (B) When haploids with a gene drive targeting *ADE2* mated with wild-type haploids in the presence of Cas9, cutting and subsequent replacement or disruption of *ADE2* produced red diploids that exclusively yielded red progeny. (C) Diploids produced by mating wild-type and *ade2*::sgRNA gene drive haploids yielded cream-colored colonies in the absence of Cas9 or when the target site was removed by recoding, but it yielded uniformly red colonies when both were present, demonstrating Cas9-dependent disruption of the wild-type *ADE2* copy. (D) Spores from 15 dissected tetrads produced uniformly red colonies on adenine-limited

plates, confirming disruption of the *ADE2* gene inherited from the wild-type parent. In the absence of the target site or Cas9, normal 2:2 segregation was observed.

Figure 12. Biased inheritance of a split Cas9-gRNA gene drive system targeting the ADE2 gene in the yeast *Saccharomyces cerevisiae* (DiCarlo et al. 2015).

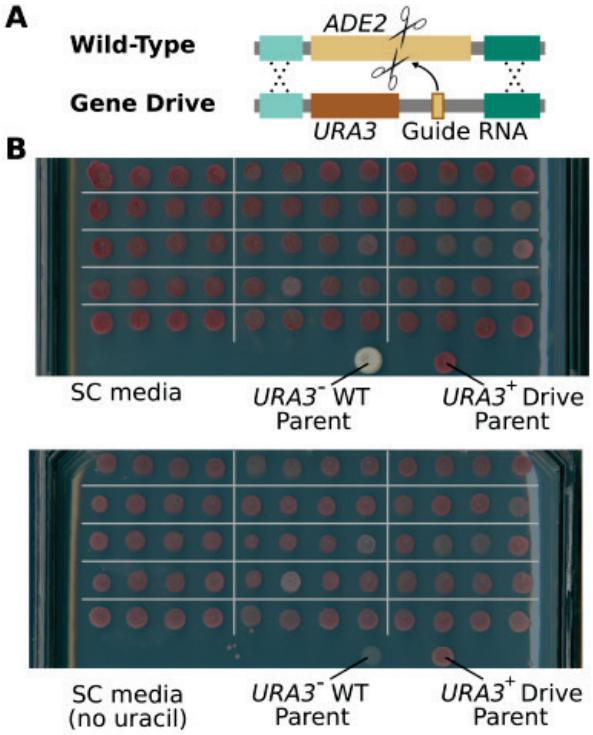


Next, researchers tested whether RNA-guided gene drives could be designed to bias the inheritance of not only the minimal drive element, but also any closely associated "cargo" gene whose spread through an existing population may be desirable. As a proof of principle, the ADE2-targeting gene drive was modified to carry URA3 as a cargo gene. Researchers inserted the *URA3* gene in *cis* to the *ade2*::sgRNA minimal guide element. *URA3* allows laboratory modified yeast strains to grow in the absence of uracil supplementation (Figure 13A). They mated these *URA3*-containing drive haploids to wild-type haploids in the presence of an episomal Cas9 plasmid, selected diploids (all of which were red), sporulated them, and dissected 18 tetrads. Diploids produced by mating wild-type URA3- haploid yeast with haploids encoding the gene drive carrying URA3 were

sporulated and tetrads dissected to isolate colonies arising from individual spores. All of these grew when replica plated onto plates lacking uracil, demonstrating that the drive successfully copied URA3 in all diploids. As was the case for the original *ADE2* gene drive, all of the sporulated haploid cells formed red colonies. Crucially, all grew normally when replica plated on uracil deficient media, indicating that *URA3* was efficiently copied with the drive (Figure 13B).

Finally, they also successfully targeted and recoded an essential gene *ADE1*. This study demonstrates that Cas9 can bias inheritance in diverse wild yeast strains over successive generations at very high efficiency, and it can guide efforts to build RNA-guided gene drives in other organisms as well as underscore the urgent need for precautionary control strategies.

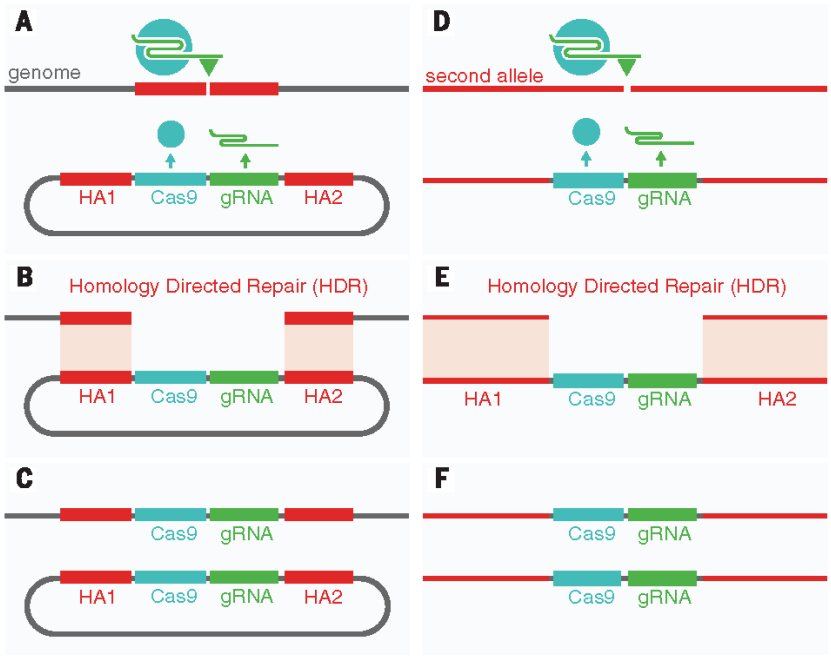
Figure 13. Gene drives and cargo genes remain intact upon copying (DiCarlo et al. 2015).



In the other study, Gantz and Bier (2015) built a gene drive consisting of three components: (1) A Cas9 gene (expressed in both somatic and germline cells), (2) a gRNA targeted to a genomic sequence of interest, and (3) homology arms flanking the Cas9-gRNA cassettes that match the two genomic sequences immediately adjacent to either side of the target cut site (Figure 14A). In such a tripartite construct, Cas9 cleaves the genomic

target at the site determined by the gRNA (Figure 14A) and then inserts the Cas9-gRNA cassette into that locus via HDR (Figure 14, B and C). Cas9 and the gRNA produced from the insertion allele then cleave the opposing allele (Figure 14D), followed by HDR-driven propagation of the Cas9-gRNA cassette to the companion chromosome (Figure 14, E and F). The authors refer to this trans-acting mutagenesis scheme as a mutagenic chain reaction (MCR).

Figure 14. Scheme outlining the mutagenic chain reaction (MCR) (Gantz and Bier 2015).

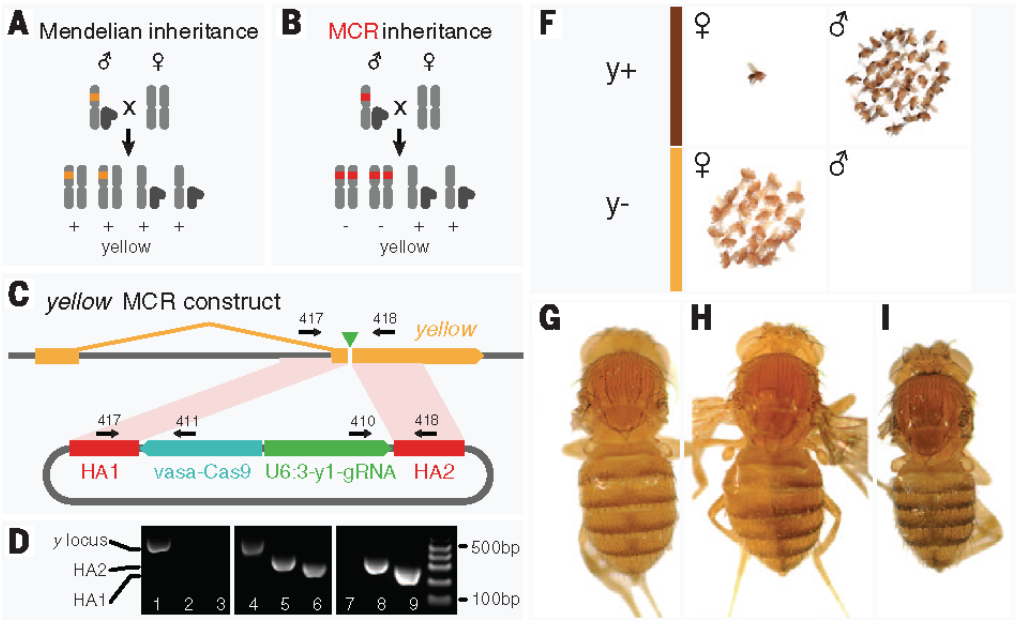


Gantz and Bier (2015) demonstrated that in *Drosophila*, a *y*-MCR construct containing a characterized efficient target sequence in the X-linked yellow (*y*) locus as the gRNA target and a vasa-Cas9 transgene as a source of Cas9 efficiently spread from its chromosome of origin to the homologous chromosome (Figure 15). The high recovery rate of full-bodied *y*- F1 and F2 female progeny from single parents containing a y^{MCR} allele detectable by PCR indicates that the conversion process is remarkably efficient in both somatic and germline lineages. This study suggests that an MCR element causing a viable insertional mutation within the coding region of a gene can provide a potent gene drive system for delivery of transgenes in disease vector or pest populations.

