CRISPRs and Cancer (Revised)

Terrence P McGarty
White Paper No 125
April, 2015

We examine CRISPRs and Cas9 as a means to treat cancers on a genetic basis. This is a new and highly effective tool for genetic engineering and holds some promise for cancer therapy. Copyright 2015 Terrence P. McGarty, all rights reserved.

Notice

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Contents

1 INTRODUCTION

CRISPRs, specifically "clustered regularly interspaced short palindromic repeats"[1], are portions of a cell's DNA which contain a particular type of short repetitions.. These specific repetitions are then followed by additional short segments of DNA which have been collected from some prior exposure to a virus phage. Namely CRISPRS are selective DNA snippets which have been garnered from viral phages which in some past period tried to attack the prior lineage of this cell. They are used to create Cas ("CRISPR associated" genes) which in turn have the capability of cleaving genes and inserting new ones.

CRISPR-Cas systems are now a useful toolkit for engineering eukaryotic cells, and especially human cells. They are also used in plant cells and that is a second tale but one worth examining as well.

As Jinek et al have recently said (Jinek et al 2014):

Although type I and III CRISPR-Cas systems rely on multiprotein complexes for crRNA-guided DNA targeting, type II systems use a single RNA-guided endonuclease, Cas9, that requires both a mature crRNA and a trans-activating crRNA (tracrRNA) for target DNA recognition and cleavage (8, 9). Both a seed sequence in the crRNA and conserved protospacer adjacent motif (PAM) sequence in the target are crucial for Cas9-mediated cleavage.

The use of the crRNA and the tracrRNA are the two key elements which we shall discuss in this process. Also the Type II CRISPR-Cas system is the one which has received the most attention.

Cas9 proteins are abundant across the bacterial kingdom, but vary widely in both sequence and size. All known Cas9 enzymes contain an HNH domain that cleaves the DNA strand complementary to the guide RNA sequence (target strand), and a RuvC nuclease domain required for cleaving the noncomplementary strand (nontarget strand), yielding double-strand DNA breaks (DSBs).

These DSB open up the DNA at a desired location. Thus if one has a specific gene to be spliced out, and to be replaced, the first step is to open the DNA at the site of that desired gene. Thus the above step is a critical first step.

In addition, Cas9 enzymes contain a highly conserved arginine-rich (Arg-rich) region previously suggested to mediate nucleic acid binding. On the basis of CRISPR-Cas locus architecture and protein sequence phylogeny, Cas9 genes cluster into three subfamilies: types II-A, II-B, and II-C. Cas9 proteins found in II-A and II-C subfamilies typically contain ~1400 and ~1100 amino acids, respectively.

The ability to program Cas9 for DNA cleavage at specific sites defined by guide RNAs has led to its adoption as a versatile platform for genome engineering . When directed to target loci in

[1] Recall that a palindrome is a collection of letters which can be read the same forwards of backwards. For example; GCATTACG.

*eukaryotes by either dual **crRNA:tracrRNA** guides or **chimeric single-guide RNAs**, Cas9 generates site-specific DSBs that are repaired either by nonhomologous end joining or by homologous recombination, which can be exploited to modify genomic sequences in the vicinity of the Cas9-generated DSBs.*

The opened DNA then can be targeted by crRNA:tracrRNA segments that remove and replace the targeted DNA or by a chimeric single-guide RNA which accomplishes this all in one step. This is the second step in CRISPR gene targeting and re-engineering. We shall discuss this a bit more later.

Furthermore, catalytically inactive Cas9 alone or fused to transcriptional activation or repression domains can be used to control transcription at sites defined by guide RNAs. Both type II-A and type II-C Cas9 proteins have been used in eukaryotic genome editing. Smaller Cas9 proteins, encoded by more compact genes, are potentially advantageous for cellular delivery using vectors that have limited size such as adeno-associated virus and lentivirus.

CRISPR, those collections of small sets of palindromic DNA inserted in the hosts original DNA, can be collectively called a process that is naturally occurring in nature and it is also a procedure that can then be implemented across a wide selection of cell types. In a sense it has been called the lower organism's immune system, a means of remembering previous attackers to the organisms such as bacteria, and a way to use that memory as a defense mechanism against future attacks. The mechanism can then be used in higher level organisms as a reverse process, a means of attacking bad genes and then inactivating them. It is in effect a trick to take what lower organisms have developed for protection and employ in higher level organisms for therapeutic purposes.

In a recent paper by Villion and Moineau the authors examine the two sides of CRISPR, the side that adds segments of foreign DNA to enable an immune type system and the side that deletes selected DNA.

To cope with this never-ending threat, microorganisms have developed a wide range of defense mechanisms.

Among them, CRISPR-Cas system is the new kid on the block as its silencing role was reported only five years ago. An outburst of articles, meetings, and reviews has since followed, arguably making it one of the hottest topics in microbiology.

CRISPR (clustered regulatory interspaced short palindromic repeats) loci are found in approximately 45% of sequenced bacterial genomes as well as 90% of archaeal ones and one genome can contain multiple CRISPR loci. Variable short regions, called spacers, separate each of the short repeats. The spacers are mainly homologous to viral or plasmid sequences. CRISPR-associated (cas) genes are often located adjacent to the CRISPR locus. The diversity and specificity of the cas operons has led to the identification of signature cas genes and to a polythetic classification scheme for CRISPR-Cas systems (types I to III, with several subtypes).

Notwithstanding their particularities, CRISPR-Cas systems operate through three general steps to provide immunity. In the adaptation stage, some cells will respond to the invasion of a phage or a plasmid by adding a new repeat-spacer unit into the CRISPR array, mostly polarized at the 5′ end. Strikingly, the spacer sequence comes from the invading nucleic acid while the newly added repeat derives from another repeat of the array.

The mechanistic details on how this adaptation/immunization occurs are still unknown but some Cas proteins are involved. The unique spacer content is now considered a sign of past challenges and can serve as a marker for strain typing. In the second step, small non-coding CRISPR RNAs (crRNAs) are generated. A long precursor CRISPR RNA is first produced from an AT-rich leader/promoter region, which is then processed within the repeats and mature into crRNAs.

Several Cas proteins participate into the biogenesis of crRNAs. Finally, in the interference stage, the crRNACas protein complex will bind to the invading nucleic acid target and cleave it, providing a defense system to the host microbe. Therefore, CRISPRCas systems are RNA-based adaptive microbial immune systems that target nucleic acid intruders.

They end with the following, the double edged sword portion:

Although already outstanding in bridging gaps in our understanding of CRISPR-Cas systems, this fascinating story does not end here. The authors investigated the possibility of using this dual-RNA system to program Cas9 to specifically cleave any desired DNA molecules. Minimal requirements to have an efficient single chimeric RNA molecule mimicking the dual RNA structure were defined and led to site specific DNA cleavage by Cas9. In fact, several different chimeric guide RNAs were engineered and used to cleave a plasmid containing the specific target and a PAM. These findings coupled to the previous observations that CRISPRCas systems can be functionally transferred from one organism to another open up exciting possibilities for gene targeting and genome editing of microbes and even higher organisms.

That is they have developed a way to reverse the process, using the mechanism now, not to add a piece of DNA, but to cleave a piece of DNA. This opens the door for many types of treatment of cancers where we may know the genetic defect and then can cut it out, cell by cell.

We examine briefly herein some of the recently discovered uses of CRISPR technology to address cancers of various types. The CRISPR approach is another tool in the toolbox of biologists which can become a means for medical application.

As Cain and Boinett state:

The CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR associated proteins) adaptive immune system is widespread in bacteria and archaea and provides heritable protection against disruptive mobile genetic elements (MGEs), such as bacteriophages and plasmids. CRISPR loci contain a series of repetitive DNA motifs separated by spacer sequences; these spacers are derived from MGEs and incorporated after exposure to each new foreign element. The CRISPR transcript is processed into small CRISPR RNAs, which are displayed on Cas protein complexes, enabling RNA-guided degradation of the foreign DNA

by Cas nucleases…. The flexibility and specificity of genome editing using CRISPR loci enables the efficient generation of mutated genotypes in diverse species. Furthermore, as CRISPR loci show strain-specific conservation at the nucleotide level, they are proving to be valuable markers for typing studies and, in conjunction with whole-genome sequencing, can provide insights into the phylogenetic relationships between different bacteria.

As reported in The Independent[2]:

The Crispr process was first identified as a natural immune defence used by bacteria against invading viruses. Last year, however, scientists led by Jennifer Doudna at the University of California, Berkeley, published a seminal study showing that Crispr can be used to target any region of a genome with extreme precision with the aid of a DNA-cutting enzyme called CAS9.

Since then, several teams of scientists showed that the Crispr-CAS9 system used by Professor Doudna could be adapted to work on a range of life forms, from plants and nematode worms to fruit flies and laboratory mice.

Earlier this year, several teams of scientists demonstrated that it can also be used accurately to engineer the DNA of mouse embryos and even human stem cells grown in culture. Geneticists were astounded by how easy, accurate and effective it is at altering the genetic code of any life form – and they immediately realized the therapeutic potential for medicine.

"The efficiency and ease of use is completely unprecedented. I'm jumping out of my skin with excitement," said George Church, a geneticist at Harvard University who led one of the teams that used Crispr to edit the human genome for the first time.

"The new technology should permit alterations of serious genetic disorders. This could be done, in principle, at any stage of development from sperm and egg cells and IVF embryos up to the irreversible stages of the disease," Professor Church said.

David Adams, a DNA scientist at the Wellcome Trust Sanger Institute in Cambridge, said that the technique has the potential to transform the way scientists are able to manipulate the genes of all living organisms, especially patients with inherited diseases, cancer or lifelong HIV infection.

