GENE DRIVES

Terrence P McGarty
White Paper No 141
December, 2016

Gene Drives are genetically engineered mechanisms which effectively insert a desired gene segment into a species. The supersaturate the species genetic makeup by inserting a gene along with a CRISPR/Cas9 or similar self-replicating mechanism.

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1 INTRODUCTION

The current capabilities of gene manipulation, also we can probably call it gene engineering, is that we have an ever growing collection of "tools". These tools allow bench folks to add this or subtract that from a cell. We can do this with a somatic cell, an existing cell in an existing organ and a germ line cell, a cell from which the organism will eventually be derived. We can add or delete genes and we can in so doing insert a mechanism which will carry on this editing process no matter what the cell becomes as the organism develops. Furthermore if we get this process working in the germ line cell then we can be assured that as the next generation proceeds this inserted tool for manipulating genes within the organism, if not now within the total species, proceeds in a dramatic manner. We lose Mendelian genetics and the tool insertion now produces a single unaltered lineage.

If this gene is of a certain type, say one which produces only a male, then by blocking in all subsequent lines of any females we can effectively wipe out this species when we have just surviving but non-producing males.

This assembly of tools by the genetic engineer has been called "gene drives". In a sense it "drives" certain genes into all members of a species. At least that is the hope. As the Broad Institute states in its licensing statements[1]:

Gene drive. This is a way to rapidly spread a new gene throughout an entire species in nature. This approach might be used to block the transmission of malaria by mosquitoes, but has the potential to disrupt ecosystems… After consulting with external experts and careful internal consideration, the Broad Institute has decided to make available non-exclusive research and commercial licenses for the use of CRISPR technology in agriculture -- but with important restrictions. These include: Gene drive: We prohibit the use of the licensed technology for gene drive.

[1] https://www.broadinstitute.org/news/licensing-crispr-agriculture-policy-considerations

2 THE PRINCIPLE

We briefly present the principles in a somewhat simplistic fashion. Yet the overall idea is also somewhat straightforward. Namely, it is the ability to utilize the set of gene manipulation tools in such a manner as to achieve dramatic results.

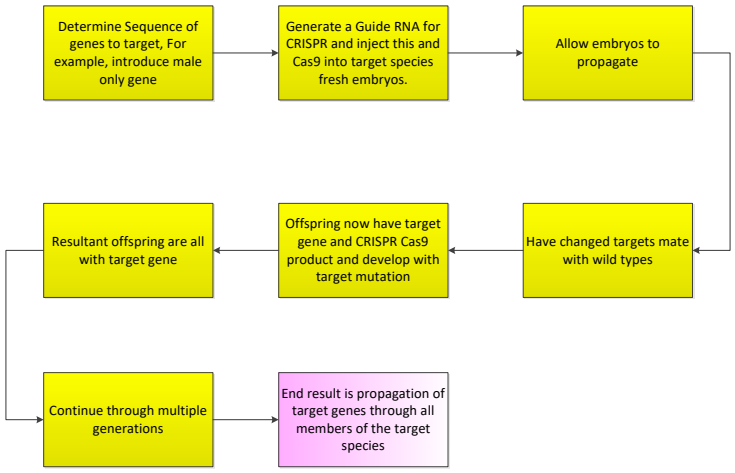
Let us begin with some comments made in early 2015 by Bohannon who notes:

In 28 December 2014, Valentino Gantz and Ethan Bier checked on the fruit flies that had just hatched in their lab at the University of California (UC), San Diego. By the classic rules of Mendelian genetics, only one out of four of the newborn flies should have shown the effects of the mutation their mothers carried, an X-linked recessive trait that causes a loss of pigmentation similar to albinism.

Instead, nothing but pale yellow flies kept emerging. "We were stunned," says Bier, who is Gantz's Ph.D. adviser. "It was like the sun rose in the west rather than the east." They hammered out a paper and submitted it to Science 3 days later….Gantz and Bier report that the introduced mutation disabled both normal copies of a pigmentation gene on the fruit fly chromosomes, transmitting itself to the next generation with 97% efficiency—a near-complete invasion of the genome. The secret of its success: an increasingly popular gene-editing toolkit called CRISPR (Science, 23 August 2013, p. 833), which Gantz and Bier adapted to give the mutation an overwhelming advantage.

The technique is the latest—and some say, most impressive—example of gene drive: biasing inheritance to spread a gene rapidly through a population, or even an entire species. At this level of efficiency, a single mosquito equipped with a parasite-blocking gene could in theory spread malaria resistance through an entire breeding population in a single season

Now this principle can be depicted as shown below.

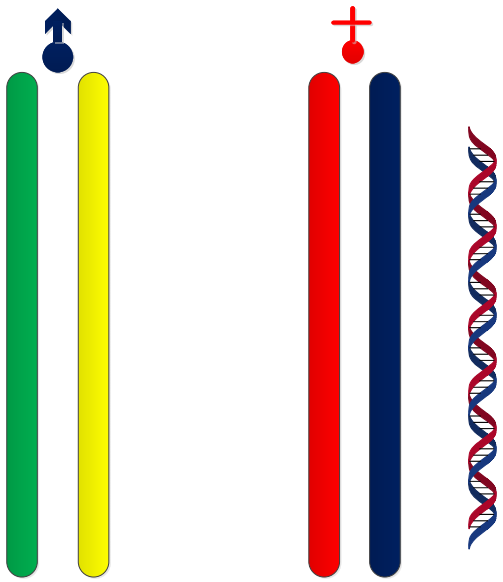


Specifically, the steps are simplified as follows:

1. Select Target Embryo
    a. Embryos are essential since we need to start with a pluripotent cell
2. Select Gene
    a. This is the key step. It is essential to know the target gene and its suppressive character
3. Select Vector
    a. This is a selection of an insert mechanism into the DNA of the embryo such as a lentivirus and reverse transcriptase
4. Select CRISPR
5. Select Cas9
    a. Cas9 is now but one of several endonucleases to cut DNA using the designed CRISPR
    b. One may seek to use others for better performance
6. Insert Gene for Change
7. Insert CRISPR/Cas9
8. Allow Embryo Maturation
9. Release Target Entity

We now cover the process in some high level detail.

Consider a male and female set of chromosome pairs as shown below. Each chromosome pair has one from each of its parents. Thus we depict four different DNA elements, each DNA a double strand. Nothing new here.



Now one question we may ask is if one of the chromosomes from a parent has a gene that results in a certain characteristic then how is it inherited downstream? Simply one can use classic Mendelian genetics and create squares and even include linkages. If but one parent has one gene change then we would expect say one fourth, the off-spring to have that trait. Furthermore, if that

offspring were to mate a wild type, namely one without that gene, then we would see the same. Actually the gene would be diluted in the total population.

Thus the question we pose is twofold:

1. How do we insert a gene which can control offspring, perhaps to the disadvantage of the species?

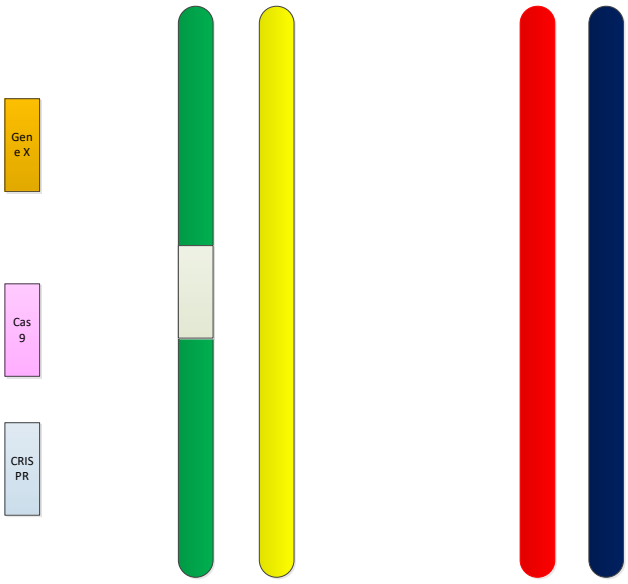
2. How can we alter the inheritance so that every subsequent off-spring will inherit the desired gene to the disadvantage of the species? This is the concept of driving genes into a species.