In Figure 15 panel A shows a Mendelian male inheritance of an X-linked trait. Panel B shows that theoretical MCR-based inheritance resulted in

the initially heterozygous allele converting the second allele, thereby generating homozygous female progeny. Panel C shows a diagram of *y*-MCR construct where two *y* locus homology arms (HA1 and HA2) flanking the *vasa*-Cas9 and y-gRNA transgenes are indicated, as are the locations of the PCR primers used for analysis of the genomic insertion site. Panel D indicates that PCR analysis of a y^+ MCR-derived F2♂ (lanes 1 to 3), y^{MCR} F1♀ (lanes 4 to 6), and y^{MCR} F1♂ (lanes 7 to 9) showed junctional bands corresponding to *y*-MCR insertion into the chromosomal *y* locus (lanes 2, 3, 5, 6, 8, and 9) and the presence (lanes 1 and 4) or absence (lane 7) of a PCR band derived from the *y* locus. Although the y^{MCR} F1♂ (carrying a single X chromosome) displays only MCR-derived PCR products (lanes 8 and 9), y^{MCR} F1♀s generate both MCR and noninsertional amplification products. Panel E summarizes F2 progeny obtained from crosses. Panel F provides low-magnification view of F2 progeny flies from an y^{MCR} ♂× y^+♀ cross. Nearly all female progeny display a y^- phenotype. Panel G shows a high magnification view of a full-bodied y^{MCR} F1♀. Panel H shows a rare 50% left-right mosaic female. Panel I shows a rare y^+ (wild-type) control fly.

Figure 15. Experimental demonstration of a CRISPR-Cas9-based gene drive in *Drosophila* (Gantz and Bier 2015).



E	y^-♂	y^-♀	mosaic♀	y^+♂	y^+♀	total
y^{MCR}♂ x y^+♀	0	40	0	50	1	91
y^{MCR}♀ x y^+♂	214	203	11	2	6	436

While the above two examples provide evidence supporting the application and optimism for RNA-guided gene drives efficiently targeting specific essential or non-essential genes in both unicellular (yeast) and multicellular (*Drosophila*) organisms, more studies examining particular drives, populations, and, more importantly, field performance in relevant ecosystems are needed. To prevent accidental and unintentional release of the genetically modified organisms into the environment, Gantz and Bier (2015) employed a barrier/physical containment method, whereas DiCarlo et al. (2015) developed a molecular containment approach. The latter is preferred and recommended for use in future gene drive development at the lab bench scale because physical methods of containment are always susceptible to human error as demonstrated by numerous containment breaches involving pathogenic viruses and bacteria (Esvelt et al. 2014).

4.6 Limitations of CRISPR-Cas9-mediated gene drives

The same fundamental limitations of gene drive systems would also apply to CRISPR-Cas9-based gene drives. Esvelt et al. (2014) summarized the following major limitations:

1. Gene drives require many generations to spread through populations. Once transgenic organisms bearing the gene drive are constructed in the laboratory, they must be released into the wild to mate with wild-type individuals in order to begin the process of spreading the drive through the wild population. The total time required to spread to all members depends on the number of drive-carrying individuals that are released, generation time of the organism, efficiency of homing, impact of the drive on individual fitness, and dynamics of mating and gene flow in the population, but, in general, it will take several dozen generations (Burt 2003; Deredec et al. 2008, 2011). Thus, drives will spread very quickly in fast-reproducing species, but only slowly in long-lived organisms.
2. Gene drives cannot affect species that exclusively practice asexual reproduction through clonal division or self-fertilization. This category includes all viruses and bacteria as well as most unicellular organisms. Highly efficient standard drives might be able to slowly spread through populations that employ a mix of sexual and asexual reproduction, such as certain plants, but drives intended to suppress the population would presumably force target organisms to reproduce asexually in order to avoid suppression.

3. Drive-mediated genome alterations are not permanent on an evolutionary timescale. While gene drives can spread traits through populations even if they are costly to each individual organism, harmful traits will eventually be outcompeted by more fit alleles after the drive has gone to fixation. Highly deleterious traits may be eliminated even more quickly, with non-functional versions appearing in large numbers even before the drive and its cargo can spread to all members of the population. Even when the trait is perfectly linked to the drive mechanism, the selection pressure favoring the continued function of Cas9 and the guide RNAs will relax once the drive reaches fixation. Maintaining deleterious traits within a population indefinitely is likely to require scheduled releases of new RNA-guided gene drives to periodically overwrite the broken versions in the environment.
4. Current knowledge of the risk management (Alphey 2014; Baldacchino et al. 2015; Scott et al. 2002; Touré et al. 2004; UNEP 2010) and containment (Benedict et al. 2008; Marshall 2009) issues associated with gene drives is largely focused on mosquito-borne diseases. Frameworks for evaluating ecological consequences are similarly focused on mosquitoes (David et al. 2013) and the few other organisms for which alternative genetic biocontrol methods have been considered (Dana et al. 2014; Hayes et al. 2014). While these examples provide an invaluable starting point for investigations of RNA-guided gene drives targeting other organisms, studies examining the particular drive, population, and associated ecosystem in question will be needed.

4.7 Biosafety, public perception and regulatory issues

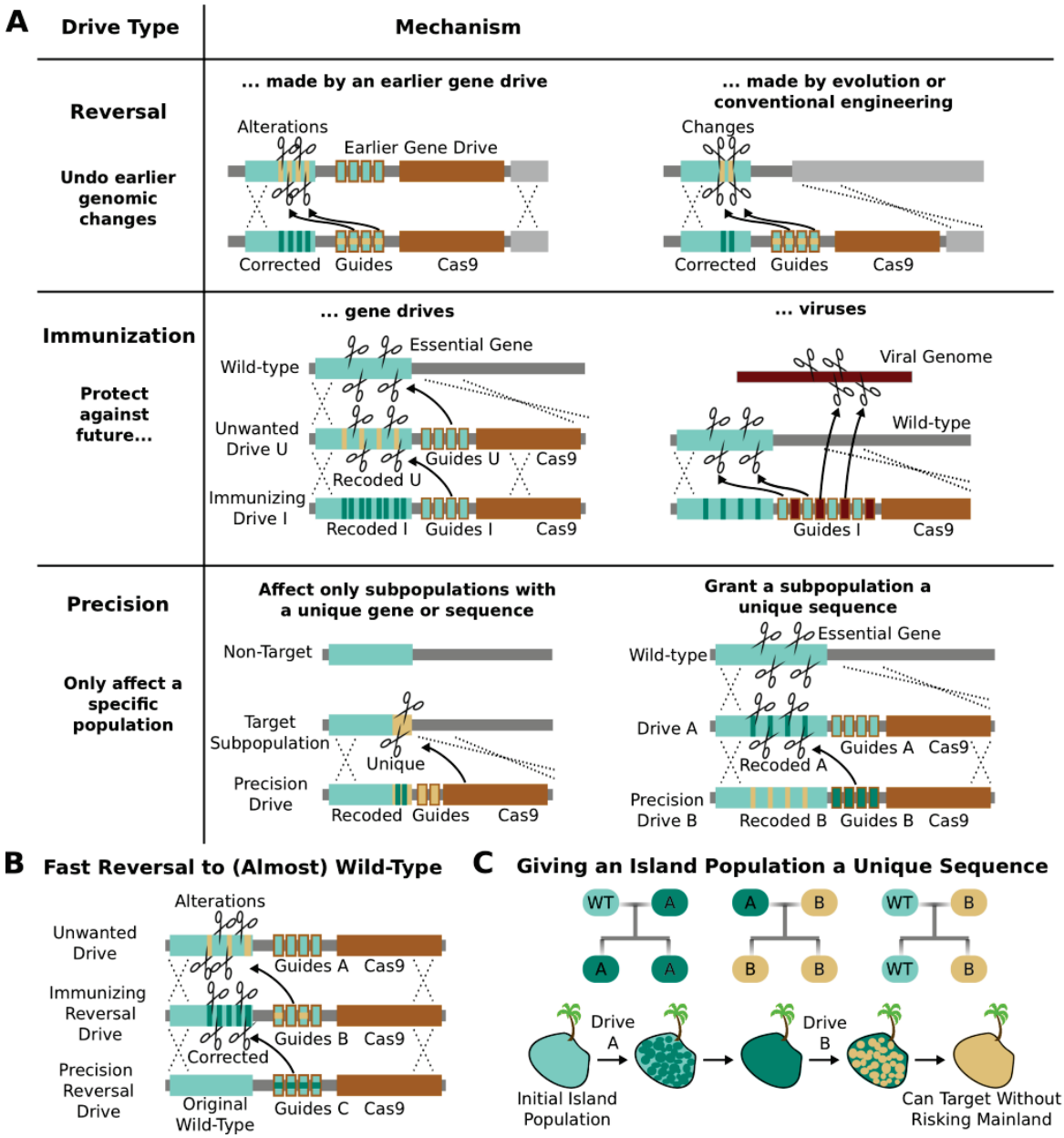
Although no CRISPR-Cas9-based gene drive has been used in combating real-world problems such as invasive species control, the potentially high efficiency and invasiveness of this technology warrant a thorough examination of biosafety, environmental risk, public perception, and regulatory issues surrounding the development and field release of CRISPR-Cas9-engineered organisms.