"This is the first time we've been able to edit the genome efficiently and precisely and at a scale that means individual patient mutations can be corrected," Dr Adams said.

"There have been other technologies for editing the genome but they all leave a 'scar' behind or foreign DNA in the genome. This leaves no scars behind and you can change the individual nucleotides of DNA – the 'letters' of the genetic textbook – without any other unwanted changes," he said.

[2] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

The essence of the above is twofold. First it is the use of CRISPR as a mechanism in prokaryotes and possibly in eukaryotes. The second is an important observation that we now have another tool for the genetic engineering tool box. The observation that in genetic engineering that many of the "tools" are artifacts of nature should not be overlooked.

2 WHAT IS A CRISPR?

We will now examine in more detail what a CRISPR is and how it functions. Let us begin by examining it in a bit more detail. As Randow et al state:

In archaea and bacteria, for example, even adaptive forms of resistance—long considered the hallmark of vertebrates—contribute to cell autonomous immunity, as exemplified by the clustered regularly interspaced short palindromic repeats (CRISPR) system, which recognizes foreign DNA in a sequence-specific manner. In metazoans, cellular self-defense synergizes with the whole-body protection provided by traditional immunity to confer pathogen resistance. Here, professional immune cells patrol their environment in search of pathogens, whereas cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection.

Cellular self-defense thus has the potential to confer antimicrobial protection on most, if not all, cells….In bacteria, foreign DNA is sensed and destroyed by the CRISPR system and restriction endonucleases. Because recognition motifs for most restriction endonucleases occur frequently in the host's own genome, these enzymes are paired with matching methyltransferases, which modify host DNA to demarcate it as "self." In eukaryotic cells, rather than being modified, DNA is largely sequestered inside the nucleus, which fosters the detection of foreign DNA in other compartments and allows the deployment of enzymes that mutate and/or degrade DNA without risk to the host genome.

Thus as noted above, the original understanding was as a bacterial self-defense system. Now as Horvath and Barrangou state also concerning the original understanding:

Microbes have devised various strategies that allow them to survive exposure to foreign genetic elements. Although out-populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments. The constant exposure to exogenous DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements. These systems maintain genetic integrity, yet occasionally allow exogenous DNA uptake and conservation of genetic material advantageous for adaptation to the environment.

Certain strategies, such as prevention of adsorption, blocking of injection, and abortive infection, are effective against viruses; other defense systems specifically target invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases. Recently, an adaptive microbial immune system, clustered regularly interspaced short palindromic repeats (CRISPR) has been identified that provides acquired immunity against viruses and plasmids.

They also state:

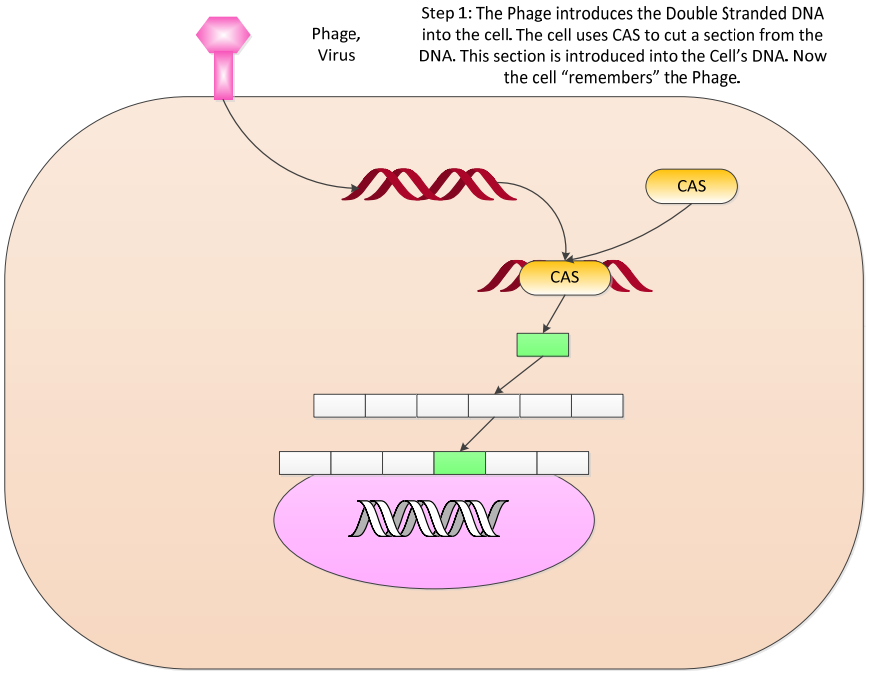
Microbes rely on diverse defense mechanisms that allow them to withstand viral predation and exposure to invading nucleic acid. In many Bacteria and most Archaea, clustered regularly interspaced short palindromic repeats (CRISPR) form peculiar genetic loci, which provide acquired immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner.

These hypervariable loci take up genetic material from invasive elements and build up inheritable DNA-encoded immunity over time. Conversely, viruses have devised mutational escape strategies that allow them to circumvent the CRISPR/Cas system, albeit at a cost. CRISPR features may be exploited for typing purposes, epidemiological studies, host-virus ecological surveys, building specific immunity against undesirable genetic elements, and enhancing viral resistance in domesticated microbes.

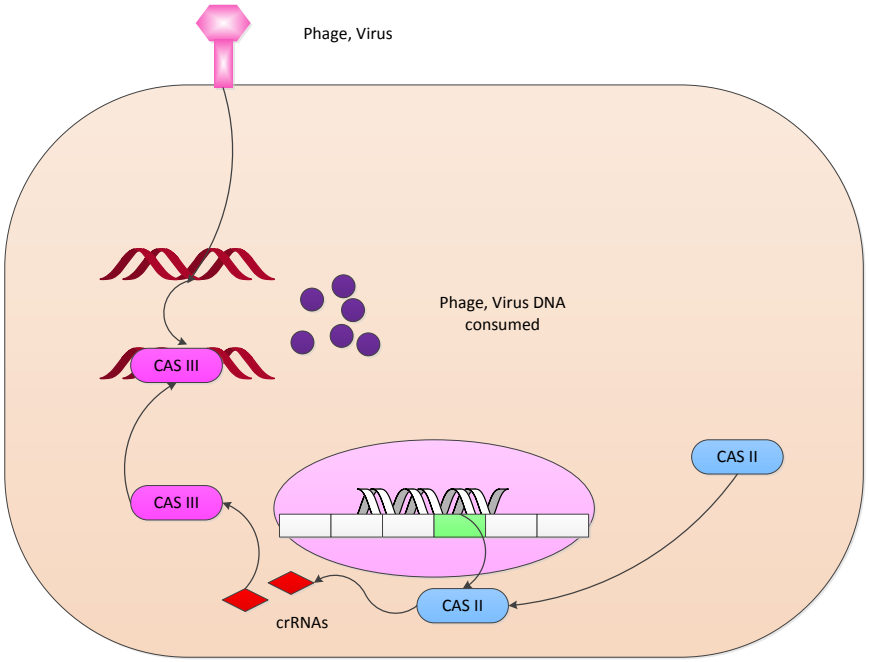
Thus we first examine how CRISPR-Cas functions in its primal environment and then we take this to human environments where we can use it as an added tool in our genetic engineering toolkit.

2.1 CRISPR DYNAMICS

We now examine some of the dynamics of the CRISPR system. We start with the use of CRISPR in a bacterial cell. We assume the cell is attacked by some viral phage and the phage sends its RNA/DNA into the cell in anticipation of replication within the host. Now from Horvath and Barrangou (as modified) we have the following description for this initial portion of the process as shown below:



The Cas protein recognizes the invading DNA and transports a portion of it to the nuclear DNA and inserts it into the cell's DNA. How specifically Cas does this task is not yet well understood. The when another phage with the same or frankly similar DNA invades again, then Cas II is activated and the section of the DNA activates a Cas II which then consumes the invading DNA.



Now the above process is a natural part of the day to day activities of bacteria. But it also is a paradigm for deal with eukaryotic cells, namely cutting and pasting genes into cells.

2.2 TYPES OF CRISPR

From Jinek et al, they discuss the three types of CRISPR systems:

There are three types of CRISPR/Cas systems.

The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA.

In contrast, type II systems process pre-crRNAs by a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA.

3 CRISPR DETAILS

Current day biotechnology is in many ways a set of tools in a large tool box that handle the what and how of manipulating genes and their products. The tool and tool box metaphor is quite powerful and descriptive. The problem oftentimes is the why and also the integration of all of these elements from a technique to a technology.

In this brief paper we examine the CRISPR element less from that of a bench technique than as a technology that can be used in gene engineering. There is a mindset being explored that differs from that of the bench biologist. As an engineering approach one asks how can this technique be moved to a useful technology, and how deeply does one have to understand the underpinnings to use it effectively and safely.

One of the challenges of genetic engineering is the ability to select a specific gene and alter it, or add another gene or delete a gene. A key step in all of these is the ability to cut and paste at specific sites, at very specific sites. Now that one can read a gene in detail and when one knows what the desired result should be, then the cut and paste side is critical. Pasting is somewhat well known, especially if we have cut at the right location. CRISPR is a tool that does just that, it is a very accurate, fast, and low cost gene cutting tool.

In this note we examine its structure from a systematic perspective. This will help understand what factors are the key factors and what elements should be understood. This is not a note for a bench biologist, it is not meant to be comprehensive. Yet unlike many of the simplified descriptions in the media I try to provide adequate depth with breath of applications.