The answer is twofold:

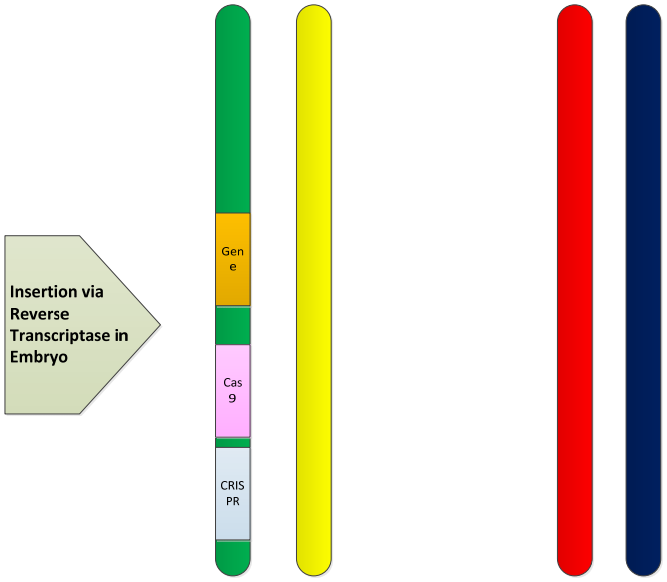
1. First we can insert the gene into an embryo via now standard gene insertion mechanisms such as lentivirus and reverse transcriptase.

2. Second, we can also insert a CRISPR to target the gene propagation and a Cas9 to cut other genes for that insertion so that we have a mechanism to cut and past the desired gene in all other chromosomes, including the wild type mating. We thus create a self-replicating gene insertion so that all off-spring will have every one of their chromosomes get an inserted gene.

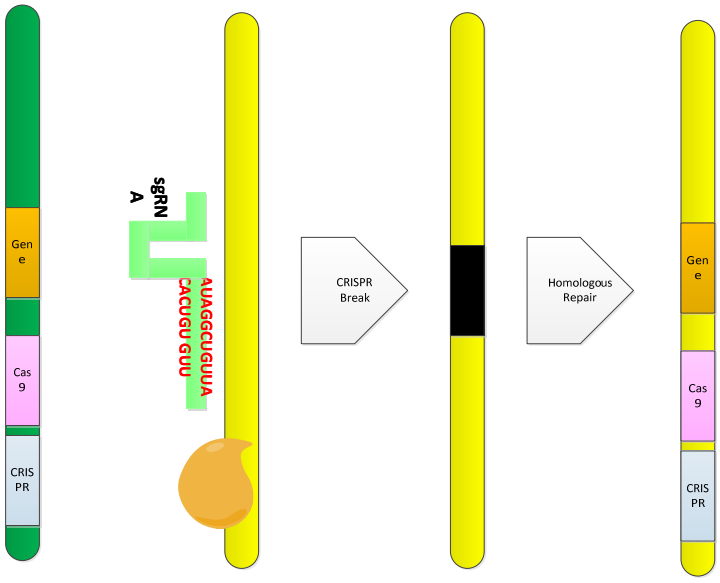
The process begins as below where we want to insert these three segments.



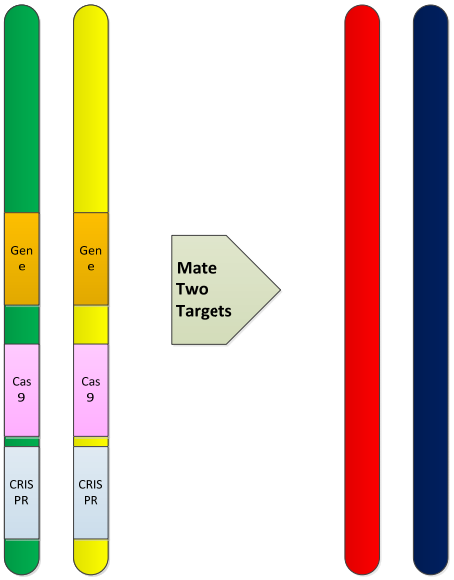
The we can proceed with the insertion of an embryo before it begins to double. That is we find say a mosquito embryo and then proceed to insert the desired genes.



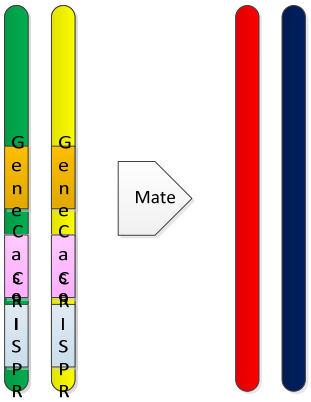
Then the CRISPR machine starts working to insert itself everywhere.

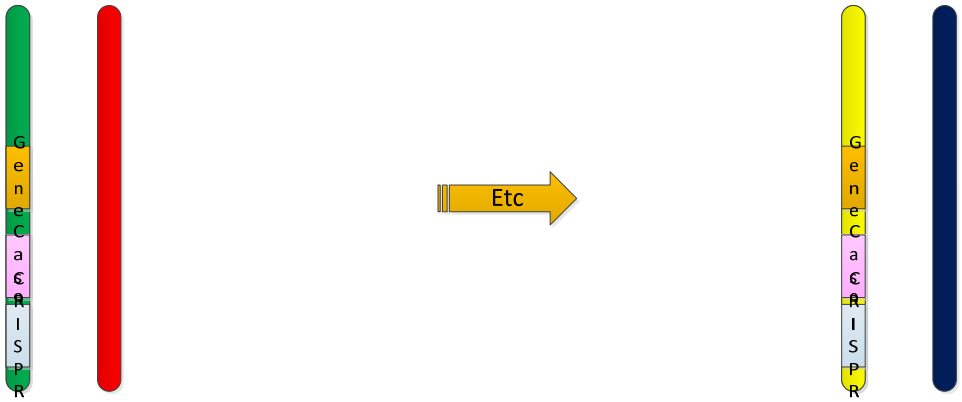


The we mate this changed vector to a wild type target as shown below.

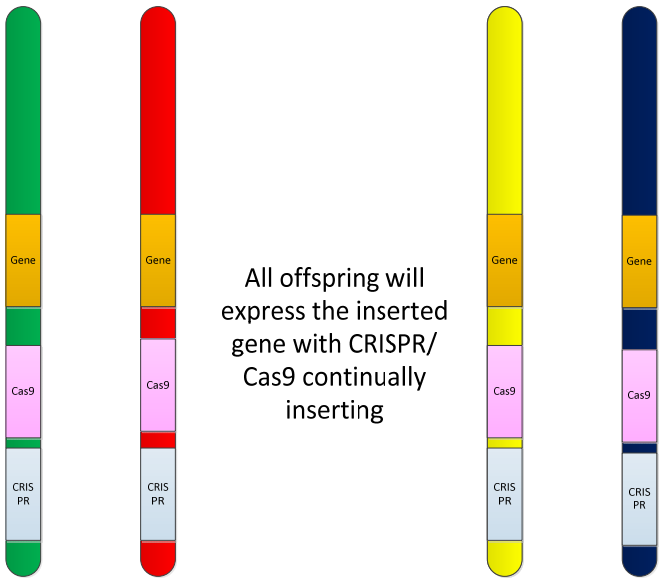


The result of the mating is as per below.





And the process is repeated again and again.



The net result in this simplistic process is the driving of the desired genes into the species by this self-replicating manner.

3 UNDERLYING ELEMENTS

We will now review some of the details of the above summarized steps. Our approach is high level and does not reflect any specific bench process. As we have noted elsewhere the development of gene manipulation "tools" has progressed at a rapid rate. It is in a sense akin to the progress in Chemistry some 150 years ago, ways to distill, separate, purify, various compounds, and in the gene space the "tools" often simple and yet to be fully appreciated, are just similar.

3.1 OUTCOME SELECTION

The first step is what we call outcome selection. What does one want to achieve and what can be achieved. Also, there is the issue of consequences. The process of Outcome Selection will most likely be controlled by some IRB process, akin to drug trials. In drug trials there is the possibility for human harm, but will this be the case with Gene Drives, in fact there could be massive human harm.

Thus, the process of Outcome Selection should be formalized, documented, reviewed and approved. The problem however is the ability for many of these Gene Drive procedures to be performed in random uncontrolled environment without any controls. That as we shall see is the most significant risk.

