Gene drive overdrive

The recent publication of a simple procedure for creating a CRISPR-Cas9-mediated gene drive has some researchers sounding the alarm. What are the risks to populations in the wild and what precautions are necessary? Laura DeFrancesco investigates.

The research world is abuzz at the first successful demonstration of a CRISPR-Cas9-mediated gene drive. Prompting a combination of breathless excitement and horror, the report of this new type of gene drive system—a synthetic genetic construct capable of propagating itself and a trait to which it is linked through a population in a few generations—has spurred debate concerning the potential risks such a construct would pose if released into the wild. This has led not only to calls for controls to ensure native species are protected but also to the convening of a US National Academy of Sciences (NAS) working group that will look into responsible use of the technology. In the meantime, the small cadre of researchers who have for decades been quietly working away applying gene drives to real world problems are wondering what all the kerfuffle is about. With RNA-directed gene drives still in their infancy and a host of questions remaining unanswered, what is the evidence that gene drives present the clear and present danger that some fear?

Fly make-over via RNA-mediated gene drive. Pictured is a rare 50% mosaic female fly, in which the *yellow* allele has been driven by the *y*-MCR element into the genome of a cell presumably at the two-cell stage, creating a fly with a yellow left half and a normal right half. Fly image provided by V. Gantz and E. Bier, UCSD.

Skirting Mendelian genetics

The concept of a gene drive dates back to 1968, when the late C.F. Curtis of the London School of Hygiene and Tropical Medicine proposed using translocations to drive anti-pathogenic genes into wild vector species[1]—where much of the interest in gene drives is still focused to this day. The rationale was that an individual heterozygous for a translocation involving two chromosomes would give rise to 50% viable offspring when crossed to a normal individual because of the imbalance created when the two translocated chromosomes segregate away from each other. However, if the translocation is homozygous, balance would be restored, albeit with a different gene order, but nonetheless, 100% would survive. The model predicts that an individual homozygous for a translocation mated to the wild type would thus eventually kick the wild-type chromosome out.

Unfortunately, at that time, there was no way to do the experiment. The only way to induce translocations was through irradiation, which creates a high background of uncharacterizable mutations. And of course back then, there was no way to link a desired (drive) gene to a translocation. It was all theoretical.

Several decades later in 2003, Austin Burt at the Imperial College, London, proposed to manipulate natural populations by targeting homing endonucleases—a kind of 'selfish DNA' that propagates horizontally with no biological purpose—to functional genes that could alter an organism's fitness or the ability to reproduce. He described various scenarios for eradicating noxious species by targeting fertility genes, for example, and modeled their behavior in the wild. Eight years later, Burt and colleagues validated the basic outlines of their model in the creation of a synthetic endonuclease-based gene drive in mosquitoes—in this case showing that the I-SceI-endonuclease, a particularly efficient homing endonuclease, can insert itself into the progeny of transgenic *Anopheles gambiae* that had been engineered to contain a cognate recognition site[2]. This was the first demonstration of a nuclease-based gene drive in an animal, and in the choice of animal, was pointing toward controlling the malaria-transmitting mosquito.

However, retargeting of a homing endonuclease to an essential gene (or anything other than its own insertion site for that matter), requires alteration of endonuclease sequence specificity by protein engineering. And although this is not impossible, it is difficult, labor intensive and slow. Most homing endonucleases have long targeting sites, making it difficult to get them to redirect cutting to the particular gene of interest.

Which is where CRISPR-Cas9 endonuclease alters the equation. With its simple, easy-to-retarget nucleic acid system for specifying target recognition sites, conferred by one or more guide RNA (gRNA) molecules, suddenly the specificity issue has become tractable. And it was precisely this feature that enabled two University of California, San Diego (UCSD) researchers, Ethan Bier and Valentino Gantz—who were looking for a way to quickly identify *Drosophila* mutants—to create a stunningly efficient gene drive. Their CRISPR-Cas9 system is capable of driving a mutation into 97% of fly progeny in just two generations. These results, published in *Science*[3] in April, prompted a chorus of concerns about the potential effects of such a drive in altering (or even eliminating) an entire population in the wild with dire consequences for ecosystems.

Sparking a firestorm

The power of CRISPR-Cas9 gene drives was not unanticipated. Last year, George Church's group at Harvard published a speculative and detailed feature describing how CRISPR-Cas9 gene drives could be constructed[4]. The salient feature of these drives is combining the gRNA, the edited (desired) version of the target gene and the endonuclease into a cassette, such that an endonuclease and edited gene are inserted together into the target gene. If introduced into the germline of a species, the elements are inherited in that stable and self-perpetuating configuration—self-perpetuating as the endonuclease and gRNA can in theory find and correct homologous, unedited versions of the target gene at each division or generation.