We also try to establish the "gene engineering" tools that this mechanism can support. Finally we discuss some of the concerns which have arisen in the use of CRISPRs.

To summarize, I refer to Mali et al who state:

Functioning of the type II CRISPR-Cas systems in bacteria.

Phase 1: in the immunization phase, the CRISPR system stores the molecular signature of a previous infection by integrating fragments of invading phage or plasmid DNA into the CRISPR locus as 'spacers'.

Phase 2: in the immunity phase, the bacterium uses this stored information to defend against invading pathogens by transcribing the locus and processing the resulting transcript to produce CRISPR RNAs (crRNAs) that guide effector nucleases to locate and cleave nucleic acids complementary to the spacer.

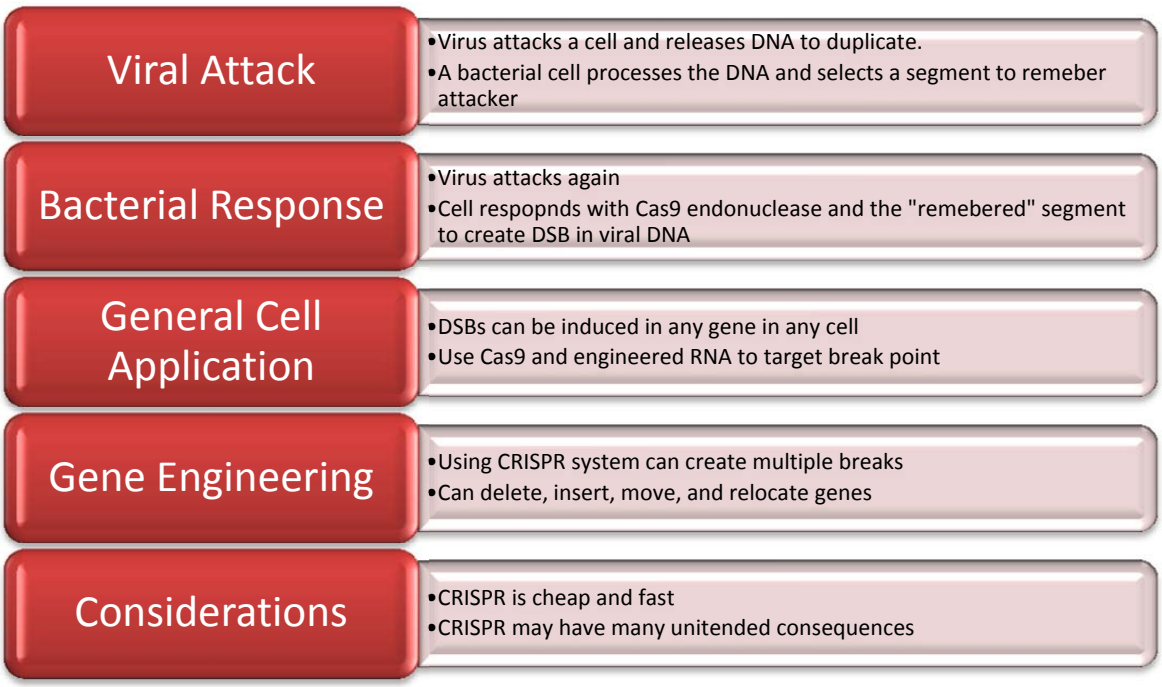
First, tracrRNAs hybridize to repeat regions of the pre-crRNA.

Second, endogenous RNase III cleaves the hybridized crRNAtracrRNA, and a second event removes the 5' end of the spacer, yielding mature crRNAs that remain associated with the tracrRNA and Cas9.

The complex cleaves complementary 'protospacer' sequences only if a PAM sequence is present.

Namely, this tool was seen developed in bacteria. The bacterium notes a section of the invading viral DNA, and then records that segment in its own DNA. Then when the virus attacks a second time, using the Cas9 nuclease protein produced by the bacteria then uses the RNA generated by the "remembered" sequences to attack the virus, and cut it so that it is made inoperable and it is digested.

Thus in the report we follow the following considerations:



3.1 BACTERIAL IMMUNOLOGY

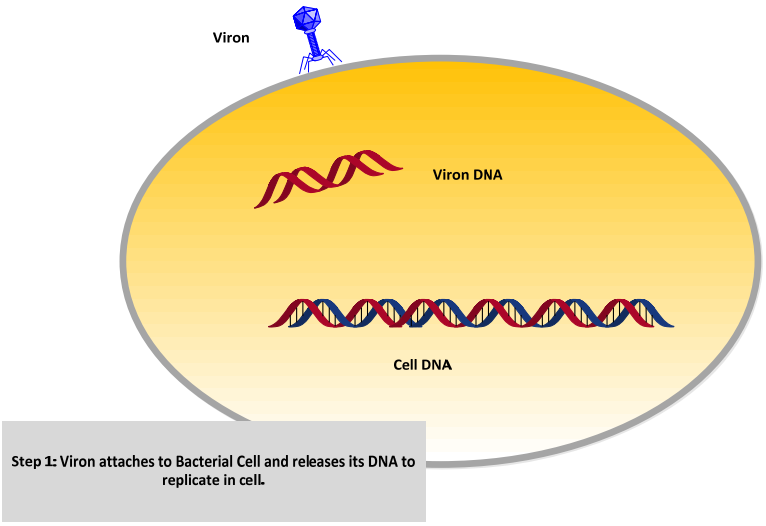
The CRISPR phenomenon comes from examining bacteria and their quasi immune response to viral attacks. Simply stated;

Bacteria have developed a technique where they can recognize a foreign viral DNA segment and then "attack" is with an enzyme and a targeted RNA segment that results in the foreign DNA being broken and becoming ineffective. This bacterial process effectively kills the DNA of the invader, stops its reproduction and induces an autophagy.

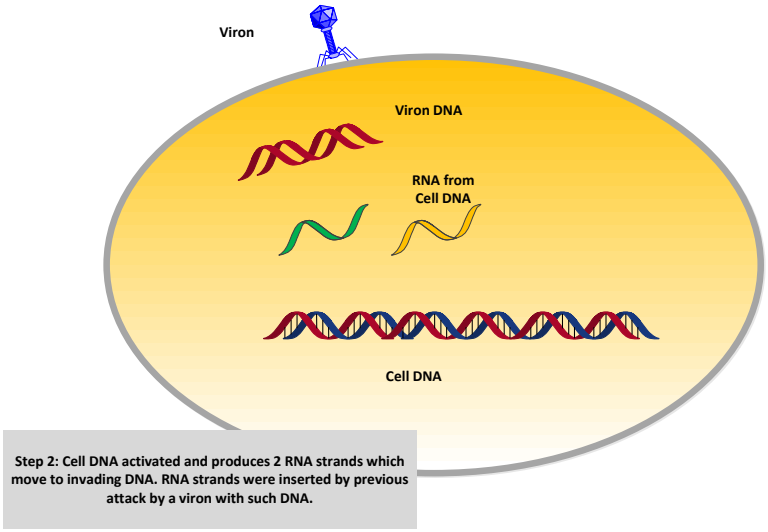
Now in discovering this process one then can take this same enzyme and modify the RNA that comes with it to match a location on some DNA we may be considering to manipulate and using this combo we can then cut DNA at a precise point anywhere we so desire. It is a powerful tool to cut DNA at a unique location. From there we can then add or delete DNA segments in a gene, in a somatic cell or in a germ line cell. It is fast and inexpensive and can be done in almost any lab.

Let us now begin with a viron attaching itself to a bacterium. We will assume that at some prior time some process has occurred where the bacteria had seen this for the first time. At this time that process is still a work in progress. But let us assume that this is a subsequent encounter and that in the process the bacteria has managed to record this prior encounter with a strand of DNA from that viron so that it can produce an RNA which is a map of some small segment of the viron's DNA.

This is a lot of assumptions but it is generally where we start with the tool. We just want to know what the tool does not how it was made or even how in any detail it does what it does. In many ways we are looking at tools as a technician, namely use this tool this way and get this result. Leave the details for someone else.

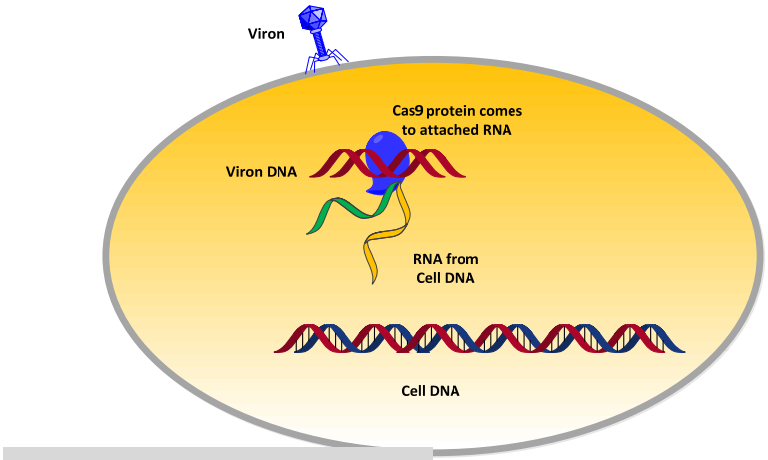


Now when the viron gets into the cell there is produced RNA from the bacteria that was RNA based upon a prior encounter with this viron. Namely this RNA released matches a segment of DNA in the viron. Also remember that a virus just wants to use a cell, any cell, to reproduce itself, which frankly is just reproducing its DNA (or RNA). If the bacteria can use this knowledge of the attacker then what can it do to stop the reproduction, and potentially the organism's death.