3.2 GENE SELECTION

Let us examine how the genes are selected. We focus first on the work of Sutton et al. They state:

In this study we have identified a set of genes with testis-specific expression or splicing. In addition to their interest from a basic biology perspective, these findings provide a basis from which to develop synthetic systems to control important pest insects via manipulation of the male germline.…Current strategies for insect control have a number of disadvantages, such as effects on non-target species and development of resistance to insecticides. Alternative synthetic biology approaches are being developed in which the control agent is a modified version of the pest insect itself. These modified insects carry a genetic system that results in the death of some or all of their descendants, so that when released modified insects mate with wild counterparts, population suppression occurs.

Such strategies require characterized modular components that can direct appropriate expression of effector sequences – protein-coding sequences or functional RNAs, for example. Conserved components that can be used across multiple species are particularly useful. However, for many applications there are few if any such components available. The goal of this study was to identify genes that could provide potential components for manipulation of the male germline in two major pest species, the mosquito Aedes aegypti (L.) and the tephritid fruit fly Ceratitis capitate…

High-throughput transcriptional profiling and subtractive hybridization studies have recently yielded several potential testis-specific transcripts in Ae. aegypti. However, to our knowledge, no studies have been performed with sufficient time resolution to determine the activity of regulatory regions at different stages of spermatogenesis. Information on insect testis-specific splicing is even more sparse; testis-specific splice forms of the genes achi and vis have been discovered in D. melanogaster, but no testis-specific splice forms have been identified, to our knowledge, in Ae. aegypti, C. capitata or any other pest insect.

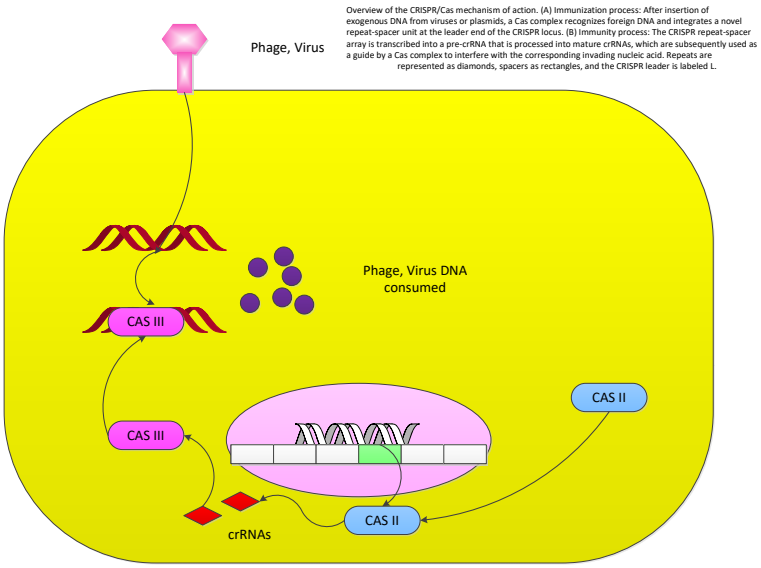
Thus there are now a multiplicity of ways to control such species. The above reduces the offspring to all male. There clearly are many others.

3.3 EMBRYO

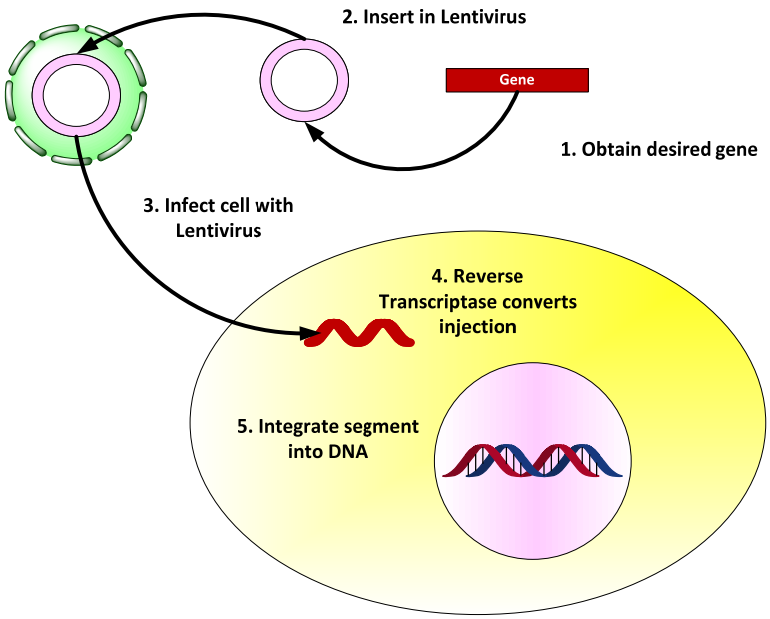
We start typically with an embryo. This is the pluripotent cell, easily targeted say in an insect population. Likewise, in plants since all cells are pluripotent we can reasonably start anywhere.

3.4 INSERTION

Insertion of the genes is performed in a standard manner. We have discussed these in our work on CAR T Cells and refer the reader to those details. We show below a simplified method of insertion using a phage and an effecting it through a reverse transcriptase. This is one of the first of the many tools we see applied here.



The above demonstrates how this applies in the context of Car insertions and the diagram depicts the lentivirus approach from the CAR T cell environment. In effect one can "infect" the embryo with the desired gene segments, get them inserted using the reverse transcriptase and have this done on one or even both chromosomes.



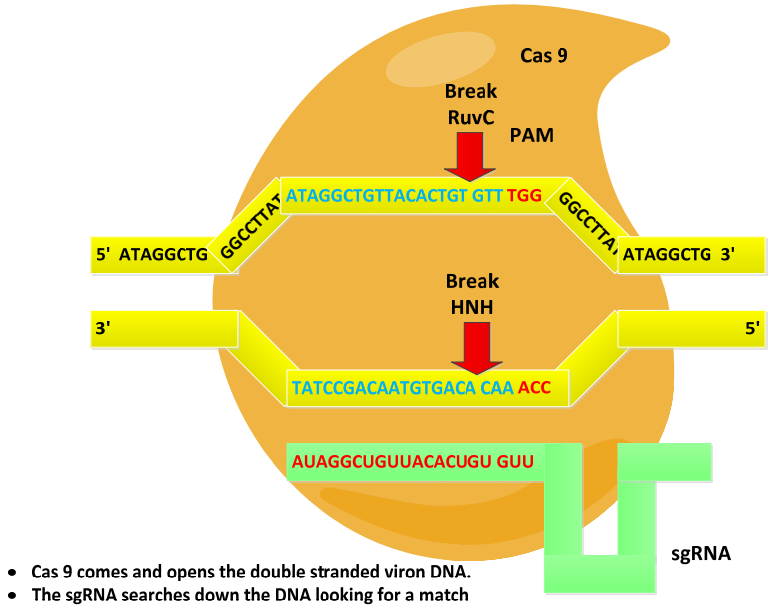
3.5 EXECUTION

We now move on towards the execution. We first consider the CRISPR issues.

3.5.1 CRISPR Cas9 System

CRISPR is an RNA guide that is used in conjunction with the Cas9 endonuclease. Simply CRISPR targets a location to cut and Cas9 does a double side break of the DNA. Then the new gene is inserted and the break repaired by a DNA repair mechanism. We details this in our report on CRISPRs and Cancer, see references.

We demonstrate the CRISPR/Cas9 system below.



3.5.2 Cpf1 System

We have examined CRISPRs for the past few years since their introduction. Initially we had a CRISPR with a Cas9 molecule which managed to cut DNA at specific spots. The CRISPR was designed to match a specific sequence and the Cas9 was able to recognize the PAM sequences and using certain portions of the Cas9 it could then "break" both strands at opposite positions of the DNA, a specific set of base pairs from the end of the PAM.

This then becomes a useful tool in an ever-growing tool-box for DNA modification. In bacteria this cut is applied to viral DNA or RNA and it is a "natural" immune system in the bacteria. In other cells, plants and animals, it enables precise and specific gene editing.