4.7.1 Biosafety

Given the potential for gene drives to alter entire wild populations and therefore ecosystems, the development of this technology must include robust safeguards and methods of control (Oye et al. 2014). In addition to the physical (Gantz and Bier 2015) and molecular (DiCarlo et al. 2015) containment measures described earlier in the two proof-of-principle

studies, the development of reversal, immunization and precision drives can reverse, prevent, and control the spread and effects of released gene drives, respectively (Figure 16). Even scientists not intending to work with gene drives should consider taking precautions, since any unintended insertion of the *cas9* gene and guide RNAs near a targeted site could generate a gene drive. When used against invasive species, two primary risks will incur with regard to undesired spread. First, rare mating events may allow the drive to affect closely related species. Using precision drives to target sequences unique to the invasive species could mitigate or eliminate this problem. Second, the suppression drive might spread from the invasive population back into the native habitat, perhaps even through intentional human action. Native populations might be protected using an immunizing drive, but doing so would risk transferring immunity back into invasive populations. Instead, one may grant the invasive population a unique sequence with a standard drive (Figure 16C), verify that these changes have not spread to the native population, and only then release a suppression drive targeting the recoded sequences while holding an immunizing drive in reserve. Another approach might utilize a sensitizing drive to render all populations newly vulnerable to a specific compound, which could then be used as a pesticide for the local control of invasive populations. Working in genetic backgrounds that are less likely to escape the laboratory and mate – such as wingless flies in the case of *Drosophila* – may also be prudent.

Figure 16. Methods of reversing, preventing, and controlling the spread and effects of gene drives.



In Figure 16 (A) reversal drives could correct or reverse genomic alterations made by an earlier drive with unexpected side effects. They might also be used to reverse conventionally engineered or evolved changes. Immunization drives could prevent other gene drives from affecting a specific population or provide a population with resistance to DNA viruses. Precision drives could exclusively spread through a subpopulation with a unique gene or sequence. (B) Together, these can quickly halt an unwanted drive and eventually restore the sequence to the original wild-type, save

for the residual Cas9 and guide RNAs. (C) Any population with limited gene flow can be given a unique sequence by releasing drives A and B in quick succession. As long as drive A does not escape into other populations before it is completely replaced by drive B, subsequent precision drives can target population B without risking spread into other populations.

4.7.2 Environmental risk management

Recently, Oye et al. (2014) recommend the following steps toward integrated management of environmental and security risks associated with Cas9-mediated gene drives:

1. Before any primary drive is released in the field, the efficacy of specific reversal drives should be evaluated. Research should assess the extent to which the residual presence of guide RNAs and/or Cas9 after reversal might affect the phenotype or fitness of a population and the feasibility of reaching individual organisms altered by an initial drive.
2. Long-term studies should evaluate the effects of gene drive use on genetic diversity in target populations. Even if genome-level changes can be reversed, any population reduced in numbers will have reduced genetic diversity and could be more vulnerable to natural or anthropogenic pressures. Genome-editing applications may similarly have lasting effects on populations owing to compensatory adaptations or other changes.
3. Investigations of drive function and safety should use multiple levels of molecular containment to reduce the risk that drives will spread through wild populations during testing. For example, drives should be designed to cut sequences absent from wild populations, and drive components should be separated.
4. Initial tests of drives capable of spreading through wild populations should not be conducted in geographic areas that harbor native populations of target species.
5. All drives that might spread through wild populations should be constructed and tested in tandem with corresponding immunization and reversal drives. These precautions would allow accidental releases to be partially counteracted.
6. A network of multipurpose mesocosms and microcosms should be developed for testing gene drives and other advanced biotechnologies in contained settings.
7. The presence and prevalence of drives should be monitored by targeted amplification or meta-genomic sequencing of environmental samples.

8. Because effects will mainly depend on the species and genomic change rather than the drive mechanism, candidate gene drives should be evaluated on a case-by-case basis.
9. To assess potentially harmful uses of drives, multidisciplinary teams of experts should be challenged to develop scenarios on deliberate misuse.
10. Integrated benefit-risk assessments informed by the actions recommended above should be conducted to determine whether and how to proceed with proposed gene drive applications. Such assessments should be conducted with sensitivity to variations in uncertainty across cases and to reductions in uncertainty over time.

4.7.3 Transparency, public discussion, and evaluation

As pointed out by Esvelt et al. (2014), CRISPR-Cas9-based gene drives are capable of self-propagating genetic and phenotypic alterations of wild populations and influencing entire ecosystems for good or for ill. As such, it is imperative that all research in this nascent field operate under conditions of full transparency, including independent scientific assessments of probable impacts and thoughtful, informed, and fully inclusive public discussions. The decision of whether or not to utilize a gene drive for invasive species control should be based entirely on the probable benefits and risks of that specific drive. That is, each drive should be judged solely by its potential outcomes, such as its ability to accomplish the intended aims, its probable effects on other species, the risk of spreading into closely related species by rare mating events, and impacts on ecosystems and human societies. As these technologies are developed, scientists must uphold the responsibility of making all empirical data and predictive models freely available to the public in a transparent and understandable format. Above all else, scientists must openly share a high level of confidence in these assessments as procedures to continue are determined.

4.7.4 Regulatory gaps.

New technologies often fit awkwardly into existing regulatory frameworks (Alphey 2014). Currently, U.S. regulations treat gene drives as veterinary medicines or toxins, whereas U.S. policies and international security regimes rely on a listed-agent-and-toxin approach (Oye et al. 2014). Neither addresses challenges posed by gene drives, hence Oye et al. (2014) called for reforms to fill the regulatory gaps. An International Symposium on Genetic Biocontrol of Invasive Fish held in Minneapolis, Minnesota in

June 2010 was an important first step towards informing the key regulatory agencies about the need for genetic biocontrol (gene drives included), status of the science, and environmental risks (Otts 2014). The next step is for researchers and agencies to build on this foundational knowledge to develop decision-making frameworks within the existing regulatory regime that ensures fair and accurate assessment of genetic biocontrol technology and its associated environmental risks.

1. U.S. environmental regulations. The responsibility for regulating animal applications of drives in the United States rests with the Food and Drug Administration (FDA). An FDA guidance issued in 2009 states that genetically engineered DNA constructs intended to affect the structure and function of an animal, regardless of their use, meet the criteria for veterinary medicines and are regulated as such. Developers are required to demonstrate that such constructs are safe for the animal. Approval of new veterinary medicines is to be based on the traditional FDA criterion "that it is safe and effective for its intended use" (Center for Veterinary Medicine (CVM)/FDA 2009). It is unclear whether these requirements can be reconciled with projected uses of drives, including suppression of invasive species. Nor is it clear how this guidance would apply to insects. The application of existing U.S. Department of Agriculture (USDA) and U.S. Environmental Protection Agency (U.S. EPA) regulations governing genetically modified organisms to gene drives is also ambiguous, with jurisdictional overlaps across the Federal Insecticide, Fungicide, and Rodenticide Act, the Toxic Substances Control Act, and the Animal and Plant Health Inspection Service (Bar-Yam et al. 2012).
2. International environmental conventions. Existing international conventions cover international movements of gene drives, but do not define standards for assessing effects, estimating damages, or mitigating harms. International movements of living modified organisms are treated under the 2003 Cartagena Protocol on Biosafety, ratified by 167 nations, not including the United States and Canada. Article 17 of the Protocol obligates parties to notify an International Biosafety Clearinghouse and affected nations of releases that may lead to movement of living modified organisms with adverse effects on biological diversity or human health. Other provisions empower nations to use border measures to limit international movements, but these measures are not likely to control diffusion of drives. The 2010 Nagoya–Kuala Lumpur Supplementary Protocol calls on Parties to adopt a process to define

rules governing liability and redress for damage from international movements (Gilna et al. 2014). Neither the process nor rules have been defined (UN 2003).