Step 2: Cell DNA activated and produces 2 RNA strands which move to invading DNA. RNA strands were inserted by previous attack by a viron with such DNA.

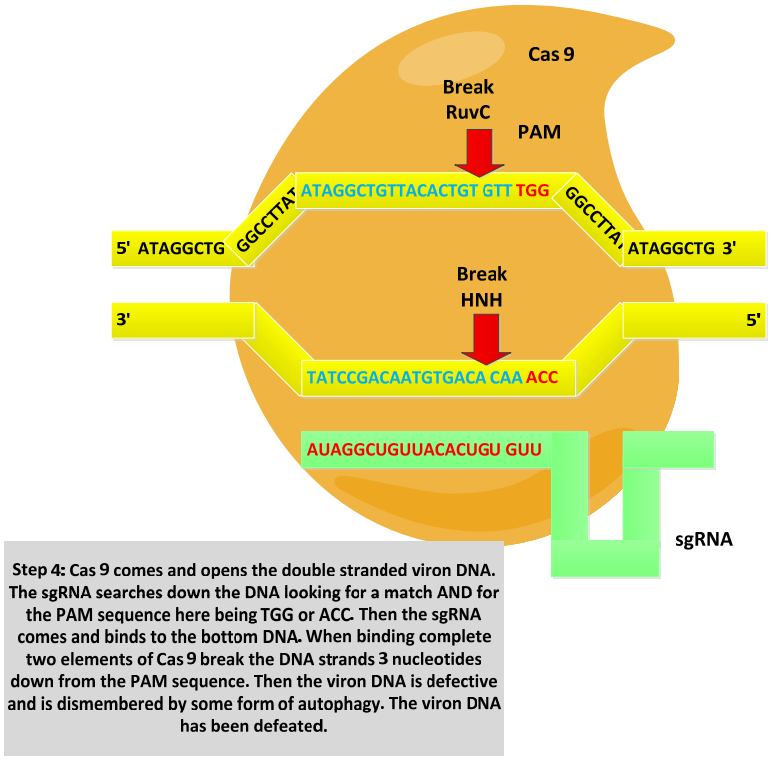
Now the RNA segments migrate to the viron DNA and along with a protein called cas9. The cas9 protein is the secret sauce of this tool.



Step 3: Cas9 protein is released and combines with viron DNA, combined RNA to create a CRISPR Cas9 cut of the viron DNA to be shown next in detail.

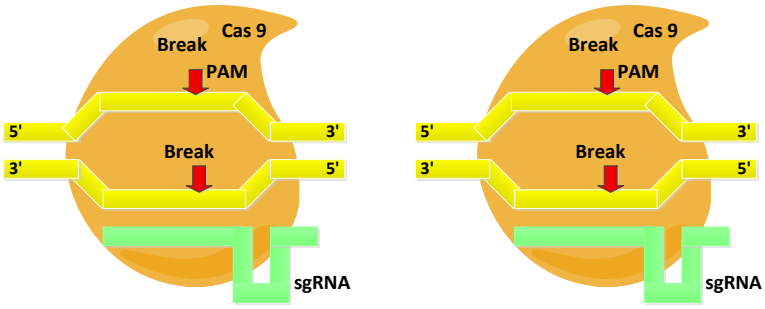
The details of the operation are depicted in the graphic below. One must recall that this tool works but its operation is not fully understood. The Cas9 protein surrounds the desired site which has been selected by a combination of two factors. The first is the PAM sequence, in this case 3 nucleotides, nt, which act as a marker and then a 20 nt long matching strand down from the PAM. This key determines where the break occurs. In a bacteria's immune like response it needs

both, the PAM to be certain it does not kill itself and the 20 nt strand which gives a good marker for a specific site. In effect we have 23 nt for specific targeting. In genetic engineering cases we select the PAM as specified by the Cas9 source and then engineer the sgRNA element. That yields a specific break site at 3 nt down from the end of the PAM. The two Cas9 fragments, RuvC and HNH are what cause the break.



Step 4: Cas 9 comes and opens the double stranded viron DNA. The sgRNA searches down the DNA looking for a match AND for the PAM sequence here being TGG or ACC. Then the sgRNA comes and binds to the bottom DNA. When binding complete two elements of Cas 9 break the DNA strands 3 nucleotides down from the PAM sequence. Then the viron DNA is defective and is dismembered by some form of autophagy. The viron DNA has been defeated.

The example below extends the above example to a double stranded break.



3.2 CRRNA AND TRACRRNA

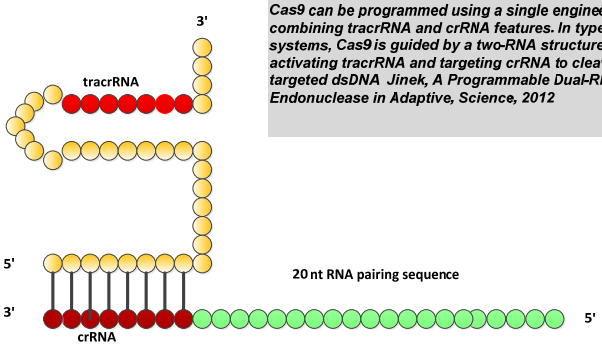
The two RNA segments, crRNA and tracrRNA can be configured in several ways. But they are the targets elements that are used to select where the break is to be. And once selected it is usually a double strand break. However single strand breaks can be accomplished as well.

As Jinek et al state:

In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short ***crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets****. Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs ….*

There are three types of CRISPR/Cas systems. The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA)*** *complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA*

We demonstrate one variation of this below. Note the tracrRNA and its binding with crRNA and the 20 nucleotide ("nt") sequence which will select out the point at which we desire a break to be made.



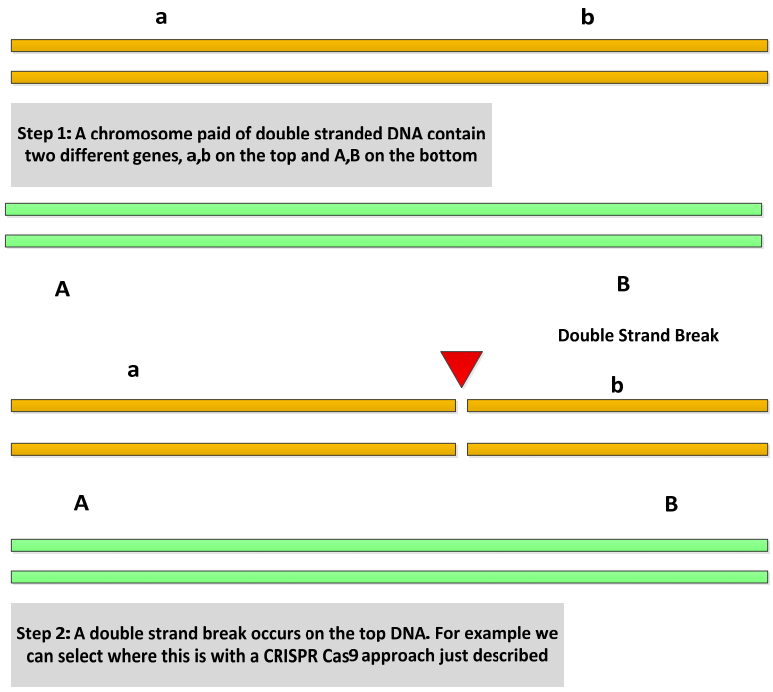
Cas9 can be programmed using a single engineered RNA molecule combining tracrRNA and crRNA features. In type II CRISPR/Cas systems, Cas9 is guided by a two-RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically – targeted dsDNA Jinek, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive, Science, 2012

3.3 GENE ENGINEERING

No we ask; given a break at the right point what do we do next? That is the beginning of gene engineering. We briefly examine homologous repair, a somewhat well understood process, which uses the other chromosome as a template. The use of templates may also be done to insert new genes as well.

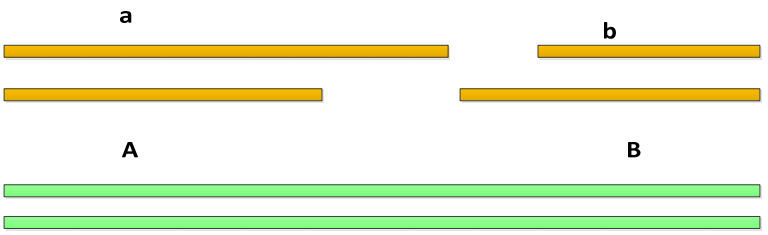
1. Let us start with a chromosome pair, one from each parent. We show this below.

2. Now we assume a double strand break, DSB, occurs on the top chromosome pair. We show this below:

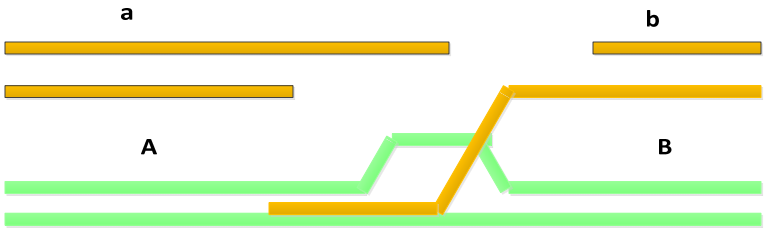


3. Next we see a shortening of strands as shown below;

4. Then we see and elongation and use of the strand in the uncut DNA as a template. This can be used for other templates as insertion mechanisms.