In the same paper, the Church team warned that under some circumstances, organisms with heritable gene drives released into the environment could spread through populations and potentially threaten wild species. It is just this potential that researchers like Burt and others have been trying to exploit for over a decade—specifically to create gene drives using various homing endonucleases that sweep through and eliminate insect populations that harbor pathogens[5].

Worried about the potential effects of a gene drive running amok in the wild, the Church team set about designing potential safety mechanisms, both physical and molecular, either to guard against release in the first place or to reverse a gene drive that has been released into a population (**Box 1**). The problem with the gene drive

Box 1 Protection from gene drive mayhem

A preprint from the Church group demonstrates gene drives in yeast[11]. In this work, the group purposefully designed a drive that would not be self-sustaining by separating the gene being driven and its gRNA from the endonuclease, which was carried on a plasmid—which they called a split drive. In a single mating cycle, they successfully drove a mutated version of the phosphoribosyl-aminoimidazole carboxylase *ADE2* locus not only into their laboratory strain, but also into a number of wild strains of yeast. This prompted the authors to outline various safety measures to guard against inadvertent spread of a gene drive into related wild populations after a release into the environment.

Physical containment measures are the first line of defense and, in fact, experiments on transgenic insects are already done under containment, according to principles laid out in US National Institutes of Health Guidelines[12]. Universities where recombinant DNA work is being done are obligated to have institutional biosafety committees to oversee experiments involving transgenic organisms. Various professional societies have also weighed in; in the case of insects, the American Committee of Medical Entomology has also published arthropod containment guidelines[13].

However, given the ease with which insects can escape from laboratories, multiple physical containment methods have been suggested—triply nested containers, for example, or positive pressure to prevent escape. Ecological barriers have also been recommended, such as working in geographical regions where no wild species exist. And molecular barriers, such as targeting genes that are not highly conserved, can reduce the likelihood of a gene drive finding a target in the wild.

Church and colleagues also devised some molecular containment methods that could be put in place either preemptively or have ready whenever the release of a gene drive is being planned. Reversal drives, which recode the target gene, could overwrite a gene drive run amok. It is also possible to immunize wild-type populations against particular unwanted gene drives by releasing individuals in which the target site is recoded and hence resistant to the drive.

described by Bier and Gantz in their *Drosophila* experiment is that all of their elements are placed on a single cassette. And as the Church group[4] warned, such a cassette construct has the potential to create a self-sustaining gene drive. It is this aspect that really set alarm bells ringing.

Several commentaries have appeared in prominent journals warning of the dangers implicit in gene drives[6,7]. An article in *Science*[8] published in July and signed by 27 researchers lays out a series of principles that should be applied when using the technology. According to Kenneth Oye, of the Departments of Political Science and Engineering Systems at MIT in Cambridge, Massachusetts, the authors were especially keen to have the article appear in *Science* (where the original Bier and Gantz paper was published) to encourage anyone interested in using the technology to think about the consequences and requisite safeguards before proceeding with experiments.

At the same time, an NAS panel has been established to try and get ahead of the technology and explore environmental and security concerns, areas of uncertainty and policy gaps. Says Oye, who gave a presentation to the NAS panel in July, "The implications of the technology are global. There are benefits that are enormous but also areas of uncertainty and risk that need to be better understood. The question is, do you wait until the technology is done in a field where there are lots of people working?"

CRISPR-Cas9 in the driver's seat

With the discovery of CRISPR-Cas9, endonuclease-based gene drives have seemingly been given a new lease on life; indeed, some are calling it a game changer. And the Bier-Gantz experiment suggests a CRISPR-Cas9-mediated drive can work quite well in *Drosophila*. It is worth noting that Bier and Gantz were not actually trying to design a gene drive, but rather a system for efficiently mutating *Drosophila*. That they succeeded is indisputable, but as they were not optimizing the system for driving a gene, the conclusions that can be drawn with respect to drives are understandably limited. In fact, the authors coined a new name for their technology—mutagenic chain reaction (MCR)—designed to conjure up parallels with another game-changing technology, polymerase chain reaction. "Both methods double DNA content at each iteration—generation for MCR, cycle for PCR," says Bier. Also, he views the gene-drive aspect of the MCR as just one part of the new non-Mendelian genetics that this technology has opened up. "There are serious constraints imposed by Mendelian genetics, and this should enable a completely new era of genetic manipulation based on getting free of those constraints," he ventures.