3. U.S. security policies. The draft U.S. Government Policy on Dual Use Research of Concern (DURC) combines a broad definition of concerns with a narrow definition of scope of oversight, the latter focusing on experiments of concern on listed pathogens and toxins (USSTO 2013). The listed-agent-toxins approach is also used in the U.S. Select Agent Rule, USDA Select Agents/Toxins, and Commerce Department export control regulations. Drives do not fall within the scope of required oversight of DURC and other listed-agent-toxin–based policies.
4. International security conventions. The United Nations (UN) Biological Weapons Convention defines areas of concern in broad terms with the intention of providing latitude to adapt to evolving technologies and threats. Article 1 bans development, production, or stockpiling of all biological agents or toxins that have no justification for prophylactic, protective, and other peaceful purposes, and weapons, equipment ,or means of delivery designed to use such agents or toxins for hostile purposes (UN 1925, 1972). However, national implementation measures defining operational oversight and Australia Group Guidelines governing exports rely on narrow lists of organisms, toxins, and associated experiments (Australia Group 2012). Gene drives and most other advanced applications of genomic engineering do not use proscribed agents or create regulated toxins and hence fall beyond the scope of operational regulations and agreements.
5. Filling the regulatory gaps. Existing frameworks for genetically modified organisms provide a basis for regulating genetic biocontrol technologies (Alphey 2014). Oye et al. (2014) suggests adopting a function-based approach that defines risks in terms of the ability to influence any key biological component, the loss of which would be sufficient to cause harm to humans or other species of interest. The agents and targets of concern with a functional approach could include DNA, RNA, proteins, metabolites, and any packages thereof. Thus, suppression drives would be covered because they would cause loss of reproductive capability in an animal population, whereas an experimental reversal drive that could only spread through engineered laboratory populations could be freely developed. Steps taken to mitigate environmental concerns will address security concerns and vice versa. Regulatory authority for each proposed RNA-guided gene drive should be granted to the agency with the expertise to evaluate the application in question.

All relevant data should be made publicly available and, ideally, subjected to peer review (Reeves et al. 2012).

4.8 Recent field releases of genetically engineered organisms for invasive species control

After due consideration, regulators in several countries have approved limited field trials of invasive disease-vector mosquitoes (Alphey et al. 2013; Alphey 2014). Successful field trials have recently been conducted for several genetic biocontrol methods (summarized in Table 2). Each trial successfully accomplished its experimental objectives, and negative consequences to human health or the environment was not identified in any of the trials. Public perception has generally been positive (Hoffman et al. 2011; Harris et al. 2012), though it is too soon to determine long-term trends. It is reasonable to be optimistic that the first field trial of CRISPR-Cas9-based gene drives will become a reality and success soon.

Table 2. Recent field trials of genetic engineering technologies for mosquito biocontrol [a].

Date	Location	Method	Outcome	Reference(s)
2005–2009	Italy	SIT	Release of irradiated male *Aedes albopictus* induced sterility in target populations; population suppression was observed in some locations	Bellini et al. 2013
2009–2010	Cayman Islands	RIDL	Males of a RIDL strain of *Aedes aegypti*, OX513A, competed successfully for mates with wild mosquitoes; sustained release of these sterile males led to strong suppression of the target wild population	Harris et al. 2011, 2012
2010	Malaysia	RIDL	RIDL OX513A males have life span and maximum dispersal similar to an unmodified comparator	Lacroix et al. 2012
2010	French Polynesia	IIT	Sustained release of *Aedes polynesiensis* males infected with a *Wolbachia* strain from *Aedes riversi* induced sterility in a target population	O'Connor et al. 2012
2011–Present	Brazil	RIDL	Sustained release of RIDL OX513A males led to strong suppression of two target wild populations; larger subsequent program in progress [b]	Carvalho et al. 2015
2011–Present	Australia	Invasive *Wolbachia*	Release of *w*Mel-infected male and female *Aedes aegypti* led to the invasion and establishment of *w*Mel *Wolbachia* in two target populations; releases in three additional areas are in progress [c]	Hoffman et al. 2011
2012–2013	Australia	Invasive *Wolbachia*	Release of *w*MelPop-infected male and female *Aedes aegypti* in two target areas; does not appear to	Eliminate Dengue Program 2012

Date	Location	Method	Outcome	Reference(s)
			have self-sustained [c]	
2013–Present	Vietnam	Invasive *Wolbachia*	Release of *w*MelPop-infected male and female *Aedes aegypti* on an island; in progress [c]	Eliminate Dengue Program 2012

a. The IIT and RIDL trials additionally confirmed the self-limiting, reversible nature of these methods (Harris et al. 2011, 2012; O'Connor et al. 2012).
b. Moscamed 2012 and Oxitec 2016 .
c. Eliminate Dengue Program 2012.
Abbreviations: IIT, incompatible insect technique; RIDL, release of insects carrying a dominant lethal gene; SIT, sterile insect technique.

4.9 A roadmap to CRISPR-Cas9-based gene drives for invasive species control

Although the CRISPR-Cas9 technology holds great promises in the gene drive approach for invasive species control, many practical difficulties must be overcome before gene drives will be in a position to alter and drive any particular gene of interest in any given species. Initial optimisms and expectations are likely to fall short simply because biological systems are complex and difficult to engineer (Esvelt et al. 2014). At the same time, there are valid concerns regarding researchers' ability to accurately predict the ecological consequences of released gene drives.

Because any consequences of releasing CRISPR-Cas9-based gene drives into the environment would be shared by the local, if not, global community, research involving gene drives capable of spreading through wild-type populations should occur only after a careful and fully transparent review process. However, basic research into gene drives and methods of controlling their effects can proceed without risking this type of spread, as long as appropriate ecological or molecular containment strategies are employed.

A great deal of information on probable ecological outcomes can be obtained without testing or even building replication-competent gene drives. For example, early studies might examine possible ecological effects by performing contained field trials with organisms that have been engineered to contain the desired change but do not possess a functional drive to spread it. Because they do involve transgenic organisms, these experiments are not completely without risk, but such transgenes are unlikely to spread in the absence of a drive.

It is recommend that all laboratories seeking to build standard gene drives capable of spreading through wild populations simultaneously create reversal drives able to restore the original phenotype. Similarly, suppression drives should be constructed in tandem with a corresponding immunizing drive. These precautions would allow the effects of an accidental release to be swift if partially counteracted. The prevalence of gene drives in the environment could, in principle, be monitored by targeted amplification or metagenomic sequencing of environmental samples. Further investigation of possible monitoring strategies will be needed.

Short Term Development Strategy. (1) Identify the potential DOD customer base involved in invasive species control and management. (2) Conduct several case studies to demonstrate the efficiency, sustainability, and cost-effectiveness of the CRISPR/Cas-based gene drive technology. (3) Make project-related information transparent and accessible using a variety of dissemination tools, including the press and the Internet. (4) Keep both, federal regulators and DOD customers, informed and engage in the case studies. (5) Provide training opportunities to all potential customers.

Long Term Development Strategy. Unlike other invasive species management strategies, gene drive technologies require a number of organismal generations to take full effects and advantages. As a genetic engineering technology, CRISPR/Cas-engineered organisms may face the same public and regulatory scrutiny as other transgenic organisms if released into the open field. As the most important component of the long-term development strategy, it is essential to establish and maintain a good perception to stakeholders, regulators, and the general public. Researchers should keep these groups fully informed of the benefits and risks associated with this powerful technology. In addition, researchers should also explore optimal approaches by integrating the gene drive technology with other management tools and technologies.

4.10 Benefits

The CRISPR/Cas-based gene drive technology provides the DOD a cost-effective, environment friendly, highly efficient, and self-sustaining tool to combat invasive species. It is expected that the application of this technology will result in significant cost savings for DOD's invasive species management and for reduced environmental liabilities caused by the use of chemical pesticides. The expected eradication of many invasive disease vectors (e.g., the yellow fever mosquito) will bring about a sharp decline in

the incidence of mosquito-borne diseases like chikungunya, dengue, West Nile, and yellow fever among military personnel, and hence provide better protection of soldier health. Other benefits include improved recreational value of military lands and increased ecosystem quality.