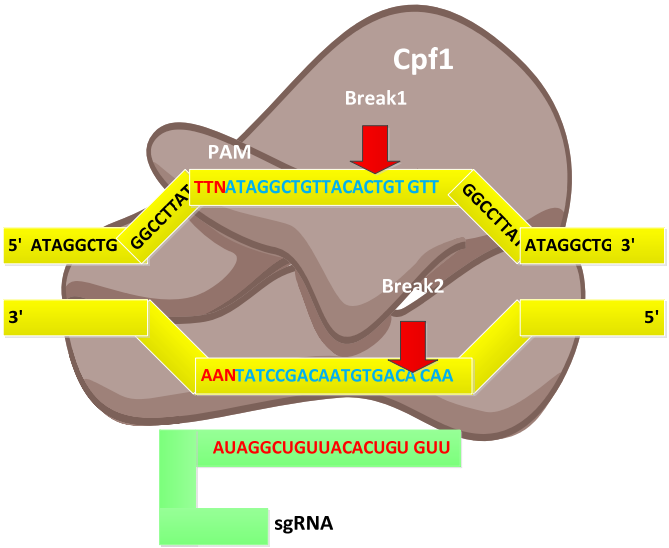
In a recent paper from Zhang's Lab at Broad they have identified another protein which acts like Cas9. This new system is called CRISPR-Cpf1 and is identified as a class 2 CRISPR system[2] . Specifically Cpf1 is a CRISPR-associated two-component RNA-programmable DNA nuclease. It functions in a manner similar to Cas9 and targeted DNA is cleaved as a 5-nt staggered cut distal to a 5′ T-rich PAM. They have also identified two Cpf1 orthologs exhibit robust nuclease activity in human cells. In the paper in Cell they state:

The microbial adaptive immune system CRISPR mediates defense against foreign genetic elements through two classes of RNA-guided nuclease effectors. Class 1 effectors utilize multi-protein complexes, whereas class 2 effectors rely on single-component effector proteins such as

[2] http://www.cell.com/cell/abstract/S0092-8674%2815%2901200-3

the well-characterized Cas9. Here, we report characterization of Cpf1, a putative class 2 CRISPR effector. We demonstrate that Cpf1 mediates robust DNA interference with features distinct from Cas9. Cpf1 is a single RNA-guided endonuclease lacking tracrRNA, and it utilizes a T-rich protospacer-adjacent motif. Moreover, Cpf1 cleaves DNA via a staggered DNA double-stranded break. Out of 16 Cpf1-family proteins, we identified two candidate enzymes from Acidominococcus and Lachnospiraceae, with efficient genome-editing activity in human cells. Identifying this mechanism of interference broadens our understanding of CRISPR-Cas systems and advances their genome editing applications.

The figure below depicts their interpretation of its functioning.



- *Cpf1 system is simpler than Cas9 in that it requires only a single RNA. The Cpf1 enzyme is also smaller than the standard SpCas9, making it easier to deliver into cells and tissues.*
- *Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving "blunt ends" that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends.*
- *Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be recut, allowing multiple opportunities for correct editing to occur.*
- *Cpf1 system provides new flexibility in choosing target sites. Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences.*
- *Cpf1 complex recognizes very different PAM sequences from those of Cas9. This could be an advantage in targeting some genomes, such as in the malaria parasite as well as in humans.*

It is worth comparing these two mechanisms. The Cas9 is a bit more rigid than Cpf1. As noted above and as discussed in the paper and elsewhere, this new protein complex does what Cas9 did but with many more attractive features.

In an MIT press release they state[3] :

The newly described Cpf1 system differs in several important ways from the previously described Cas9, with significant implications for research and therapeutics, as well as for business and intellectual property:

First: In its natural form, the DNA-cutting enzyme Cas9 forms a complex with two small RNAs, both of which are required for the cutting activity. The Cpf1 system is simpler in that it

[3] http://mcgovern.mit.edu/news/news/system-for-genome-editing-could-increase-power-of-genome-engineering/

requires only a single RNA. The Cpf1 enzyme is also smaller than the standard SpCas9, making it easier to deliver into cells and tissues.

Second, and perhaps most significantly: Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving "blunt ends" that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends. This is expected to help with precise insertion, allowing researchers to integrate a piece of DNA more efficiently and accurately.

Third: Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be recut, allowing multiple opportunities for correct editing to occur.

Fourth: The Cpf1 system provides new flexibility in choosing target sites. Like Cas9, the Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences. The Cpf1 complex recognizes very different PAM sequences from those of Cas9. This could be an advantage in targeting some genomes, such as in the malaria parasite as well as in humans.

The above four properties are quite compelling and worthy of note. Cas9 did have the problem of cutting at opposite sites and trusting that a competent and non-aberrant re-fusion was made. This discovery, assumedly after hundreds of attempts, opens the door on another dimension of the CRISPR world.

As is noted in Xcomony they state[4] :

… the Cpf1 work is still in its infancy. It's well behind CRISPR/Cas9—which researchers have used to make changes in the cells of all types of organisms, including humans. Several companies are working with CRISPR/Cas9 to create therapeutics for genetic disease. None have reached clinical trials yet.

The issue here is just how extensive is Cpf1 development and how readily available is the technology. The above presentation seems to imply an early stage. They continue:

But work with CRISPR/Cas9 to modify the human germline—eggs, sperm, and embryos—is also coming faster than expected, sparking ethical concerns. An international summit on the topic is scheduled for December in Washington, DC.

Meanwhile, researchers around the world are working to find new versions of Cas9, or new enzymes entirely, like Cpf1, to make the whole enterprise easier. "There is little doubt that… there are additional systems with distinctive characteristics that await exploration and could further enhance genome editing and other areas of biotechnology as well as shed light on the

[4] http://www.xconomy.com/boston/2015/09/25/crispr-update-could-make-gene-edits-easier-discoverers-say/

evolution of these defense systems," Zhang (pictured above speaking at a 2014 Xconomy event) and his coauthors write in the Cell paper.

In other words, Cpf1 is the tip of the iceberg. I'll outline three differences between Cpf1 and Cas9 that the paper's authors have highlighted as potentially important for the field. First, for those unfamiliar with CRISPR and gene editing, it helps to think of these enzymes as molecular scissors. Bacteria use them in the wild to defend themselves against invading viruses, cutting up the viral RNA and storing the pieces in a kind of immune system memory bank.

It was only in recent years that the natural system has been modified and harnessed as a gene editing tool. The enzyme—a protein—and its guide—made from RNA—need to be sent into a cell (that's one difficult trick) and hit the right spot (that's another difficult trick).

The following is the Xconomy author's description. It is a restatement of what was in the MIT release but rephrases the key differences:

Here's why Zhang and his co-authors think Cpf1 could have advantages over Cas9:

—Cpf1 only uses one strand of RNA as a guide to reach its target gene. Cas9 uses two strands. A single-strand system might lead to simpler, cheaper designs and easier delivery of the enzyme-guide complex into cells.

—Once delivered into the cell's nucleus, Cpf1 makes staggered double-stranded cuts in the target DNA, whereas Cas9 cuts both DNA strands in the same location. This could be important, Zhang and colleagues write, because the staggered ends make it easier to insert a new gene after the old one is removed. That could help get around one of the hurdles of Cas9: Scientists say using Cas9 to replace an old gene with a new one has proven far more difficult than simply cutting out a gene.

—When Cpf1 homes in on a gene, it actually makes the cut off to the side, relatively speaking—farther down the DNA strand. (Imagine your friend holding a string in the exact location that needs snipping. You don't cut her finger; you cut off to the side.) Zhang and colleagues write that this could be a "potentially useful feature" because it preserves the target site for subsequent rounds of editing.

The off-setting of the splices is a significantly better method. It gives the "sticky" ends approach and tends to much fewer errors. This alone could make this much more attractive.

In a Nature discussion of these results they state[5] :

But now one of the technique's pioneers thinks that he has found a way to make CRISPR even simpler and more precise. In a paper published in Cell on 25 September, a team led by synthetic biologist Feng Zhang of the Broad Institute in Cambridge, Massachusetts, reports the discovery of a protein1 called Cpf1 that may overcome one of CRISPR-Cas9's few limitations; although

[5] http://www.nature.com/news/alternative-crispr-system-could-improve-genome-editing-1.18432

the system works well for disabling genes, it is often difficult to truly edit them by replacing one DNA sequence with another.