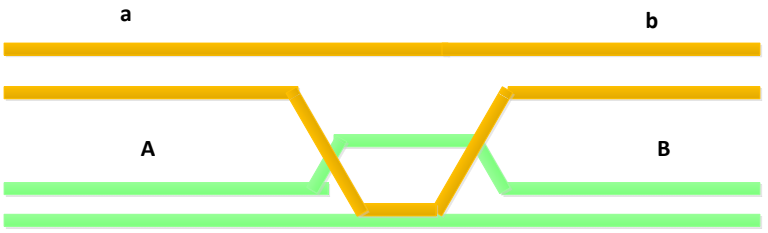
Step 3: The cut strands are shortened in top and bottom branches



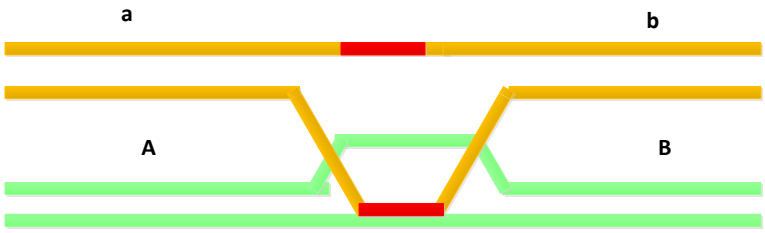
Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

5. We see both top and bottom expanding and a crossover occurring.

6. We can see this also as an insertion mechanism.



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.

Now this is a simple reconstruction of the process. Details are in Watson et al.

4 APPLICATIONS

There are a large set of applications for this tool. We consider some here but it is anticipated that there will be many more. There is a balance between correcting gene defects in such disorders as muscular dystrophy, thalassemia, sickle cell anemia, or cystic fibrosis, and cancers such as those involving BRCA genes. There are also applications in the field of plant genetics which are extensive. Plants are pluripotent; that is, a single cell can regenerate almost any plant. Thus adding or extracting a gene can dramatically change a plant's characteristics. We have examined some of these opportunities elsewhere for horticultural plants.

4.1 GENE EXTRACTION

The simplest application is gene extraction. Using two DSBs at the desired locations we can accurately extract a gene.

4.2 GENE INSERTION

Gene insertion is a major step. A template must be available and an insertion point specified. It is also important to understand the location of any promoter genes or suppressor genes. Just inserting may not always work. There is also the issue of methylation and acetylation as well as miRNA interference.

4.3 SOMATIC APPLICATIONS

As the body matures or as a result of a genetic defect, we often see genetic changes which result in less than benign results.

4.4 GERMLINE APPLICATIONS

This is the most concerning application. Recently a group of researchers have indicated their concern and we shall discuss it later. However, one can take a sperm and ovum, cut-and-paste a new set of genes, and then allow them to combine and we have developed putatively a new species.

4.5 TYPES OF APPLICATIONS

There have been a multiplicity of papers on various applications We have indicated some general ones above but the Zhang Lab at MIT has performed a great deal worth examining

From the work of Cox et al,

The specific type of genome editing therapy depends on the nature of the mutation causing disease.

(a) In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of indels in the gene of interest often results in frameshift mutations that create premature stop codons resulting in a nonfunctional protein product or nonsense-mediate decay of transcripts, suppressing gene function. Gene disruption may also be used to introduce protective loss of- function mutations into wild-type genes to generate a therapeutic effect (Box 1).

(b) In NHEJ gene correction, two DSBs targeted to both sides of a pathogenic expansion or insertion may be resolved by NHEJ, causing a therapeutic deletion of the intervening sequences. This form of treatment would require multiplexed targeting of disease-causing mutations.

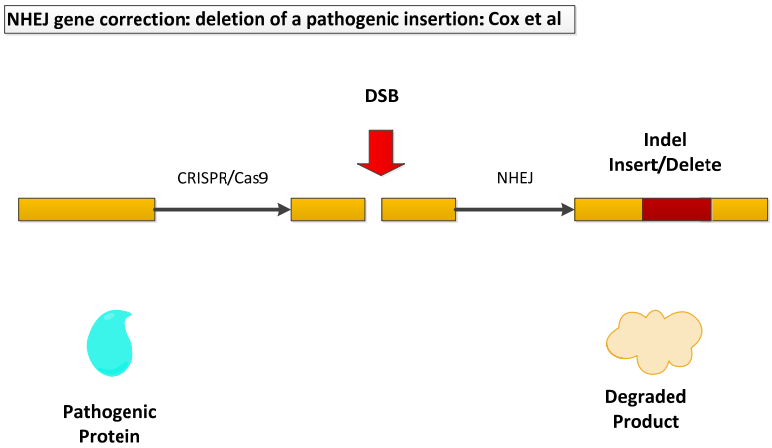
(c) HDR gene correction can be used to correct a deleterious mutation. A DSB is induced near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function.

(d) An alternative to gene correction is HDR gene addition, which introduces a therapeutic transgene into a predetermined locus. This may be the native locus, a safe harbor locus or a non-native locus. A DSB is induced at the desired locus, and an HDR template containing sequence similarity to the break site, a promoter, a transgene and a polyadenylation sequence is introduced to the nucleus. HDR repair restores gene function in the target locus, albeit without true physiological control over gene expression.

We graphically demonstrate some of these below:

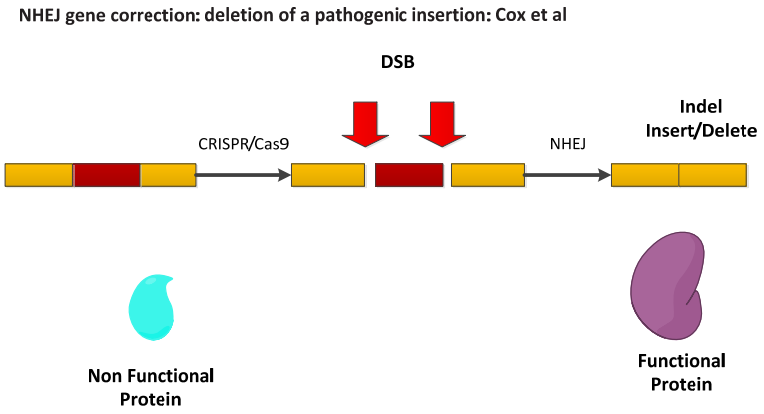
4.5.1 Deletion

The first cases below are a non-homologous break and joining of some insertion/deletion ("indel") to degrade the production of a protein. This is a simple double stranded break.



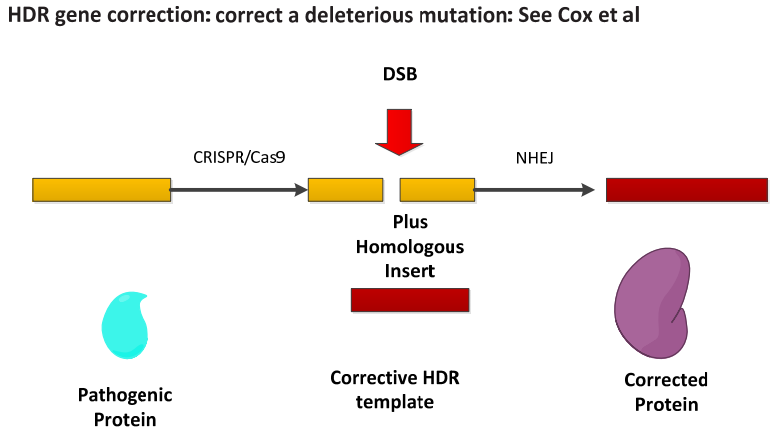
4.5.2 Correction

The second is again one using a DSB but this time a specific section is deleted and it requires two DSBs to be initiated. This may be an application where we seek a functional protein. It may be possible to employ this in a fusion gene process as well.



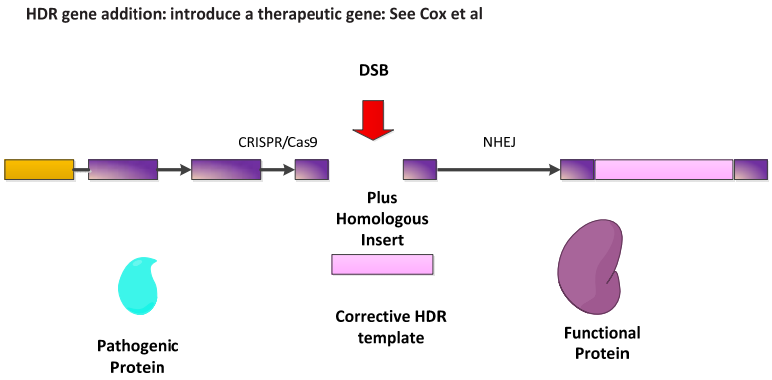
4.5.3 Homologous Correction

The next two applications are for homologous changes where we have a template to reconstruct the gene of the type we have previously discussed. The example below is a simple correction process with a single DSB.



4.5.4 Homologous Insertion

The final example below is for a complicate deletion and insertion. Again it uses a template and a homologous rebinding.



4.5.5 Ex Vivo and In Vivo

There are several dimensions in applying this tool to humans. They can be inserted ex vivo or in vivo. As Cox et al state:

… in ex vivo editing therapy, cells are removed from a patient being treated, edited and then re-engrafted. For this mode of therapy to be successful, the target cells must be capable of surviving outside the body and homing back to target tissues after transplantation.

… in vivo therapy involves genome editing of cells in situ. For in vivo systemic therapy, delivery agents that are relatively agnostic to cell identity or state would be used to effect editing in a wide range of tissue types. Alternatively, targeted in vivo therapy may also be achieved through targeted local injection of viral vectors to the affected tissue or through the systemic injection of viral vectors with inherent tropism for specific diseased tissues, such as the eye brain, or muscle.