5 Summary

As initially proposed in 2003 by Austin Burt of Imperial College London, naturally occurring selfish genetic elements (e.g., transposable elements, meiotic drive genes, homing endonuclease genes, and *Wolbachia*-induced cytoplasmic incompatibility) spread at the expense of their hosts and can be harnessed to build effective and self-sustaining gene drives for invasive species control. A gene drive refers to a mechanism that spreads the desired gene and its effect (e.g., sterility or inability to transmit a pathogen) into the target population in a timeframe that is appropriate for disease control or population eradication. Historically, gene drive development has been hampered by the difficulty of engineering homing endonucleases to cut new target sequences and the instability of earlier targeted genome editing techniques such as ZFN and TALEN. As a technological breakthrough in genome engineering, the CRISPR system uses RNA as its DNA-homing mechanism, which makes it low cost, high efficiency, and easy to implement, as well as dramatically shortens the design-build-test cycle for gene drive development. The high efficiency, robustness, and versatility of the CRISPR/Cas9 technology also make it feasible to alter multiple loci at the target gene that prevent mutations from blocking the spread of the drive. It also allows quick development of multiple types of drives such as precision, immunization, and reversal drives to precisely target a specific subpopulation, protect a population from any future gene drive "invasions," and overwrite previously released drives in case of unanticipated effects, respectively. While relying on exactly the same copying/homing mechanism as naturally existing homing endonucleases, CRISPR/Cas9-mediated gene drives can sweep a "deleterious" gene through a population exponentially faster than the normal Mendelian inheritance, and thus suppress or eradiate the target invasive species. CRISPR/Cas9-based gene drives are poised to become a self-sustaining, highly efficient, environmentally benign, and cost-effective alternative for invasive species biocontrol. Meanwhile, as a novel and powerful biotechnology, there is an urgent need for precautionary biosafety strategies, inclusive public engagement, ecological risk assessment, and regulatory reform in advance of the field release of genetically engineered organisms containing CRISPR-Cas9-mediated gene drives.

References

Addgene. 2015. CRISPR history and background. https://www.addgene.org/crispr/reference/history/

Alphey, L. 2014. Genetic control of mosquitoes. *Annu Rev Entomol*. 59:205-24.

Alphey, L., A. McKemey, D. Nimmo, O.M. Neira, R. Lacroix, K. Matzen, and C. Beech. 2013. Genetic control of *Aedes* mosquitoes. *Pathog Glob Health* 107(4):170-9.

Australia Group. 2012. Guidelines for transfers of sensitive chemical or biological items. www.australiagroup.net/en/guidelines.html.

Baker, D.A., T. Nolan, B. Fischer, A. Pinder, A. Crisanti, and S. Russell. 2011. A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, *Anopheles gambiae*. *BMC Genomics* 12(1):296.

Baldacchino, F, B. Caputo, F. Chandre, A. Drago, A. Della Torre, F. Montarsi, and A. Rizzoli. 2015. Control methods against invasive *Aedes* mosquitoes in Europe: a review. *Pest Manag Sci*. 71(11): 1471-1485.

Bar-Yam, S., J. Byers-Corbin, R. Casagrande, F. Eichler, A. Lin, M. Oesterreicher, P. Regardh, R.D. Turlington, and K.A. Oye. 2012. The Regulation of synthetic biology: A guide to United States and European Union regulations, rules, and guidelines. SynBERC and iGEM version 9.1. Emeryville, CA: Synthetic Biology Engineering Research Center. http://synberc.org/sites/default/files/Concise%20Guide%20to%20Synbio%20Regulation%20OYE%20Jan%202012_0.pdf.

Bax, N.J., and R.E. Thresher. 2009. Ecological, behavioral, and genetic factors influencing the recombinant control of invasive pests. *Ecol Appl*. 19(4):873-888.

Beck, K.G., K. Zimmerman, J.D. Schardt, J. Stone, R.R. Lukens, S. Reichard, J. Randall, A.A. Cangelosi, D. Cooper, and J.P. Thompson. 2008. Invasive species defined in a policy context: Recommendations from the Federal Invasive Species Advisory Committee. *Invasive Plant Science and Management*. 1(4):414-421.

Belfort, M., and R.J. Roberts. 1997. Homing endonucleases: keeping the house in order. *Nucleic Acids Res*. 25(17):3379-88.

Bellini, R., A. Medici, A. Puggioli, F. Balestrino, and M. Carrieri. 2013. Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. *J Med Entomol*. 50(2):317-25.

Benedict, M., P. D'Abbs, S. Dobson, M. Gottlieb, L. Harrington, S. Higgs, A. James, S. James, B. Knols, J. Lavery, S. O'Neill, T. Scott, W. Takken, and Y. Toure. 2008. Guidance for contained field trials of vector mosquitoes engineered to contain a gene drive system: recommendations of a scientific working group. *Vector-Borne Zoonotic Dis*. 8(2):127-166.

Benfey, T. J. 2011. The physiology of triploid fish. In *Encyclopedia of fish physiology: from genome to environment*, ed. A.P. Ferrell, 2009-2015. New York, NY: Academic Press.

Bikard, D., C. W. Euler, W. Jiang, P.M. Nussenzweig, G.W. Goldberg, X. Duportet, V.A. Fischetti, and L.A. Marraffini. 2014. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat Biotechnol*. 32(11):1146-1150.

Bikard, D., W. Jiang, P. Samai, A. Hochschild, F. Zhang, and L.A. Marraffini. 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res*. 41(15):7429-7437.

Bolotin, A., B. Quinquis, A. Sorokin, and S.D. Ehrlich. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 151(8):2551-2561.

Bourtzis ,K., S. L. Dobson, Z. Xi, J.L. Rasgon, M. Calvitti, L.A. Moreira, H.C. Bossin, R. Moretti, L.A. Baton, G.L. Hughes, P. Mavingui, and J.R. Gilles. 2014. Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Trop*. 132: S150-S163.

Brouns, S.J., M.M. Jore, M. Lundgren, E.R. Westra, R.J. Slijkhuis, A.P. Snijders, M.J. Dickman, K.S. Makarova, E.V. Koonin, and J. van der Oost. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*. 321(5891):960-964.

Burt, A. 2003. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society of London B: Biol Sci*. 270(1518): 921-928.

Burt, A. 2014. Heritable strategies for controlling insect vectors of disease. *Philos Trans R Soc of Lond B: Biol Sci*. 369(1645):20130432.

Burt, A., and R. Trivers. 2005. Genes in conflict: The biology of selfish genetic elements. Cambridge, MA: Harvard University Press.

Carvalho, D.O., A.R. McKemey, L. Garziera, R. Lacroix, C.A. Donnelly, L. Alphey, A. Malavasi, and M.L. Capurro. 2015. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Negl Trop Dis*. 9(7):e0003864.

Center for Veterinary Medicine (CVM), Food and Drug Administration (FDA), U.S. and Department of Health and Human Services. 2009. Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs: Final Guidance. Docket No. FDA-2008-D-0394. Rockville, MD: Food and Drug Administration. www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM113903.pdf.

Centers for Disease Control and Prevention (CDC). 2015. West Nile virus. http://www.cdc.gov/westnile/index.html

Chan, Y.S., D. A. Naujoks, D. S. Huen, and S. Russell. 2011. Insect population control by homing endonuclease-based gene drive: an evaluation in *Drosophila melanogaster*. Genetics. 188(1):33-44.

Chan, Y-S, D. S. Huen, R. Glauert, E. Whiteway, and S. Russell. 2013a. Optimising homing endonuclease gene drive performance in a semi-refractory species: the *Drosophila melanogaster* experience. PloS One 8: e54130.

Chan, Y.S., R. Takeuchi, J. Jarjour, D.S. Huen, B.L. Stoddard, and S. Russell. 2013b. The design and in vivo evaluation of engineered I-OnuI-based enzymes for HEG gene drive. *PLoS One*. 8(9):e74254.

Chen, B., L.A. Gilbert, B.A. Cimini, J. Schnitzbauer, W. Zhang, G.W. Li, J. Park, E.H. Blackburn, J.S. Weissman, L.S. Qi, and B. Huang. 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155(7):1479-1491.

Cheng, A.W., H. Wang, H. Yang, L. Shi, Y. Katz, T.W. Theunissen, S. Rangarajan, C.S. Shivalila, D.B. Dadon, and R. Jaenisch. 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res*. 23(10):1163-1171.