The CRISPR/Cas9 system evolved as a way for bacteria and archaea to defend themselves against invading viruses. It is found in a wide range of these organisms, and uses an enzyme called Cas9 to cut DNA at a site specified by 'guide' strands of RNA. Researchers have turned CRISPR/Cas9 into a molecular-biology powerhouse that can be used in other organisms. The cuts made by the enzyme are repaired by the cell's natural DNA-repair processes.
Good, better, best?

CRISPR is much simpler than previous gene-editing methods, but Zhang thought there was still room for improvement.

So he and his colleagues searched the bacterial kingdom to find an alternative to the Cas9 enzyme commonly used in laboratories. In April, they reported that they had discovered a smaller version of Cas9 in the bacterium Staphylococcus aureus2. The small size makes the enzyme easier to shuttle into mature cells — a crucial destination for some potential therapies.

The team was also intrigued by Cpf1, a protein that looks very different from Cas9, but is present in some bacteria with CRISPR. The scientists evaluated Cpf1 enzymes from 16 different bacteria, eventually finding two that could cut human DNA.

They also uncovered some curious differences between how Cpf1 and Cas9 work. Cas9 requires two RNA molecules to cut DNA; Cpf1 needs only one. The proteins also cut DNA at different places, offering researchers more options when selecting a site to edit. "This opens up a lot of possibilities for all the things we could not target before," says epigeneticist Luca Magnani of Imperial College London.

Cpf1 also cuts DNA in a different way. Cas9 cuts both strands in a DNA molecule at the same position, leaving behind what molecular biologists call 'blunt' ends. But Cpf1 leaves one strand longer than the other, creating a 'sticky' end. Blunt ends are not as easy to work with: a DNA sequence could be inserted in either end, for example, whereas a sticky end will only pair with a complementary sticky end.

"The sticky ends carry information that can direct the insertion of the DNA," says Zhang. "It makes the insertion much more controllable."

Zhang's team is now working to use these sticky ends to improve the frequency with which researchers can replace a natural DNA sequence. Cuts left by Cas9 tend to be repaired by sticking the two ends back together, in a relatively sloppy repair process that can leave errors. Although it is possible that the cell will instead insert a designated, new sequence at that site, that kind of repair occurs at a much lower frequency. Zhang hopes that the unique properties of how Cpf1 cuts may be harnessed to make such insertions more frequent.

In contrast, we also have an article in The Economist which states[6] :

CRISPR-Cpf1 may also be better than CRISPR-Cas9 in other ways. Cpf1 is a smaller and simpler enzyme (known technically as an endonuclease) than Cas9, which means it will be easier to deliver to the cells whose genes need modifying. And its slightly offset cuts to double-stranded DNA will help researchers to insert genetic patches more efficiently and accurately.

Its discovery also raises the question of how many other endonuclease-based systems are out there in the world's bacteria. Viral infection is a serious threat to these microbes, and the natural job of both CRISPR-Cas9 and CRISPR-Cpf1 is to recognize viral genes and chop them up before they can do any harm. Conversely, viruses are constantly evolving to escape the antiviral systems' attentions, meaning bacteria need to generate new ones. The chances are good, therefore, that CRISPR-Cas9 and CRISPR-Cpf1 are not alone. …

The tools to carry out that exploration now exist. CRISPR-Cpf1, for instance, was found not by searching in bacteria directly, but by scrutinizing a published database of bacterial genetic sequences, which yielded two species that contain it. Further searches might be equally rewarding—and the more gene-editing systems are discovered, the harder it will be to monopolies their use.

Despite the optimism of those who think the new techniques may calm qualms about genetic engineering, however, some people are bound to have ethical worries—certainly when it comes to applying them to human beings. Earlier this year, for example, when Chinese scientists used CRISPR-Cas9 gene editing on a human embryo (albeit one that was unviable, and could not therefore have developed into a person) there was much brouhaha and several calls for a moratorium on this line of inquiry.

There may not only be ethical worries but as we have discussed previously there is a weaponization approach also readily available. In a report in Nature World they state[7] :

The CRISPR/Cas9 system evolved as a way for bacteria and archaea to defend themselves against invading viruses. It is found in a wide range of these organisms, and uses an enzyme called Cas9 to cut DNA at a site specified by 'guide' strands of RNA. Researchers have turned CRISPR/Cas9 into a molecular-biology powerhouse that can be used in other organisms. The cuts made by the enzyme are repaired by the cell's natural DNA-repair processes…

The newly described Cpf1 system differs in several important ways from the previously described Cas9, with significant implications for research and therapeutics, as well as for business and intellectual property.

[6] http://www.economist.com/news/science-and-technology/21668031-scientists-have-found-yet-another-way-edit-genomes-suggesting-such-technology-will

[7] http://www.natureworldreport.com/2015/09/cpf1-for-precise-genome-engineering/

In its natural form, the DNA-cutting enzyme Cas9 forms a complex with two small RNAs, both of which are required for the cutting activity. The Cpf1 system is simpler in that it requires only a single RNA.

Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving 'blunt ends' that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends.

Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be re-cut, allowing multiple opportunities for correct editing to occur.

The Cpf1 system provides new flexibility in choosing target sites. Like Cas9, the Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences.

Finally, in a discussion in Wired the reporting is as follows[8] :

The discovery comes at a time when CRISPR/Cas9 is sweeping through biology labs. So revolutionary is this new genome editing technique that rival groups, who each claim to have been first to the tech, are bitterly fighting over the CRISPR/Cas9 patent. This new gene-editing protein called Cpf1—and maybe even others yet to be discovered—means that one patent may not be so powerful after all…

Many different proteins are associated with CRISPR. But in the early 2010s, Emmanuelle Charpentier, who was studying the flesh-eating bacteria Streptococcus pyogenes, stumbled onto one with special powers. Her bacteria happen to carry Cas9 proteins, which have the remarkable ability to precisely cut DNA based on a RNA guide sequence. In 2012, Charpentier and UC Berkeley biologist Jennifer Doudna published a paper describing the CRISPR/Cas9 system and speculated about its genome editing capabilities. And they filed a patent application. Much more on that patent later.

The patent issue is something we spoke about when the PTO pushed the Broad version through in less than six months, an unheard of process time.

While Cas9 has driven thousands of lab experiments and millions of dollars in funding for startups trying to capitalize on the technology, Cpf1 has remained relatively obscure. This study drags Cpf1 into the limelight. "It's a very comparable to Cas9 and it has a few different features which could be quite useful," says Dana Carroll, a biochemist at the University of Utah.

That's because Cas9 isn't perfect, despite its hype as a laser-precise genome editing tool. Cpf1 offers some slight advantages. For example, when it cuts double-stranded DNA, it snips the two strands in slightly different locations, resulting in overhang that molecular biologists call "sticky ends." Sticky ends can make it easier to insert a snippet of new DNA—say, a different version of

[8] http://www.wired.com/2015/09/war-genome-editing-just-got-lot-interesting/

a gene—though the Cell paper does not actually show data directly comparing Cas9 and Cpf1 when inserting DNA.

Cpf1 is also physically a smaller protein, so it may be easier to put into human cells. It requires only one RNA molecule instead of two, with Cas9. But it's not a rival so much as a complementary tool: The two proteins favor binding to different locations in the genome, so together, they might allow more flexibility in where scientist want to cut.

The writer then returns to the patent issues:

Not long after Doudna and UC Berkeley filed a patent, the Broad Institute and MIT filed their own patent on behalf of Zhang for the CRISPR/Cas9 system. Zhang had been working on actually showing that CRISPR/Cas9 can edit mammalian genomes in mammalian cells, an application he published in 2013 and says he came up with independently. The Broad's and MIT's attorney paid a fee to accelerate their application. Ultimately, the US Patent and Trademark Office awarded the patent to Zhang, MIT, and the Broad Institute. The University of California, obviously unhappy with the decision, filed an application for an interference proceeding to get the USPTO to reconsider. That process is ongoing.

But biotech companies have raced ahead to develop therapeutics and techniques with the system. Feng and Doudna have since licensed their technology to rival companies, Editas and Caribou. Charpentier also cofounded Crispr Therapeutics in Switzerland. Whoever wins the patent dispute will have a monopoly on CRISPR/Cas9 technology, the hottest new thing in biotech.