Now we can see this as applied in somatic or germ line cells.

	Ex Vivo	***In Vivo***
Somatic	One may consider this approach in certain hematopoietic cell lines.	Insertion into targeted somatic cells of modified genes may produce or delete expressions of genes. Various means of insertion are possible.
Germ Line	This is a somewhat routine procedure for certain reproductive processes. However the viability and sustainability of such cells may have issues.	Each of the germ line cells may be separately dealt with insertions or deletions and then the two combined. This method also permits the insertion of CRISPR Cas9 genes themselves to assure continual propagation of the desired change. However this may be a very complex approach since it deals with in vivo germ line cells where injecting the cells may not function completely.

5 CRISPR AND CANCER TREATMENT

Thus one may ask if one knows that some gene has been the cause of a cancer, can we then treat the cells with a CRISPR-Cas system to delete the gene and replace it with a normal wild type. If we have a procedure to do this then perhaps this is a therapeutic approach. It does, of course beg the question of how this is accomplished even if we have the chimeric Cas delivery system. We also must ask if we have identified all the genes. There are also many other such questions. Yet this has become a focal point of interest.

In a recent paper by Yin et al the authors discuss the delivery of a CRISPR-Cas9 mediated cutting and reintroduction of a gene into liver cells by means of an injection process. The result was conversion of the errant gene cells into normal wild type cells. They utilized the backwards flow of the CRISPER-Cas9 approach for cutting and injection. This potentially paves the way for substantial progress in alternative targeted gene replacement and return to normal states. As Yin et al state:

We demonstrate CRISPR-Cas9–mediated correction of a Fah mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype. Our study indicates that CRISPR-Cas9–mediated genome editing is possible in adult animals and has potential for correction of human genetic diseases.

From Gene News[3] we have a more detailed discussion worthy of note regarding the above recent report:

MIT scientists report the use of a CRISPR methodology to cure mice of a rare liver disorder caused by a single genetic mutation. They say their study … offers the first evidence that this gene-editing technique can reverse disease symptoms in living animals. CRISPR, which provides a way to snip out mutated DNA and replace it with the correct sequence, holds potential for treating many genetic disorders, according to the research team.

"What's exciting about this approach is that we can actually correct a defective gene in a living adult animal," says Daniel Anderson, Ph.D., the Samuel A. Goldblith associate professor of chemical engineering at MIT, a member of the Koch Institute for Integrative Cancer Research, and the senior author of the paper.

The recently developed CRISPR system relies on cellular machinery that bacteria use to defend themselves from viral infection. Researchers have copied this cellular system to create gene-editing complexes that include a DNA-cutting enzyme called Cas9 bound to a short RNA guide strand that is programmed to bind to a specific genome sequence, telling Cas9 where to make its cut.

[3] http://www.genengnews.com/gen-news-highlights/crispr-reverses-disease-symptoms-in-living-animals-for-first-time/81249682/

At the same time, the researchers also deliver a DNA template strand. When the cell repairs the damage produced by Cas9, it copies from the template, introducing new genetic material into the genome. Scientists envision that this kind of genome editing could one day help treat diseases such as hemophilia, Huntington's disease, and others that are caused by single mutations.

For this study, the researchers designed three guide RNA strands that target different DNA sequences near the mutation that causes type I tyrosinemia, in a gene that codes for an enzyme called FAH. Patients with this disease, which affects about 1 in 100,000 people, cannot break down the amino acid tyrosine, which accumulates and can lead to liver failure. Current treatments include a low-protein diet and a drug called NTCB, which disrupts tyrosine production.

In experiments with adult mice carrying the mutated form of the FAH enzyme, the researchers delivered RNA guide strands along with the gene for Cas9 and a 199-nucleotide DNA template that includes the correct sequence of the mutated FAH gene.

"Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells," wrote the investigators. "Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype."

While the team used a high pressure injection to deliver the CRISPR components, Dr. Anderson envisions that better delivery approaches are possible. His lab is now working on methods that may be safer and more efficient, including targeted nanoparticles.

The above described an interesting I vivo approach to the editing and insertion of a specific gene in a specific location. Although this is of interest, it is limited to a very specific site and also using a difficult delivery mechanism.

As to more extensive editing capabilities we examine Zhang et al who state:

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. CRISPR/Cas9 comprises of a non-specific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA), which can guide Cas9 to cleave DNA and generate double-strand breaks at target sites. Subsequent cellular DNA repair process leads to desired insertions, deletions or substitutions at target sites.

The specificity of CRISPR/Cas9-mediated DNA cleavage requires target sequences matching crRNA and a protospacer adjacent motif (PAM) locating at downstream of target sequences. Here, we review the molecular mechanism, applications and challenges of CRISPR/Cas9-mediated genome editing and clinical therapeutic potential of CRISPR/Cas9 in future.

The above stresses the strong point of CRISPR-Cas9, namely its specificity. It can target specific DNA, assuming we know what to target. It can then replace that with a substitute, assuming we

know that the substitute does no harm and in fact is positively therapeutic. From Pandika we have[4] the following lengthy discussion regarding the evolution of this specific result:

Because a Nobel Prize winner says this breakthough is better than his breakthrough. Jennifer Doudna has always had an explorer's spirit. It's what led the UC Berkeley molecular and cell biology professor to engineer a cheaper, easier way to correct DNA defects. Her game-changing technology takes a mysterious bacterial genetic code and transforms it into a powerful tool for cutting and pasting bits of genetic material – meaning not only could the entire field of gene therapy be revived, but her genome-editing tool could one day be used to treat a range of diseases, from cancer and AIDS to hereditary disorders like Down syndrome and Huntington disease.

Every time we see some new tool for the toolkit the immediate tendency is to label it as a cure for cancer.

Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose….The bacterial enzyme Cas9 is the engine of RNA-programmed genome engineering in human cells. Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors…

"I wasn't actively trying to go in any particular direction," she said. That willingness to wander, to maybe even get a little lost, could be how she was able to make a creative break from earlier genome-editing technologies. Doudna "certainly didn't set out to discover a genome editing tool by any stretch of the imagination." It all began with a puzzle she couldn't resist solving, thanks largely to her father. When Doudna was growing up, the literature professor got her hooked on one of his favorite pastimes —decoding short pieces of encrypted text, or cryptograms.

In 2005, a colleague presented Doudna with a genetic cryptogram — weird repetitive RNA sequences tucked in the genomes of many of the bacteria she studied. Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose.

Sure enough, scientists discovered that CRISPRs played an important role in immunity: they recognize the DNA of viral invaders for the bacteria to chop up and fight off. But how did this search-and-destroy mechanism work? Teaming up with Umea University molecular biologist Emmanuelle Charpentier, Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors. A CRISPR RNA fragment hooks up with Cas9 to precisely target the DNA of an invading virus, which it then cuts and destroys.

Here's where it gets really complicated. Martin Jinek, a postdoctoral researcher in Doudna's lab, found that Cas9 in bacteria needs two RNA guide strands – this sent the gears in their heads turning. What if they could engineer the system to require only a single, programmable RNA strand? Then biologists could use it to easily target and cut any DNA sequence. Doudna felt "a chill of excitement." Maybe they could link the two RNA strands into one, and loop it in on

[4] http://www.ozy.com/rising-stars-and-provocateurs/jennifer-doudna/4690.article

itself—mimicking a double-stranded structure. Those chills were warranted: Doudna's lab and other groups successfully used this simplified CRISPR system to modify genes in bacteria, plant and animal cells.

One early form of CRISPR-based gene therapy could involve editing the genes responsible for blood disorders like sickle-cell anemia in bone marrow cells, growing them into mature blood cells and injecting them back into patients.

However, the application needs a more effective insertion system. It also needs to demonstrate that it does not wander and affect other genes.

Little more than a year after Doudna first described CRISPR in the journal Science, the cut-and-paste technology has yielded promising results in labs around the world. Last month, researchers from the Netherland's Utrecht institute reported in Cell Stem Cell that CRISPR corrected the gene mutation responsible for cystic fibrosis in stem cells developed from two children with the life-threatening disease. Doudna believes a clinical trial of CRISPR-based gene therapy could begin in less than a decade.

As is all too often the case, any prediction of clinical application may be much too speculative. Single gene targeting may become the first step, albeit even there one must be cautious.

Doudna experienced "many frustrations" getting CRISPR to work in human cells. But she knew if she succeeded, CRISPR would be "a profound discovery" — and maybe even a powerful gene therapy technique.

We knew if the system could be made to work in human cells, it would be a really profound discovery.

"I hope you're sitting down," an excited colleague told Doudna in an unexpected phone call. "CRISPR is turning out to be absolutely spectacular in [Harvard geneticist] George Church's hands." He had even gotten it to work in human cells. Thrilled, Doudna immediately contacted Church. They shared their results, and both published studies in January 2013 showing that CRISPR can cut, delete and replace genes in human cells. University of Massachusetts biologist Craig Mello, who shared the 2006 Nobel Prize for another genome editing tool, hails Doudna's CRISPR technique as a "tremendous breakthrough," even admitting that "in many ways it's better" than his own technique.

Other techniques can also edit genes at specific DNA regions. But they require scientists to engineer a separate protein for each target site. In contrast, CRISPR only needs the Cas9 protein, allowing it to correct multiple defects at once. Besides being cheaper and easier to use, CRISPR is also much more precise, reducing the risk of off-target modifications introducing dangerous mutations. As a result, it could help revive the gene therapy field, whose early clinical failures — including patient deaths — led some to dismiss it as overhyped.