Citorik, R.J., M. Mimee, and T.K. Lu. 2014. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat Biotechnol*. 32(11):1141-1145.

Dana, G.V., A.M. Cooper, K.M. Pennington, and L.S. Sharpe. 2014. Methodologies and special considerations for environmental risk analysis of genetically modified aquatic biocontrol organisms. *Biol Invasions*. 16(6):1257-1272.

David, A.S., J.M. Kaser, A.C. Morey, A.M. Roth, and D.A. Andow. 2013. Release of genetically engineered insects: a framework to identify potential ecological effects. *Ecol Evol*. 3(11):4000-4015.

Deltcheva, E,, K. Chylinski, C.M. Sharma, K. Gonzales, Y. Chao, Z.A. Pirzada, M.R. Eckert, J. Vogel, and E. Charpentier. 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471(7340):602-607.

Deredec ,A., A. Burt, and H.C.J. Godfray. 2008. The population genetics of using homing endonuclease genes in vector and pest management. *Genetics* 179(4): 2013-2026.

Deredec, A., H.C.J. Godfray, and A. Burt. 2011. Requirements for effective malaria control with homing endonuclease genes. *Proc. Natl Acad. Sci.* 108(43): E874–E880.

DiCarlo, J.E., A. Chavez, S.L. Dietz, K.M. Esvelt, and G.M. Church. 2015. RNA-guided gene drives can efficiently bias inheritance in wild yeast. Boston Massachusetts: Harvard Medical School, Department of Genetics. http://biorxiv.org/content/early/2015/01/16/013896

Eliminate Dengue Research Program. 2012. Eliminate dengue our challenge. Clayton, Victoria, Australia: Institute of Vector-borne Disease, Monash University. www.eliminatedengue.com/progress.

Esvelt, K.M., P. Mali, J.L. Braff, M. Moosburner, S.J. Yaung, and G.M. Church. 2013. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods*. 10(11):1116-1121.

Esvelt, K.M., A.L. Smidler, F. Catteruccia, and G.M. Church. 2014. Concerning RNA-guided gene drives for the alteration of wild populations. ELife 3: e03401.

Farzadfard, F., S.D. Perli, and T.K. Lu. 2013. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth Biol*. 2(10):604-613.

Galizi, R., L.A. Doyle, M. Menichelli, F. Bernardini, A. Deredec, A. Burt, B.L. Stoddard, N. Windbichler, and A. Crisanti. 2014. A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nat Commun*. 5(3977).

Gantz, V.M., and E. Bier. 2015. Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science*. 348(6233):442-444.

Gao, H., J. Smith, M. Yang, S. Jones, V. Djukanovic, M.G. Nicholson, A. West, D. Bidney, S.C. Falco, D. Jantz, and L.A. Lyznik. 2010. Heritable targeted mutagenesis in maize using a designed endonuclease. *The Plant Journal* 61(1):176-187.

Gentile, J.E., S.S. Rund, G.R. Madey. 2015. Modelling sterile insect technique to control the population of *Anopheles gambiae*. *Malar J*. 14:92.

Gilbert, L.A., M.H. Larson, L. Morsut, Z. Liu, G.A. Brar, S.E. Torres, N. Stern-Ginossar, O. Brandman, E.H. Whitehead, J.A. Doudna, W.A. Lim, J.S. Weissman, and L.S. Qi. 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 154(2):442-451.

Gilna, B., J. Kuzma, and S.S. Otts. 2014. Governance of genetic biocontrol technologies for invasive fish. *Biol Invasions* 16(6):1299-1312.

Gould, F., and P. Schliekelman. 2004. Population genetics of autocidal control and strain replacement. *Annu Rev Entomol* 49(1):193-217.

Gratz, S.J., J. Wildonger, M.M. Harrison, and K.M. O'Connor-Giles. 2013. CRISPR/Cas9-mediated genome engineering and the promise of designer flies on demand. *Fly* 7(4):249-255.

Guilinger, J.P., D.B. Thompson, and D.R. Liu. 2014. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat Biotechnol*. 32(6):577-582.

Gutierrez, J.B., and J. Teem. 2006. A model describing the effect of sex-reversed YY fish in an established population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. *J Theor Biol*. 241(2):333-341.

Hale, C.R., P. Zhao, S. Olson, M.O. Duff, B.R. Graveley, L. Wells, R.M. Terns, and M.P. Terns. 2009. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell. 139(5):945-956.

Hamilton, W.D. 1967. Extraordinary sex ratios. *Science* 156(3744):477-488.

Hardy, C.M., L.A. Hinds, P.J. Kerr, M.L. Lloyd, A.J. Redwood, G.R. Shellam, and T. Strive. 2006. Biological control of vertebrate pests using virally vectored immunocontraception. *J Reprod Immunol*. 71(2):102-111.

Harris, A.F., A.R. McKemey, D. Nimmo, Z. Curtis, I. Black, S.A. Morgan, M.N. Oviedo, R. Lacroix, N. Naish, N.I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C.A. Donnelly, W.D. Petrie, and L. Alphey. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotechnol*. 30(9):828-830.

Harris, A.F., D. Nimmo, A.R. McKemey, N. Kelly, S. Scaife, C.A. Donnelly, C. Beech, W.D. Petrie, and L. Alphey. 2011. Field performance of engineered male mosquitoes. *Nat Biotechnol*. 29(11):1034-1037.

Hayes, K.R., B. Leung, R. Thresher, J.M. Dambacher, and G.R. Hosack. 2014. Meeting the challenge of quantitative risk assessment for genetic control techniques: a framework and some methods applied to the common Carp (*Cyprinus carpio*) in Australia. *Biol Invasions*. 16(6):1273-1288.

Hodgins, K.A., L. Rieseberg, and S.P. Otto. 2009. Genetic control of invasive plants species using selfish genetic elements. *Evol Appl*. 2(4):555-569.

Hoffman, A., B. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P.H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y.S. Leong, Y. Dong, H. Cook, J. Axford, A.G. Callahan, N. Kenny, C. Omodei, E.A. McGraw, P.A. Ryan, S.A. Ritchie, M. Turelli, and S.L. O'Neill. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*. 476(7361):454-7.

Horvath, P, and Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. *Science*. 327(5962):167-170.

Hsu, P.D., E.S. Lander, and F. Zhang. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 157(6):1262-1278.

Hu, J., Y. Lei, W.K. Wong, S. Liu, K.C. Lee, X. He, W. You, R. Zhou, J.T. Guo, X. Chen, X. Peng, H. Sun, H. Huang, H. Zhao, and B. Feng. 2014. Direct activation of human and mouse Oct4 genes using engineered TALE and Cas9 transcription factors. *Nucleic Acids Res*. 42(7):4375-4390.

ISSG (Invasive Species Specialist Group). 2007. Comparing U.S. Animal import list to global invasive species data. Auckland, New Zealand: School of Geography, Geology, and Environmental Sciences. http://www.defenders.org/publications/comparing_u.s._animal_import_list_to_global_invasive_species_data.pdf

Jiang, W., and L.A. Marraffini. 2015. CRISPR-Cas: New tools for genetic manipulations from bacterial immunity systems. *Annu Rev Microbiol*. 69:209-228.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 337(6096):816-821.

Kapuscinski, A.R., and L.M. Sharpe. 2014. Introduction: genetic biocontrol of invasive fish species. *Biol Invasions*. 16(6):1197-1200.

Kelly, S. 2002. Invasive plant impacts. Chapter 1 in CIPM (ed.), Invasive Plant Management: CIPM Online Textbook. Bozeman, MT: Center for Invasive Plant Management (CIPM). http://www.weedcenter.org/education/adult.html/

Kistler, K.E., L.B. Vosshall, and B.J. Matthews. 2014. Genome-engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. http://biorxiv.org/content/biorxiv/early/2014/12/30/013276.full.pdf

Koike-Yusa, H., Y. Li, E.P. Tan, M.D.C. Velasco-Herrera, and K. Yusa. 2014. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*. 32(3):267-273.

Kondo, S., and R. Ueda. 2013. Highly improved gene targeting by germline-specific Cas9 expression in *Drosophila*. *Genetics*. 195(3):715-721.

Konermann, S., M.D. Brigham, A.E. Trevino, J. Joung, O.O. Abudayyeh, C. Barcena, P.D. Hsu, N. Habib, J.S. Gootenberg, H. Nishimasu, O. Nureki, and F. Zhang. 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 517(7536):583-588.