But with Cfp1, the stakes of that specific patent dispute go down. A lab or company could use Cfp1 without infringing on the CRISPR/Cas9 patent. "It takes power away from whoever the winner is going to be," says Jacob Sherkow, a NYU law professor. (Zhang has indicated the rights to Cpf1 may not necessarily go to the company he cofounded, Editas.) Whether a CRISPR/Cfp1 system is patentable as a separate invention—Sherkow says it probably is—perhaps isn't even relevant because its very existence means Cas9 is no longer the only game in town.

This latter observation is of significant value. Namely Cpf1 if it is truly better makes Cas9 battles of less value. It is of continuing interest to follow the dimensions of this new "tool box" available to those of us working on gene changes.

3.5.3 Other Endonucleases

The Scientist reports the identification of new enzymes to effect CRISPR targeting. Recall that CRISPR is a targeting RNA sequence and the enzyme, such as Cas9 is used to cut and then allow splicing of segments. CRISPR targets the gene position and the enzyme does the cutting. Cas9 does DSB or double stranded breaks. Other enzymes allow for sticky ends.

As The Scientist states[9]:

[9] http://www.the-scientist.com/?articles.view/articleNo/47845/title/New-CRISPR-Cas-Enzymes-Discovered/

Banfield's team searched the genomes for sequences that were both near cas1, which encodes a conserved CRISPR protein, and close to characteristic sequence repeats. The researchers found sequences for Cas9 in two archaeal genomes extracted from the Richmond Mine in Iron Mountain, California.

Previously, archaea were known to use class 1 CRISPR systems, but class 2 had only been identified in bacteria. "We don't really know how it performs, because that has not been achieved in the laboratory yet," said Banfield. "Archaea have different biology. The fact that [my collaborators] haven't yet managed to show its function probably means there are components of the system that we don't yet know about." The group also uncovered new types of Cas proteins from groundwater and soil bacteria, dubbed CasX and CasY. "They're really small, especially CasX," said Banfield. "That means it's potentially more useful."

CasX is made up of only 980 amino acids, whereas other Cas enzymes are larger. For instance, the commonly used Cas9 from Staphylococcus pyogenes contains 1,368 amino acids, while a smaller one from S. aureus is made up of 1,053 amino acids (CasY is around 1,200 amino acids). "This is important biotechnologically, because if you look at from the angle of genome editing, the delivery of small genes into cells is much easier than the delivery of large genes," ... In partnership with UC Berkeley's Jennifer Doudna, Banfield's team demonstrated that CasX and CasY are functional.

The researchers introduced CRISPR-CasX and CRISPR-CasY into E. coli, finding that they could block genetic material introduced into the cell.

The article mentioned above appears in Nature by Burnstein et al. The article states:

CRISPR-Cas systems provide microbes with adaptive immunity by employing short sequences, termed spacers, that guide Cas proteins to cleave foreign DNA. Class 2 CRISPR-Cas systems are streamlined versions in which a single Cas protein bound to RNA recognizes and cleaves targeted sequences. The programmable nature of these minimal systems has enabled their repurposing as a versatile technology that is broadly revolutionizing biological and clinical research.

However, current CRISPR-Cas technologies are based solely on systems from isolated bacteria, leaving untapped the vast majority of enzymes from organisms that have not been cultured. Metagenomics, the sequencing of DNA extracted from natural microbial communities, provides access to the genetic material of a huge array of uncultivated organisms. Here, using genome-resolved metagenomics, we identified novel CRISPR-Cas systems, including the first reported Cas9 in the archaeal domain of life.

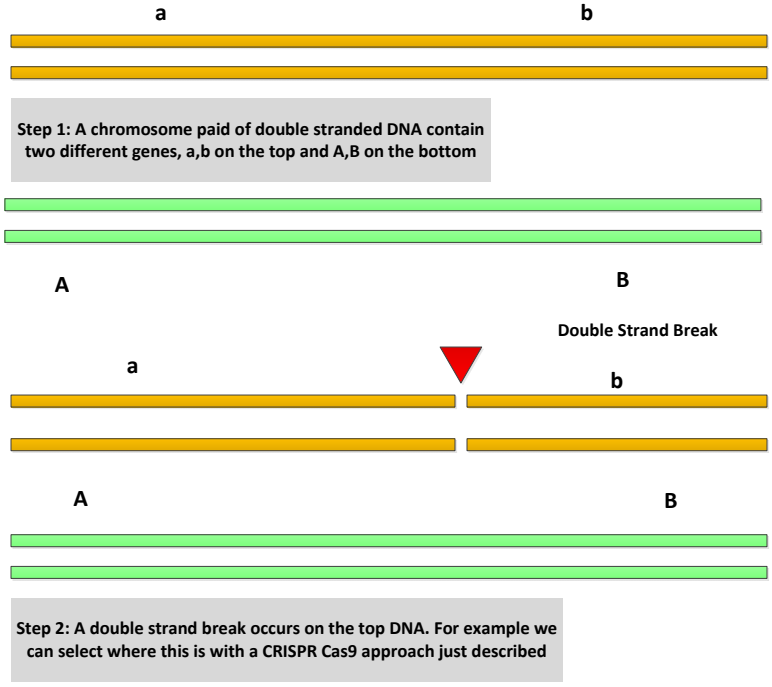
This divergent Cas9 protein was found in little-studied nanoarchaea as part of an active CRISPR-Cas system. In bacteria, we discovered two previously unknown systems, CRISPR-CasX and CRISPR-CasY, which are among the most compact systems yet identified. Notably, all required functional components were identified by metagenomics, enabling validation of robust in vivo RNA-guided DNA interference activity in E. coli. Interrogation of environmental

microbial communities combined with in vivo experiments allows access to an unprecedented diversity of genomes whose content will expand the repertoire of microbe-based biotechnologies.

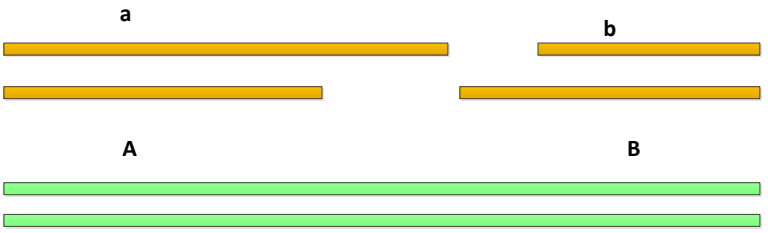
The targets and capabilities continue to expand.

3.6 GERM LINE INCLUSION

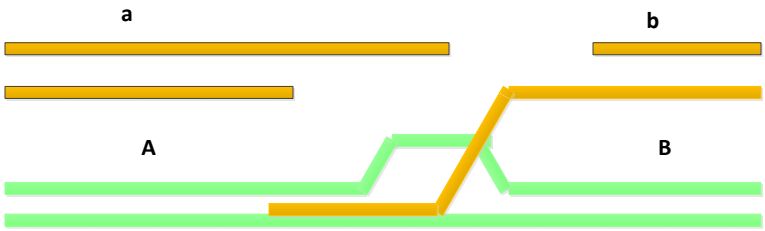
The following is a brief graphic summary of DSB repair. The first step the DS and then a single break of a single DS.



The next step is the shortening of one end and then the opening of the opposite chromosome DNA and the use of it for repair.

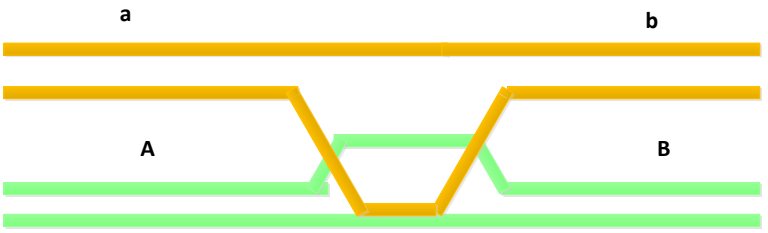


Step 3: The cut strands are shortened in top and bottom branches

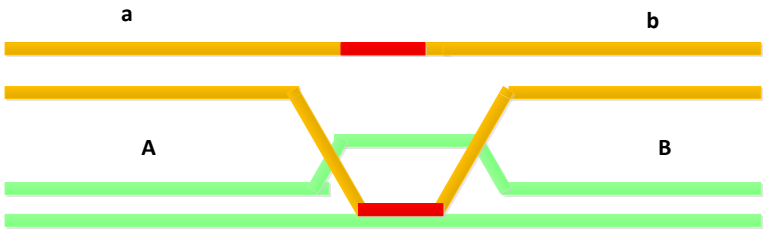


Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

Finally the repair is achieved and the other end similarly repaired.