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene. Understanding

and exploiting how Cas9 avoids these close matches "is an active area of investigation," Doudna said. Still, CRISPR is "a real game-changer," Mello told the Independent. "It's incredibly powerful."[5]

Indeed the observations above detail some of the powers of CRISPR-Cas9 complex.

Now one would think that perhaps this could become a therapeutic as applied to various cancers. Consider it use as a kinase inhibitor in CML. Would it work there by targeting the aberrant kinases? What of an application in melanoma with a BRAF V400 mutation? Can we cut and paste back the proper genetic sequence? If so, how do we deliver the elements of the process, especially in a metastatic case? Furthermore, how do we determine what genes must be modified, and does that mean that we not only customize it for a patients but also for cells? Finally how do we know that there are not some deleterious sequellae from this cutting and pasting process, what if we "miss" the gene in some cell and start an secondary malignancy?

These are all reasonable questions that lead us to examine the CRISPR process in further detail

As recently stated by Stephen et al:

We are also optimistic that completely different approaches to treating cancer will contribute to eliminating Ras cancers, including new ways of knocking down/out genes using RNAi and CRISPR technologies and delivering these payloads to tumors, as well as new ways of deploying the immune system.

In this respect, it is noteworthy that anti-CTLA-4 therapy appears to be equally effective in treating melanoma driven by N-Ras or B-Raf; therefore, Ras cancers may not be excluded from these approaches as they have been from others. All of these considerations lead us to be optimistic about future prospects of finally delivering the knockout punch.

As Way et al state:

Synthetic biology is a young discipline with the declared goal of rationally engineering biological systems through approaches similar to those used by engineers to build bridges and send people to the moon. This field has rapidly developed over the past 15 years from its initial conceptualization by a few academics and government program managers into a sizeable field whose meetings attract large numbers of participants. Recently, new tools have emerged that should allow specific integration at desired sites in the genome. For example, methods based on zinc-finger, TALE, and CRISPR fusions to nucleases can be used to generate double-strand breaks at specific sites in the genome. The questions remain—where should we integrate, and how can we avoid effects of adjacent sequences?

[5] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

6 OBSERVATIONS

Having given a high level description of this tool we can make several key observations. Amongst them is the recent concern as to the potential abuse of the process.

CRISPR Cas 9 is a new technique to cut and splice genes. We had written about it about a year ago regarding its use in cancer treatment and also regarding the patent so quickly issues. Now David Baltimore, a highly respected scientist, and colleagues have in Science suggested a prudent set of steps as to its use in humans. It is reminiscent of the concerns some 40 years ago regarding recombinant DNA.

6.1 CONTROLS TO USE

Recently several groups of researchers have become concerned regarding the unintended consequences of CRISPRs. For example David Baltimore et al recommend:

In the near term, we recommend that steps be taken to:

1) Strongly discourage, even in those countries with lax jurisdictions where it might be permitted, any attempts at germline genome modification for clinical application in humans, while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. (In countries with a highly developed bioscience capacity, germline genome modification in humans is currently illegal or tightly regulated.) This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums in which experts from the scientific and bioethics communities can provide information and education about this new era of human biology, the issues accompanying the risks and rewards of using such powerful technology for a wide variety of applications including the potential to treat or cure human genetic disease, and the attendant ethical, social, and legal implications of genome modification.

3) Encourage and support transparent research to evaluate the efficacy and specificity of CRISPR-Cas9 genome engineering technology in human and nonhuman model systems relevant to its potential applications for germline gene therapy. Such research is essential to inform deliberations about what clinical applications, if any, might in the future be deemed permissible.

4) Convene a globally representative group of developers and users of genome engineering technology and experts in genetics, law, and bioethics, as well as members of the scientific community, the public, and relevant government agencies and interest groups—to further consider these important issues, and where appropriate, recommend policies.

Baltimore et al have a significant point. Not only can this be significant on a person by person basis but it also has the potential to be weaponized. The technology is out there, thousands are now proficient in it, the cost is low and the means for distribution is high.

Clearly a sensible effort in collaboration with others is essential. The problem is that with much of science, the genie is out of the box.

The system used by bacteria to defend against a virus attacking is the CRISPR Cas 9 system. An interesting use of a protein, enzyme, and a DNA segment that can open DNA at desired locations and cut and insert new segments of DNA. We have been discussing this for well over a year now and have discussed its potential and its risks.

Now along come researchers who instead of doing this in somatic cells do it in germline cells, thus changing the potentially maturing entity. Thus each cell has this changed gene or genes.

In a recent Nature article the authors state[6]:

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells....In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited. At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germ-line modification, we encourage an open discussion around the appropriate course of action.

Now this point is well made. Germline cell changes introduce all sorts of issues. Not only is there the issue of what this new gene will do, we hardly have begun to understand gene interactions, but the issues of epigenetic factors such as methylation dramatically change the risks.

Then again you do have the techno-advocates in Technology Review, who state[7]:

When I visited the lab last June ... proposed that I speak to a young postdoctoral scientist named ..., a Harvard recruit from Beijing who'd been a key player in developing a new, powerful technology for editing DNA, called CRISPR-Cas9. With ...had founded a small company to engineer the genomes of pigs and cattle, sliding in beneficial genes and editing away bad ones. As I listened to ..., I waited for a chance to ask my real questions: Can any of this be done to human beings? Can we improve the human gene pool? The position of much of mainstream science has been that such meddling would be unsafe, irresponsible, and even impossible. But ... didn't hesitate. Yes, of course, she said. In fact, the Harvard laboratory had a project to determine how it could be achieved.

[6] http://www.nature.com/news/don-t-edit-the-human-germ-line-1.17111

[7] http://www.technologyreview.com/featuredstory/535661/engineering-the-perfect-baby/

She flipped open her laptop to a PowerPoint slide titled "Germline Editing Meeting." Here it was: a technical proposal to alter human heredity. "Germ line" is biologists' jargon for the egg and sperm, which combine to form an embryo. By editing the DNA of these cells or the embryo itself, it could be possible to correct disease genes and to pass those genetic fixes on to future generations. Such a technology could be used to rid families of scourges like cystic fibrosis. It might also be possible to install genes that offer lifelong protection against infection, Alzheimer's, and ... told me, maybe the effects of aging.

These would be history-making medical advances that could be as important to this century as vaccines were to the last.

The problem is, as the writers in Nature and in Science led by David Baltimore, has noted, the germ line modifications could be unwieldy.

Just because we have a new technology is no reason to let is loose. The problem with this technology is that it not only can be weaponized but that it can be done in a basement lab. This not building a nuclear weapon. This is potentially setting the world afire.

The again there is the issue of Government regulation. In an interesting piece in Xconomy the author remarks[8]:

But researchers' and investors' fear that a patchwork of regulation would cripple biotechnology in the United States did not disappear right away. Biologist Thomas Maniatis of Harvard left his home lab to work on the techniques in tighter-security conditions at Cold Spring Harbor Laboratory in New York.

Others went abroad. Biogen, founded in 1978, put its first major lab in Geneva, Switzerland. This was a time of intense concern about environmental dangers from the chemical industry in particular and science in general. It took some years for biologists to gain respect among local state, and federal officials for their sense of responsibility in the recombinant DNA maelstrom of the mid-1970s. But politicians did accept that biotechnology was a significant new industry that other countries, like Japan, might seize if America dropped the ball.

A valid point, but in the 70s we worried about errant scientists. Now we are terrified about terrorist post docs! One wonders what would be worse; the Government Regulators or the Terrorist?

6.2 CURRENT APPLICATIONS

There are many areas where this technology may have immediate use. There is a report in Genome Research of CRISPR being used to correct β-Thalassemia. They state[9]:

[8] http://www.xconomy.com/boston/2015/03/26/amid-gene-editing-worry-a-return-to-biotechs-1st-asilomar-moment/2/

[9] http://genome.cshlp.org/content/early/2014/07/30/gr.173427.114.abstract

β-thalassemia, one of the most common genetic diseases worldwide, is caused by mutations in the human hemoglobin beta (HBB) gene. Creation of human induced pluripotent stem cells (iPSCs) from β-thalassemia patients could offer an approach to cure this disease. Correction of the disease-causing mutations in iPSCs could restore normal function and provide a rich source of cells for transplantation.

In this study, we used the latest gene-editing tool, CRISPR/Cas9 technology, combined with the piggyBac transposon to efficiently correct the HBB mutations in patient-derived iPSCs without leaving any residual footprint.

No off-target effects were detected in the corrected iPSCs, and the cells retain full pluripotency and exhibit normal karyotypes. When differentiated into erythroblasts using a monolayer culture, gene-corrected iPSCs restored expression of HBB compared to the parental iPSCs line.

Our study provides an effective approach to correct HBB mutations without leaving any genetic footprint in patient-derived iPSCs, thereby demonstrating a critical step toward the future application of stem cell-based gene therapy to monogenic diseases.