Lacroix, R., A.R. McKemey, N. Raduan, L.K. Wee, W.H. Ming, T. G. Ney, A.A.S. Rahidah, S. Salman, S. Subramaniam, O. Nordin, A.T.N. Hanum, C. Angamuthu, S.M. Mansor, R.S. Lees, N. Naish, S. Scaife, P. Gray, G. Labbé, C. Beech, D. Nimmo, L. Alphey, S.S. Vasan, L.H. Lim, A.N. Wasi, and S. Murad. 2012. Open field release of genetically engineered sterile male *Aedes aegypti* in Malaysia. *PLoS One* 7(8):e42771.

Ledford, H. 2015. CRISPR, the disruptor. *Nature*. 522(7554):20-24.

Long, C., J.R. McAnally, J.M. Shelton, A.A. Mireault, R. Bassel-Duby, E.N. Olson. 2014. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science*. 345(6201):1184-1188.

Louda, S.M., D. Kendall, J. Connor, and D. Simberloff. 1997. Ecological effects of an insect introduced for the biological control of weeds. *Science* 277(5329):1088-1090.

Makarova, K.S., D.H. Haft, R. Barrangou, S.J. Brouns, E. Charpentier, P. Horvath, S. Moineau, F.J. Mojica, Y.I. Wolf, A.F. Yakunin, J. van der Oost, and E.V. Koonin. 2011. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol*. 9(6):467-477.

Mali, P., J. Aach, P.B. Stranges, K.M. Esvelt, M. Moosburner, S. Kosuri, L. Yang, and G.M. Church. 2013. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol*. 31(9):833-838.

Marshall, J.M. 2009. The effect of gene drive on containment of transgenic mosquitoes. *J Theor Biol*. 258(2):250-265.

Miller, J.C., S. Tan, G. Qiao, K.A. Barlow, J. Wang, D.F. Xia, X. Meng, D.E. Paschon, E. Leung, S.J. Hinkley, G.P. Dulay, K.L. Hua, I. Ankoudinova, G.J. Cost, F.D. Urnov, H.S. Zhang, M.C. Holmes, L. Zhang, P.D. Gregory, and E.J. Rebar. 2011. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol*. 29(2):143-8.

Mojica, F.J., C. Díez-Villaseñor, J. García-Martínez, and C. Almendros. 2009. Short motif sequences determine the targets of the prokaryotic CRISPR defence [*sic*] system. *Microbiology*. 155(3):733-740.

Mojica, F.J., C. Díez-Villaseñor, J. García-Martínez, and E. Soria. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol*. 60(2):174-182.

Molloy, D.P., D.A. Mayer, M.J. Gaylo, J.T. Morse, K.T. Presti, P.M. Sawyko, A.Y. Karatayev, L.E. Burlakova, F. Laruelle, K.C. Nishikawa, and B.H. Griffin. 2013. *Pseudomonas fluorescens* strain CL145A - a biopesticide for the control of zebra and quagga mussels (Bivalvia: Dreissenidae). *J Invertebr Pathol*. 113(1):104-114.

Moscamed. 2012. Projecto Aedes transgênico. http://www.moscamed.org.br/2012/projeto-aedes/1

Muñoz, I.G., J. Prieto, S. Subramanian, J. Coloma, P. Redondo, M. Villate, N. Merino, M. Marenchino, M. D'Abramo, F.L. Gervasio, S. Grizot, F. Daboussi, J. Smith, I. Chion-Sotinel, F. Pâques, P. Duchateau, A. Alibés, F. Stricher, L. Serrano, F.J. Blanco, and G. Montoya. 2011. Molecular basis of engineered meganuclease targeting of the endogenous human RAG1 locus. *Nucleic Acids Res*. 39(2):729-743.

Myers, J.H., and D. Bazely. 2003. Ecology and Control of Introduced Plants. Cambridge Massachusetts: Cambridge University Press.

National Archives and Records Administration. 1999. Invasive species. Executive Order 13112. *Federal Register* 64(25): 6183-6186.

Nishimasu, H., F.A. Ran, P.D. Hsu, S. Konermann, S.I. Shehata, N. Dohmae, R. Ishitani, F. Zhang, and O. Nureki. 2014. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell. 156(5):935-949.

O'Connor, L., C. Plichart, A.C. Sang, C.L. Brelsfoard, H.C. Bossin, and S.L. Dobson. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: field performance and infection containment. *PLoS Negl Trop Dis*. 6(11):e1797.

Otts, S.S. 2014. U.S. regulatory framework for genetic biocontrol of invasive fish. *Biol Invasions* 16(6):1289-1298Oye, K.A., K. Esvelt, E. Appleton, F. Catteruccia, G. Church, T. Kuiken, S.B. Lightfoot, J. McNamara, A. Smidler, and J.P. Collins. 2014. Regulating gene drives. *Science* 345(6197): 626-628.

Oxitec. 2016. Friendly *Aedes aegypti* project. http://www.oxitec.com/programmes/brazil/

Paix, A., Y. Wang, H.E. Smith, C.Y. Lee, D. Calidas, T. Lu, J. Smith, H. Schmidt, M.W. Krause, and G. Seydoux. 2014. Scalable and versatile genome editing using linear DNAs with microhomology to Cas9 sites in *Caenorhabditis elegans*. *Genetics* 198(4):1347-1356.

Pennisi, E. 2013. The CRISPR craze. *Science* 341(6148): 833-836.

Pennisi E. 2014. U.S. researchers call for greater oversight of powerful genetic technology. *Science Insider*.

Pimental, D. 2002. Biological invasions: Economic and environmental costs of alien plant, animal and microbe species. Boca Raton, FL: CRC Press.

Pimentel, D., R. Zuniga, and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52(3):273-288.

Pourcel, C., G. Salvignol, and G. Vergnaud. 2005. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 151 (3):653-663.

Rath, D., L. Amlinger, A. Rath, and M. Lundgren. 2015. The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*. 117: 119-128.

Reeves, R.G., J.A. Denton, F. Santucci, J. Bryk, and F.A. Reed. 2012. Scientific standards and the regulation of genetically modified insects. *PLoS Negl Trop Dis*. 6(1): e1502.

Sakuma, T., and K. Woltjen. 2014. Nuclease-mediated genome editing: At the front-line of functional genomics technology. *Dev Growth Differ*. 56(1): 2-13.

Sander, J.D., and J.K. Joung. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol*. 32(4):347-355.

Sapranauskas, R., G. Gasiunas, C. Fremaux, R. Barrangou, P. Horvath, V. Siksnys. 2011. The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res*. 39(21):9275-9282.

Scott, T.W., W. Takken, B.G.J. Knols, C. Boëte. 2002. The ecology of genetically modified mosquitoes. *Science* 298(5591):117-119.

Shalem, O., N.E. Sanjana, E. Hartenian, X. Shi, D.A. Scott, T.S. Mikkelsen, D. Heckl, B.L. Ebert, D.E. Root, J.G. Doench, and F. Zhang. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343(6166):84-87.

Shearer, J.F., N.E. Harms, and M. Graves. 2013. Impact of Invasive Species on Department of Defense Installations in the Chesapeake Bay Watershed. ERDC/EL LR-13-1. Vicksburg, MS: U.S. Army Engineer Research and Development Center.

Simoni, A., C. Siniscalchi, Y.S. Chan, D.S. Huen, S. Russell, N. Windbichler, and A. Crisanti. 2014. Development of synthetic selfish elements based on modular nucleases in *Drosophila melanogaster*. *Nucleic Acids Res*. 42(11):7461-7472.

Sinkins, S.P., and F. Gould. 2006. Gene drive systems for insect disease vectors. *Nat Rev Genet*. 7(6):427-435.

Stein, B.A., L.S. Kutner, and J.S. Adams, eds. 2000. Precious heritage: The Status of Biodiversity in the United States. New York, NY: Oxford University Press.

Sternberg, S.H., and J.A. Doudna. 2015. Expanding the biologist's toolkit with CRISPR-Cas9. *Mol Cell*. 58(4):568-574.

Sternberg, S.H., S. Redding, M. Jinek, E.C. Greene, and J.A. Doudna. 2014. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507(7490):62-67.

Stoddard, B.L. 2011. Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure* 19(1):7-15.

Takeuchi, R., M. Choi, and B.L. Stoddard. 2014. Redesign of extensive protein–DNA interfaces of meganucleases using iterative cycles of in vitro compartmentalization. *Proc Natl Acad Sci*. 111(11):4061-4066.

Takeuchi, R., A.R. Lambert, A.N. Mak, K. Jacoby, R.J. Dickson, G.B. Gloor, A.M. Scharenberg, D.R. Edgell, and B.L. Stoddard. 2011. Tapping natural reservoirs of homing endonucleases for targeted gene modification. *Proc. Natl Acad Sci*. 108(32):13077-13082.