Step 5: Bottom completes and top finishes

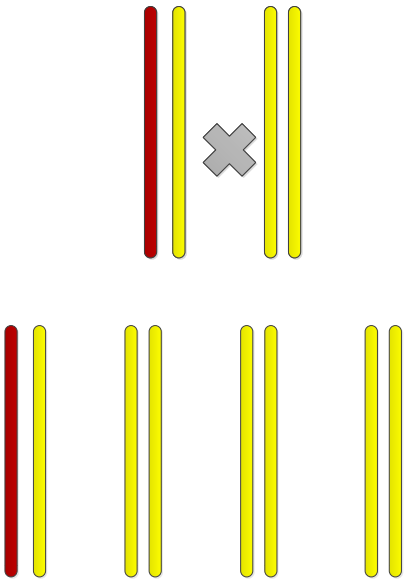


Step 6: A selected gene can be inserted and added, or it can equally well be removed by a two cut process.

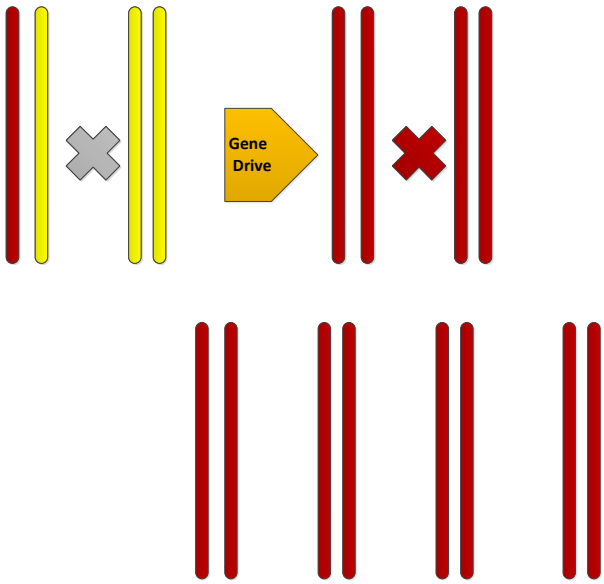
One can imagine the many possible faults in this process. This process, the Cas9 single strand break, the specificity of the Guide RNA all add up to levels of uncertainty of this process.

3.7 DISTRIBUTION AND EVOLUTION

We now examine the consequences of this approach. First in a Mendelian world as shown below we have four possible offspring. Three of the four express wild type. One will have the mutant gene. If we continue to cross with wild types, then the percent of the mutant will decrease and disappear unless there is some reason for preferentially self-breeding or a natural selection process. Thus in classic Mendelian worlds things are such that the mutant gene will stay suppressed to a reasonable degree.



Now in our Gene Drive world, things are quite different. Remember we have that CRISPR/Cas addition which keeps inserting the mutant gene wherever it can. This we show below.



Thus instead of one in four off springe being mutant now we have any and all off spring being mutant. These mutants mate and their off spring are again all mutant. In no time at all this mutant strain spreads across the species. If the mutant strain is a male only off spring the result is a spreading loss of species until it is completely gone.

That is unless Nature takes some new twist and turn.

4 APPLICATIONS

There clearly is a plethora of applications for Gene Drives. We explore some of them here.

4.1 DISEASE CONTROL

Many diseases are vector borne. If one could control or eliminate the vector then the disease would disappear. Malaria, Zika, Dengue, and others fall in this group. This most likely will expand to a wide variety of vector borne pathogens.

4.2 PLANT ENGINEERING

Plant bio-engineering has been around now for almost 30 years. This can be a highly productive area for both food stock and horticultural varieties. For example, many invasive species can be controlled while retaining their beneficial effects.

As Barkate and Stephens note:

Different omics platforms have opened the flood gate of potential disease resistance genes that need a more efficient validation pipeline than earlier gene manipulation tools like gene silencing.

Plant–pathogen omics data could be improved even further by reducing the background noise in the biological samples. This can now be achieved, for example, by performing cell-type specific RNA or chromatin profiling with novel tools like INTACT. Cell-type enrichment will help monitor the dynamics of post-translational modifications during plant–pathogen interactions. CRISPR–Cas9 technology has revolutionized gene manipulation capabilities in many species including crops. The multitude of functions that can be performed with CRISPR– Cas9 and its many derivatives make it a molecular tool that will open new opportunities in the complicated world of plant–pathogen interactions and help design durable crop resistance to pathogens.

Only the gene editing function of CRISPR–Cas9 has so far been used in plants and pathogens. However, the future use of dCas9-based tools will also help to unmask the master regulators of disease resistance. GT tools will help integrate omics data in order to fully understand and improve crop defense mechanisms. The complexity of the plant microbiome with good and bad microbes is beginning to be unraveled. CRISPR–Cas9 tools will help future studies of plant–pathogen interactions to transcend individual genes …

4.3 HUMAN MODIFICATION

Imagine if you will that a woman has some genetic disease. Or desires some trait. Then by "gene driving" in the egg it would be possible to modify any of these. Just what human modifications are possible is left to the imagination at this stage.

5 OBSERVATIONS

We can now make a set of observations regarding Gene Drives.

5.1 SAFETY

The National Academy of Sciences NAS report states:

The potential for gene drives to spread throughout a population, to persist in the environment, and to cause irreversible effects on organisms and ecosystems calls for a robust method to assess risks. Environmental assessments and environmental impact statements required by the National Environmental Protection Act, though widely acknowledged as valuable in other contexts, are inappropriate tools to characterize the risks of gene-drive modified organisms. Instead, ecological risk assessment would be beneficial in the context of gene drive research, because this method can be used to estimate the probability of immediate and long-term environmental and public health harms and benefits. Ecological risk assessment allows comparisons among alternative strategies, incorporates the concerns of relevant publics, and can be used to identify sources of uncertainty, making it well-suited to inform research directions and support public policy decisions about emerging gene-drive technologies. Two key features of ecological risk assessments are the ability to trace cause-and-effect pathways and the ability to quantify the probability of specific outcomes.

This approach could also potentially be built into a structured, adaptive process to oversee the release and management of gene-drive modified organisms in the environment. As of May 2016, no ecological risk assessment has yet been conducted for a gene-drive modified organism. Some amount of uncertainty is unavoidable. There is currently sufficient knowledge to begin constructing ecological risk assessments for some potential gene-drive modified organisms, including mosquitoes and mice. In some other cases it may be possible to extrapolate from research and risk analyses focused on other genetically-modified organisms and non-indigenous species. However, laboratory studies and confined field tests (or studies that mimic confined field tests such as large cage trials and greenhouse studies) represent the best approaches to reduce uncertainty in an ecological risk assessment, and are likely to be of greatest use to risk assessors.

This assembly of tools by the genetic engineer has been called "gene drives". In a sense it "drives" certain genes into all members of a species. At least that is the hope. As the Broad Institute states in its licensing statements[10]:

Gene drive. This is a way to rapidly spread a new gene throughout an entire species in nature. This approach might be used to block the transmission of malaria by mosquitoes, but has the potential to disrupt ecosystems… After consulting with external experts and careful internal consideration, the Broad Institute has decided to make available non-exclusive research and commercial licenses for the use of CRISPR technology in agriculture -- but with important

[10] https://www.broadinstitute.org/news/licensing-crispr-agriculture-policy-considerations

restrictions. These include: Gene drive: We prohibit the use of the licensed technology for gene drive.

As The Scientist notes[11]:

The United Nations (UN) biodiversity meeting, held in Mexico this month, could have ended poorly for scientists working on gene drives, genetic elements that can perpetuate specific mutations and may help cull dangerous mosquito populations. But in spite of environmental activists pushing the UN to ban gene drives, citing the risk of accidental release, the UN's final agreement—penned December 16—merely urged caution in testing gene drives, Nature reported. Overall, the organization broadly supported further research in synthetic biology. "I'm very relieved," Andrea Crisanti, a molecular parasitologist at Imperial College London who works with gene drives, told Nature. "It would have been a disaster for developing the technology. "By engineering mutations that render organisms infertile or less infectious, then perpetuating these mutations with gene drives, scientists may be able to reduce the occurrence of certain mosquito-borne illnesses and cull invasive species. Gene drives have already been tested in yeast, fruit flies, and mosquitoes, and may soon be enlisted in the fight against malaria. One team hopes to conduct field trials in Africa as soon as 2024.