In their paper, Bier and Gantz targeted a single locus, chosen because it is particularly susceptible to mutagenesis, and used a strain of flies with a bias toward a certain type of DNA repair—homologous recombination. Homologous recombination allows insertion and copying of the template (in the case of the Bier and Gantz paper, the mutated *yellow* gene). Interestingly, another type of DNA repair, nonhomologous end joining, would have a different outcome, destroying the recognition site and creating resistance to insertion and copying. In their experimental design, Bier and Gantz favored copying, in line with their aim; in nature, this would not be expected to be the case. In this respect, the high efficiency of conversion (97%) in their flies may not be surprising.

Researchers working on gene drives in mosquito have also been quick to take up CRISPR-Cas9. Anthony James at the University of California, Irvine, has been working with various systems for driving resistance to the malaria-parasite plasmodium into mosquito populations, not reducing mosquito population *per se*, but rather removing the insect's ability to transmit the parasite. Before the recent CRISPR-Cas9 work, James was principally focused on transposable elements, such as *piggyBac*, as they have the ability to copy themselves into genomes and spread through populations. The difficulty, he found, was that their recognition sites can be as short as a few nucleotides, so they appear to insert almost randomly. What's more, the frequency with which they remobilize is too low. CRISPR-Cas9 solves the problem. "In addition to having all the properties of homing endonucleases, in terms of being able to mobilize and copy itself in, you could actually direct it to the place of your choice. It has gRNA, so you have this extra level of control," says James.

Burt has been working with various schemes to alter sex ratios as a way of controlling *Anopheles gambiae* populations. With protein engineer Barry Stoddard at the Fred Hutchinson Cancer Research Center in Seattle, he and Andrea Crisanti of Imperial College, London, reported last year on an endonuclease (I-PpoI) that targets ribosomal RNA genes exclusively on the X chromosome of *A. gambiae* (a system they term an 'X-chromosome shredder'). The researchers created a version of the endonuclease that is active only during spermatogenesis, such that only Y-bearing sperm (and thus males) make it to the next generation. In caged experiments, they got greater than 95% male offspring[9]. Burt and his colleague Andrea Crisanti are now trying their hand at removing mosquito female fertility genes using a variety of targeted nucleases, including CRISPR-Cas9, transcription factor activator-like effector nucleases (TALENs) and meganucleases.

Is going wild the same as going rogue?

Thus far, gene drives have been created that

work in yeast, *Drosophila* and mosquitoes in the laboratory setting. Each of these has three characteristics that enable a productive gene drive: a sexual reproductive cycle, a short generation time and a reasonably facile genetic system. With mammalian species that have long generation times, like humans and livestock, it would take many decades, if not centuries, for a gene drive to become fixed in a population. And because the reproduction and genetics of agricultural crops are subject to rigorous oversight from a handful of large agriculture companies, they are not apt to be overtaken by a gene drive gone wild.

In the microbial context of haploid bacteria and viruses, gene drives are largely irrelevant and genetic systems already exist (e.g., plasmids and phage) to move genes horizontally through a population, if the intent is to spread a trait through transfection.

Invasive species—such as herbicide-resistant weeds, or animals, like the African carp in the US—are commonly mentioned in discussions of potential applications for gene drives. However, in each case, the relative activities of homologous recombination and nonhomologous end-joining will likely ultimately determine whether a gene drive would work. Carp, with a reproductive cycle of several years, might just be at the outer limits of effective gene drives, according to Kevin Esvelt, a researcher at the Wyss Institute at Harvard Medical School, who works on yeast gene drives. Ron Thresher, a population ecologist at CSIRO in Hobart, Australia, agrees that with longer generation times, the period before a gene drive can become fixed is prolonged. And with carp, the release of a single or small number of individuals would require on the order of a 100 years to become fixed in the population, he says, by which time, the drive could be reversed or some other intervention might become possible (**Box 1**).

Opinions differ as to what would happen were one of these transgenic organisms released into the environment where related species exist. Experience with transgenic organisms suggests that they would fare poorly. "I've always made the assumption that whatever I do to a wild-type mosquito would make it less fit. It's just the idea that whatever is being selected in nature, at least for the time and place, is the most fit thing out there," James says. Esvelt claims that with gene drives, that's no longer the case. "Gene drives distort inheritance in their favor and consequently can spread, even when costly," he says. Both experimental and natural gene drives have, in fact, successfully spread through populations, even given a fitness cost; P-elements (a transposable element) in flies have spread through wild populations of *Drosophila*, for example[10]. Indeed, this is why gene drive systems seemed a necessity for the mosquito work.

Putting fitness arguments aside, there is the fact that natural populations are more diverse genetically than laboratory strains. Polymorphisms in the population at the cleavage site could make the gene drive less likely to find homology, although the Cas9 nuclease does accommodate some mismatching. For CalTech's Bruce Hay, who created a *Drosophila* gene drive using microRNA, any worst-case scenario is likely to be short-lived. "The worst possible thing is that these things would go through their doubling, they'll go into the wild a little bit, and then these errors will start to happen. If CRISPR is in a gene, very quickly the mutant versions that are functional and can't be cleaved will experience positive selection and basically the gene drive will die."