Taylor, G.K., H. Petrucci, A.R. Lambert, S.K. Baxter, J. Jarjour, and B.L. Stoddard. 2012. LAHEDES: the LAGLIDADG homing endonuclease database and engineering server. *Nucleic Acids Res*. 40(W1):W110-W116.

Tebas, P., D. Stein, W.W. Tang, I. Frank, S.Q. Wang, G. Lee, S.K. Spratt, R.T. Surosky, M.A. Giedlin, G. Nichol, M.C. Holmes, P.D. Gregory, D.G. Ando, M. Kalos, R.G. Collman, G. Binder-Scholl, G. Plesa, W.T. Hwang, B.L. Levine, and C.H. June. 2014. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 370(10):901-910.

Terns, M.P., and R.M. Terns. 2011. CRISPR-based adaptive immune systems. *Microbiol*. 14(3):321-327.

Terns, R.M., and M.P. Terns. 2014. CRISPR-based technologies: prokaryotic defense weapons repurposed. *Trends in Genet*. 30(3):111-118.

Thomas, D.D., and C.A. Donnelly, R.J. Wood, and L.S. Alphey. 2000. Insect population control using a dominant, repressible, lethal genetic system. *Science* 287(5462):2474-24766.

Thresher, R.E. 2007. Genetic Options for the Control of Invasive Vertebrate Pests: Prospects and Constraints. In proceedings of the USDA National Wildlife Research Center Symposia – Managing Vertebrate Invasive Species, 1 August, Lincoln, Nebraska, Paper 52. Lincoln, Nebraska: University of Nebraska. http://digitalcommons.unl.edu/nwrcinvasive/52

Thresher, R.E. 2008. Autocidal technology for the control of invasive fish. *Fisheries*. 33(3):114-121.

Thresher, R.E., P. Grewe, S. Whyard, J. Patil, C.M. Templeton, A. Chaimongol, C.M. Hardy, L.A. Hinds, and R. Dunham. 2009. Development of repressible sterility to prevent the establishment of feral populations of exotic and genetically modified animals. *Aquaculture*. 290(1):104-109.

Thresher, R.E., K. Hayes, N.J. Bax, J. Teem, T.J. Benfey, and F. Gould. 2014. Genetic control of invasive fish: technological options and its role in integrated pest management. *Biol Invasions*. 16(6):1201-1216.

Thyme, S.B., S.J. Boissel, S.A. Quadri, T. Nolan, D.A. Baker, R.U. Park, L. Kusak, J. Ashworth, and D. Baker. 2014. Reprogramming homing endonuclease specificity through computational design and directed evolution. *Nucleic Acids Res.* 42(4):2564-2576

Touré, Y.T., A.M.J. Oduola, J. Sommerfeld, and C.M. Morel. 2004. Biosafety and risk assessment in the use of genetically modified mosquitoes for disease control. In *Ecological aspects for application of genetically modified mosquitoes, Vol.2* (Wageningen UR Frontis Series), eds. W. Takken and T.W. Scott, 217-222. Dordrecht, Netherlands: Kluwer Academic Publishers.

Tsai, S.Q., N. Wyvekens, C. Khayter, J.A. Foden, V. Thapar, D. Reyon, M.J. Goodwin, M.J. Aryee, and J.K. Joung. 2014. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nat Biotechnol.* 32(6):569-576.

Tu, M., C. Hurd, and J.M. Randall. 2001. Weed Control Methods Handbook: Tools & Techniques for Use in Natural Areas. Paper 533. Logan, UT: Utah State University-All U.S. Government Documents (Utah Regional Depository). http://digitalcommons.usu.edu/govdocs/533

United Nations (UN). 1925. Geneva Protocol. New York, NY: United Nations Office of Disarmament Affairs (UNODA). www.un.org/disarmament/WMD/Bio/1925-Geneva-Protocol/

UN. 1972. Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction. New York, NY: United Nations Office of Disarmament Affairs (UNODA). www.un.org/disarmament/WMD/Bio/

UN. 2000. Cartagena protocol on biosafety. In Proceedings, *Covention on Biological Diversity*, 29 January. Montreal, Canada, 2: 1-23. https://treaties.un.org/doc/Treaties/2000/01/20000129%2008-44%20PM/Ch_XXVII_08_ap.pdf

United Nations Environmental Programme (UNEP). 2010. Final report of the ad hoc technical expert group on risk assessment and risk management under the cartagena protocol on biosafety. In proceedings, *Convention on Biological Diversity*, 20-23 April, Ljubljana, Slovenia, 1-40.http://www.cbd.int/doc/meetings/bs/bsrarm-02/official/bsrarm-02-05-en.pdf

Urnov, F.D., E.J. Rebar, M.C. Holmes, H.S. Zhang, and P.D. Gregory. 2010. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*. 11(9):636-646.

U.S. Science and Technology Office (USSTO). 2013. United States Government policy for institutional oversight of life sciences dual use research of concern. Draft Notice. *Federal Register* 78(36): 12369-12372. https://www.federalregister.gov/articles/2013/02/22/2013-04127/united-states-government-policy-for-institutional-oversight-of-life-sciences-dual-use-research-of-concern.

Voytas, D.F., and C. Gao. 2014. Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biol.* 12(6):e1001877.

Wang, H, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, and R. Jaenisch. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153(4):910-918.

Wang, T., J.J. Wei, D.M. Sabatini, and E.S. Lander. 2014a. Genetic screens in human cells using the CRISPR-Cas9 system. Science. 343(6166):80-84.

Wang, Y., X. Cheng, Q. Shan, Y. Zhang, J. Liu, C. Gao, J.L. Qiu. 2014b. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol. 32(9):947-951.

Weldy, T. 2008. Invasive species management on military lands. In *Conserving Biodiversity on Military Lands: A Guide for Natural Resources Managers*, eds. N. Benton, J.D. Ripley, and F. Powledge, 118-127. Arlington, VA: Department of Defense Legacy Program, NatureServe. http://www.dodbiodiversity.org/ch7/Chapter.7.Invasive_Species.pp118-127.pdf.

World Health Organization (WHO). 2015. Vector-borne diseases. www.who.int/mediacentre/factsheets/fs387/en/

Wikipedia. 2015. CRISPR. https://en.wikipedia.org/wiki/CRISPR.

Wiedenheft, B., S.H. Sternberg, and J.A. Doudna. 2012. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*. 482(7385):331-338.

Windbichler, N., P.A. Papathanos, and A. Crisanti. 2008. Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genet*. 4(12):e1000291.

Windbichler, N., M. Menichelli, P.A. Papathanos, S.B. Thyme, H. Li, U.Y. Ulge, B.T. Hovde, D. Baker, R.J. Monnat, Jr., A. Burt, and A. Crisanti. 2011. A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature*. 473(7346): 212-215.

Yin, H., W. Xue, S. Chen, R.L. Bogorad, E. Benedetti, M. Grompe, V. Koteliansky, P.A. Sharp, T. Jacks, and D.G. Anderson. 2014. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol*. 32(6):551-553.

Yosef, I., M.G. Goren, and U. Qimron. 2012. Proteins and DNA elements essential for the CRISPR adaptation process in Escherichia coli. *Nucleic Acids Res*. 40(12):5569-5576.

Zhou, Y., S. Zhu, C. Cai, P. Yuan, C. Li, Y. Huang, and W. Wei. 2014. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature*. 509(7501):487-491.

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14. ABSTRACT

Applications of genetic engineering-based technologies to the control of invasive species are emerging as an important area of innovation. As a mechanism to spread the desired genes and associated traits into the target population, gene drive is recognized as a promising genetic biocontrol strategy to combat invasive species. Unlike existing technologies that depend on the cumbersome custom-making of new proteins for each DNA target, the CRISPR system uses RNA as its DNA-homing mechanism, which makes it low cost, high efficiency, easy to implement, and it dramatically shortens the design-build-test cycle for gene drive development. The CRISPR/Cas9 technology can alter multiple loci at the target gene that prevent mutations from blocking the spread of the drive. It can also be used to develop multiple types of gene drives such as precision, immunization and reversal drives to precisely target a specific subpopulation, protect a population from any future gene drive "invasions," and overwrite previously released drives in case of unanticipated effects, respectively. Poised to become a self-sustaining, highly efficient, environmentally benign, and cost-effective alternative for invasive species control, CRISPR/Cas9-mediated gene drives can sweep a "deleterious" gene through a population exponentially faster than the normal Mendelian inheritance, and thus suppress or eradicate the target invasive species.

15. SUBJECT TERMS

Invasive species; Biocontrol; Genetic control; CRISPR/Cas9; Gene drive; Military lands; Nonindigenous pests; Pests--Biological control; Genetics; Military bases; Environmental protection

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