In a similar fashion Nature states[12]:

When the CBD last met in South Korea in 2014, gene drives were a largely theoretical idea. They are genetic elements that can quickly spread through sexually reproducing populations. In general, an organism's two copies of a gene — known as alleles — each have a 50% chance of being passed on to its offspring. This limits the pace at which a genetic modification can spread through a population. But gene-drive technology tilts the odds, so that a specific change to one allele is inherited by a higher proportion of progeny. In theory, an entire population could quickly carry the same modification. In the past two years, researchers have lab-tested gene drives in yeast, fruit flies and mosquitoes that are based on a gene-editing technology called CRISPR–Cas9. Crisanti's team, for instance, is working on gene drives in the malaria-carrying mosquito Anopheles gambiae that perpetuate mutations causing females to become infertile. Spread of this mutation could mean that mosquito populations plummet to levels that do not support the transmission of malaria. The researchers' project, called Target Malaria, has attracted tens of millions of dollars in funding, and the scientists hope to conduct field trials in Africa as early as 2024. Other groups are developing gene drives to quell island rodents and other pests.

This development takes the next step and it presents a rather double edged sword. It is essential to be watched as we move forward.

[11] http://www.the-scientist.com/?articles.view/articleNo/47854/title/UN-Rejects-Calls-for-Moratorium-on-Gene-Drive-Research/

[12] http://www.nature.com/news/gene-drive-moratorium-shot-down-at-un-biodiversity-meeting-1.21216

5.2 ACCURACY AND REPEATABILITY

As we noted previously there are many points of error or failure in these processes. It would be a worthwhile effort if one could develop an error model for this and similar sets of processes. CRISPR errors, insertion errors, DSB repair errors, errors in mitosis, and the list goes on. Not to mention the errors that normally occur to cells. We have also left out such effects as epigenetic methylation effects which can have an overwhelming effect.

5.3 INTER SPECIES SPREADING

One of the concerns is what we term inter-species spread. namely it is possible that the same or different gene transfer may occur from the target species to other species. The risk is low if into a somatic cell perhaps but is significant if into a germ line cell or embryo. Human females for example may have their eggs infiltrated or males the sperm and then result when reproduction occur is infiltration of the new species.

5.4 GENE PERTURBATIONS

The embryo as it progresses may change genes in different generations. The CRISPR may be altered and the result is cutting of the wrong location. Furthermore the replication on the matching chromosome may not work effectively. Double stranded breaks are serious and as we have noted may result in malignancies.

5.5 UNDERSTANDING THE DYNAMICS

As Marshall and Hay note:

Gene drive systems are genetic elements capable of spreading into a population even if they confer a fitness cost to their host. We consider a class of drive systems consisting of a chromosomally located, linked cluster of genes, the presence of which renders specific classes of offspring arising from specific parental crosses unviable. Under permissive conditions, a number of these elements are capable of distorting the offspring ratio in their favor. We use a population genetic framework to derive conditions under which these elements spread to fixation in a population or induce a population crash. Many of these systems can be engineered using combinations of toxin and antidote genes, analogous to Medea, which consists of a maternal toxin and zygotic antidote. The majority of toxin–antidote drive systems require a critical frequency to be exceeded before they spread into a population. Of particular interest, a Z-linked Medea construct with a recessive antidote is expected to induce an all-male population crash for release frequencies above 50%. We suggest molecular tools that may be used to build these systems, and discuss their relevance to the control of a variety of insect pest species, including mosquito vectors of diseases such as malaria and dengue fever.

The above work was written before the utilization of the CRISPR techniques. The CRISPR approach as one can see is much more aggressive and self-replicating.

5.6 WEAPONIZING

One always asks; what is the potential of this technology for harm? Not only accidental harm but deliberate harm, namely weaponized. The answer is a resounding; it has great potential. First the technology is not that sophisticated. Second, the details are in the open literature. Third, the facilities are minimal. Fourth, thousands of graduate students have the capability now and it will wind its way down to the High School level. Fifth, it can easily be spread.

Overall this is just another example of a genie getting out of the jar. We have seen how poorly Governments have deal with Cyber threats, and the Bio threats of this type and others is overwhelming. We all too often focus on Apps and purloined passwords when the implementation of these schemes is all the more important.

5.7 ECOLOGICAL CONCERNS

In banishing a species, deliberately and completely, one makes potentially a massive ecological change. Let us say we eliminate the disease vector mosquitos. o we fully understand any and all unintended consequences. DDT had eliminated much of these vectors then there was the effect of DDT on secondary species and mankind decided to let millions of humans die off to save tens of millions of other species. Are there tradeoffs here as well?

The NAS study does examine the issue of dispersal. They state:

The promise of gene drives is based on the potential spread of the desired gene through an entire area occupied by a species or population. The spread itself occurs via the movement of individuals or gametes from one location to another, with subsequent mating and reproduction. The spread of genes via movement between populations is called gene flow. Understanding the role of gene flow is critical for determining how rapidly a gene drive will spread among populations, whether the goal is to move the drive into additional populations or, conversely to limit its spread.

Understanding gene flow is also vital for estimating the likelihood that the gene drive may move into a non-target population. The diversity of gene flow patterns are influenced by three main factors: the stage of the life cycle in which the movement of individual organisms among populations is most likely, the type of movement through which individuals carry genes among populations, and the spatial scale over which movement typically occurs. Gene flow may occur by the movement of either whole organisms or gametes. For many species, "typical" movement of an individual occurs in specific life cycle stages. For example, in many organisms, movement occurs via dispersal of fertilized eggs, seeds, or spores (as in fungi, ferns, and mosses, for example.).

By contrast, in many animals, movement among populations is most likely when juveniles or young adults of one gender disperse from the area of their birth to establish themselves

elsewhere (Graw et al., 2016). In these cases, social interactions can play a critical role in determining individual movement, where an individual settles, and whether movement results in breeding and actual gene flow. The stage of the life cycle in which gene flow occurs can influence the rate at which genes move from one population into another. For example, the passive dispersal of fertilized eggs and seeds can introduce substantial numbers of genes from one population into another, whereas the dispersal of juvenile or adult individuals in search of new habitat will generate much lower rates of gene exchange.

In contrast, many plants and some marine invertebrates disperse primarily through the movement of gametes rather than whole organisms. The most familiar example is wind-borne pollen, which can transport genes across long distances . In many cases, especially when pollen movement is facilitated by insect pollinators, the movement of genes can be quite circumscribed. Gene flow via gametes is fundamentally different from gene flow via movement of individual organisms in two ways. First, it represents sexual transfer of a haploid genome rather than the movement of a diploid genome. Second, it offers a greater possibility of gene flow among closely related species. For example, gamete dispersal can move engineered genes from a target organism into a wild or domesticated relative more quickly and at a higher rate than might occur in hybridization via the movement of seeds among locations (O'Connor et al., 2015). There are four broad types of movement that produce gene flow.

First, individuals move via human assisted dispersal. Human-assisted dispersal is well-recognized as a common avenue for the introduction of unwanted invasive species (Fonzi et al., 2015), but humans also move genotypes from one area to another. This can be accidental, as in the transport of marine organisms in ballast or purposeful, as in the enhancement of game or fishery populations. Human-assisted movement can produce high or low rates of gene flow, depending upon the numbers of individuals transported.

Second, individuals move in response to disruptive events. These can include evacuation in response to wildfires or other sources of rapid habitat destruction or fragmentation. Individuals in aquatic systems can also be transported among locations by flooding events such as flash flooding of streams or sheet flows across large areas…

5.8 FUTURE ISSUES

As I have noted again and again. Silicon Valley apps pale to what bio tech is doing. In bio tech, there is a potentially deadly field at play and any country and enter the game. The cost is low, expertise is required, and somehow we seem to be providing it in our Institutions.

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