From theory to practice

Not everyone is jumping on the CRISPR-Cas9 bandwagon. Andy Scharenberg of the Seattle Children's Research Institute and the University of Washington, Seattle, who collaborates with Stoddard on engineering nucleases, rejects the notion that RNA-guided nucleases are necessarily the optimal platform for gene targeting applications, including gene drives. "Do you really want to use a nuclease that has to be programmed with a special RNA every time it is used? Or that could be reprogrammed by accident in an organism thus producing unpredictable effects? Or would you rather have a preprogrammed nuclease that works as a protein alone, and whose cleavage specificity is pretty well fixed?" he says. And whereas Stoddard admits the process of engineering proteins, including meganucleases, is labor intensive, he also points out that for some applications, where the highest standards of activity and specificity are required, the performance of a nuclease in a living system outweighs any advantage from the standpoint of ease of manufacturing. "Relative to how long biological and genetic assays take for gene drive systems, a difference between needing a hundred or two hundred days to make a perfect nuclease, versus only needing one day to make a poorly behaved nuclease is not a particularly significant issue," he says.

And although CRISPR-Cas9 might be the flavor of the month, several different enzyme architectures are also already out there that need to be considered. All will need to be judged according to several criteria, such as the ease of engineering, their activity in the cellular context, their specificity of cleavage and the type of repair stimulated by their cleavage. "CRISPRs are out in front with respect to ease of engineering, and their rapid adoption by the community is great in terms of speeding up the rate at which we learn useful things about mosquito genetics in the laboratory. But when it comes to having a safe and effective intervention in nature, the other criteria are also going to be important, and I don't think we can say definitively right now how the different architectures compare," says Austin Burt.

Bier finds the prospects of getting a gene drive to work in the field daunting. "You begin to appreciate how hard it is likely to be to do it in the wild. If this works as we are hoping it does, I will be surprised if the modeling that has been done theoretically, and even carried out in the lab, translates to how it works in the field where you have discontinuous populations of organisms that have different cycles," he says. Work with population suppression technologies, for example, shows that the effects can be transient. "You just wipe out a local group of insects, and then that's it for the spread of the vector. Insects from a distant place that haven't been affected fly back in," says Bier.

But Esvelt, who also appeared before the NAS panel in July, feels that the responsible thing to do is weigh all the possibilities before embarking on a line of experimentation involving a gene drive. "We need to paint the utopian vision of how we could solve a particular problem, and that's why we are working on it. We also need to give the horror stories about all the ways it could possibly go wrong, and we need to invite other people to do the same. We need to recognize that the answer to the question of whether we should use a gene drive in a particular instance—'no' has to be an acceptable answer," he says.

However, Scharenberg thinks it's equally important that any discussion of risks should be tempered by parallel discussions of many known mechanisms that will act to limit the spread of gene drive alleles. Otherwise, "a lay person perusing the recent risk-focused gene drive literature could be led to believe that gene drive systems have a near-term potential to turn the world into the proverbial "grey goo" of dystopian nanotechnology fame, which I don't think serves the field well," he says.

1. Curtis, C.F. *Nature* **218**, 368–369 (1968).
2. Windbichler, N. *et al. Nature* **473**, 212–215 (2011).
3. Gantz, V.M. & Bier, E. *Science* **348**, 442–444 (2015).
4. Esvelt, K. *et al. Elife* e03401 (2014).
5. Marshall, J.M. & Hay, B.A. *PLoS One* **9**, e102694 (2014).
6. Oye, K.A. *et al. Science* **345**, 626–628 (2014).
7. Lunshof, J. *Nature* **521**, 127 (2015).
8. Akbari, O.S. *et al. Science* **349**, 927–929 (2015).
9. Galizi, R. *et al. Nat. Commun.* **5**, 3977 (2014).
10. Engels, W.R. *BioEssays* **14**, 681–686 (1992).
11. DiCarlo, J.E. *et al.* Preprint at http://biorxiv.org/content/early/2015/03/19/013896 (2015).
12. Department of Health and Human Services. NIH guidelines for research involving recombinant or synthetic nucleic acid molecules. http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines (HHS, November 2013).
13. ASTMH-ACME Draft Committee. *Arthropod Containment Guidelines* (ver. 3.1). http://www.astmh.org/AM/Template.cfm?Section=ACME&Template=/CM/ContentDisplay.cfm&ContentID=1444 (The American Committee of Medical Entomology of the American Society for Tropical Medicine and Hygiene; 20 December 2001).