We have considered Cancer applications in a separate note in 2014. Cox et al present the following summary Table:

Disease type	*Nuclease platform*	*Therapeutic strategy*
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence
HIV	ZFN and CRISPR	NHEJ-mediated inactivation of CCR5
Duchene muscular dystrophy (DMD)	CRISPR and TALEN	NHEJ-mediated removal of stop codon, and HDR-mediated gene correction
Hepatitis B virus (HBV)	TALEN and CRISPR	NHEJ-mediated depletion of viral DNA
SCID	ZFN	HDR-mediated insertion of correct gene sequence
Cataracts	CRISPR	HDR-mediated correction of mutation in mouse zygote
Cystic fibrosis	CRISPR	HDR-mediated correction of CFTR in intestinal stem cell organoid
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver

6.3 OTHER CRISPR VEHICLES

In Nature (Ran et al 2015) we have an article demonstrating a variant on the now standard CRISPR cas9 vehicle. As they first note:

Type II CRISPR-Cas systems require only two main components for eukaryotic genome editing: a Cas9 enzyme, and a chimeric sgRNA derived from the CRISPR RNA (crRNA) and the noncoding trans-activating crRNA (tracrRNA). Analysis of over 600 Cas9 orthologues shows that these enzymes are clustered into two length groups with characteristic protein sizes of approximately 1,350 and 1,000 amino acid residues, respectively

Thus the classic source is Streptococcus pyogenes and as noted:

The RNA-guided endonuclease Cas9 has emerged as a versatile genome-editing platform. However, the size of the commonly used Cas9 from Streptococcus pyogenes (SpCas9) limits its utility for basic research and therapeutic applications that use the highly versatile adeno-associated virus (AAV) delivery vehicle.

But the same vehicle with a Cas9 is in many other bacteria and they note:

Here, we characterize six smaller Cas9 orthologues and show that Cas9 from Staphylococcus aureus (SaCas9) can edit the genome with efficiencies similar to those of SpCas9, while being more than 1 kilobase shorter. We packaged SaCas9 and its single guide RNA expression cassette into a single AAV vector and targeted the cholesterol regulatory gene Pcsk9 in the mouse liver.

Thus we have a variant but the same functionality. They conclude regarding in vivo changes:

Here, we develop a small and efficient Cas9 from S. aureus for in vivo genome editing. The results of these experiments highlight the power of using comparative genomic analysis in expanding the CRISPR-Cas9 toolbox. Identification of new Cas9 orthologues, in addition to structure-guided engineering, could yield a repertoire of Cas9 variants with expanded capabilities and minimized molecular weight, for nucleic acid manipulation to further advance genome and epigenome engineering. ...

We examined these sites in liver tissue transduced by AAV-SaCas9 and did not observe any indel formation within the detection limits of in vitro BLESS and targeted deep sequencing. Importantly, the off-target sites identified in vitro might differ from those in vivo, which need to be further evaluated by the applications of BLESS or other unbiased techniques such as those published during the revision of this work. Finally, we did not observe any overt signs of acute toxicity in mice at one to four weeks after virus administration. ....these findings suggest that in vivo genome editing using SaCas9 has the potential to be highly efficient and specific.

This is an interesting next step.

6.4 CRISPR CONCERNS

The system used by bacteria to defend against a virus attacking is the CRISPR Cas 9 system. An interesting use of a protein, enzyme, and a DNA segment that can open DNA at desired locations and cut and insert new segments of DNA. We have been discussing this for well over a year now and have discussed its potential and its risks.

Now along comes researchers who instead of doing this in somatic cells do it in germline cells, thus changing the potentially maturing entity. Thus each cell has this changed gene or genes.

In a recent Nature article the authors state:

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells....In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited. At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germ-line modification, we encourage an open discussion around the appropriate course of action.

Now this point is well made. Germline cell changes introduce all sorts of issues. Not only is there the issue of what this new gene will do, we hardly have begun to understand gene interactions, but the issues of epigenetic factors such as methylation dramatically change the risks.

Frankly I miss Michael Circhton, in this case he would have clearly shown us the mistakes we could be making with an unruly unleashing of this technology. Jurassic Park would be a walk in the park as compared to what these could unleash. Imagine correcting those few genes in Apes and the other close to man mammals and see what we could get!

The again you do have the advocates in Technology Review, that somewhat unidentifiable magazine sent to MIT alumni and others, that states:

When I visited the lab last June, ... proposed that I speak to a young postdoctoral scientist named ..., a Harvard recruit from Beijing who'd been a key player in developing a new, powerful technology for editing DNA, called CRISPR-Cas9. With ..., ...had founded a small company to engineer the genomes of pigs and cattle, sliding in beneficial genes and editing away bad ones. As I listened to ..., I waited for a chance to ask my real questions: Can any of this be done to human beings? Can we improve the human gene pool? The position of much of mainstream science has been that such meddling would be unsafe, irresponsible, and even impossible. But ... didn't hesitate. Yes, of course, she said. In fact, the Harvard laboratory had a project to determine how it could be achieved. She flipped open her laptop to a PowerPoint slide titled "Germline Editing Meeting." Here it was: a technical proposal to alter human heredity. "Germ line" is biologists' jargon for the egg and sperm, which combine to form an embryo. By editing the DNA of these cells or the embryo itself, it could be possible to correct disease genes and to pass those genetic fixes on to future generations. Such a technology could be used to rid families of scourges like cystic fibrosis. It might also be possible to install genes that offer lifelong protection against infection, Alzheimer's, and, ... told me, maybe the effects of aging. These

would be history-making medical advances that could be as important to this century as vaccines were to the last.

The problem is, as the writers in Nature and in Science noted, led by David Baltimore, have noted, the germ line modifications could be unwieldy.

Just because we have a new technology is no reason to let is loose. The problem with this technology is that it not only can be weaponized but that it can be done in a basement lab. This not the building of a nuclear weapon. This is potentially setting the world afire.

The again there is the issue of Government regulation. In an interesting piece in Xconomy the author remarks:

But researchers' and investors' fear that a patchwork of regulation would cripple biotechnology in the United States did not disappear right away. Biologist Thomas Maniatis of Harvard left his home lab to work on the techniques in tighter-security conditions at Cold Spring Harbor Laboratory in New York. Others went abroad. Biogen, founded in 1978, put its first major lab in Geneva, Switzerland. This was a time of intense concern about environmental dangers from the chemical industry in particular and science in general. It took some years for biologists to gain respect among local state, and federal officials for their sense of responsibility in the recombinant DNA maelstrom of the mid-1970s. But politicians did accept that biotechnology was a significant new industry that other countries, like Japan, might seize if America dropped the ball.

A valid point, but in the 70s we worried about errant scientists. Now we are terrified about terrorist post docs! One wonders what would be worse; the Government Regulators or the Terrorist?

CRISPR Cas 9 is a new technique to cut and splice genes. We had written about it about a year ago regarding its use in cancer treatment and also regarding the patent so quickly issues. Now David Baltimore, a highly respected scientist, and colleagues have in Science suggested a prudent set of steps as to its use in humans. It is reminiscent of the concerns some 49 years ago regarding recombinant DNA.

Baltimore et al recommend:

In the near term, we recommend that steps be taken to:

1) Strongly discourage, even in those countries with lax jurisdictions where it might be permitted, any attempts at germline genome modification for clinical application in humans, while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. (In countries with a highly developed bioscience capacity, germline genome modification in humans is currently illegal or tightly regulated.) This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums in which experts from the scientific and bioethics communities can provide information and education about this new era of human biology, the issues accompanying the risks and rewards of using such powerful technology for a wide variety of applications including the potential to treat or cure human genetic disease, and the attendant ethical, social, and legal implications of genome modification.

3) Encourage and support transparent research to evaluate the efficacy and specificity of CRISPR-Cas9 genome engineering technology in human and nonhuman model systems relevant to its potential applications for germline gene therapy. Such research is essential to inform deliberations about what clinical applications, if any, might in the future be deemed permissible.

4) Convene a globally representative group of developers and users of genome engineering technology and experts in genetics, law, and bioethics, as well as members of the scientific community, the public, and relevant government agencies and interest groups—to further consider these important issues, and where appropriate, recommend policies.

Baltimore et al have a point. Not only can this be significant on a person by person basis but it also has the potential to be weaponized. The technology is out there, thousands are now proficient in it, the cost is low and the means for distribution is high.

Clearly a sensible effort in collaboration with others is essential. The problem is that with much of science, the genie is out of the box.

6.5 ISSUE OF CONCERN

As noted there may be many unintended consequences which have yet to be explored. We examine a few of them here and we are certain many more will arise in the future. This is a powerful technology, one that is inexpensive and fast acting, and one which if in th wrong hands can be used for less than benign purposes.

6.5.1 Accuracy of Cutting

How accurate are these breaks? There are two elements. One is the actual targeting and that seem to be excellent. The other would be the equivalent of a false targeting. Namely targeting an identical string at the wrong place.

6.5.2 Promoters and Other Interactions

We know that just having the right gene does not mean that it is expressed. Thus promoters and similar interactions must be considered. Gene expression is oftentimes a complicated process.

6.5.3 Methylation Factors

Methylation is well known to play active roles in gene expression. In the cut and paste mode we may change methylation profiles. This could dramatically change gene expression, since one gene product may be another gene promoter.

6.5.4 Other Epigenetic Factors

There are many other epigenetic factors including acetylation, miRNA, lncRNAs and the like. It is uncertain how these factors can be influenced in this process.

6.6 SOME QUESTIONS

The CRISPR-Cas9 system has proven to be a workable in vivo editing mechanism for specific gene cut and paste situations. However there are several key questions that seem to hang over it. None are so severe as to cause substantial concern but in toto they clearly indicate potential but substantial work is still required, especially in the area of cancer therapeutics.

We thus present and discuss several such questions:

1. Can the CRISPR-Cas9 system target the correct sets of aberrant cancer genes?

The issue here is that in many cancers we have a multiple set of genes which are aberrant. To make it even more complex, there are cancer cells with different mixtures of mutated or inoperable genes. How thus does one target this broad and varying complex. A single genetic mutation is one thing an broad complex set of changes is another.

2. Can CRISPER-Cas9 system be delivered in vivo in a more effective manner?

The current delivery mechanism is targeted at specific cells in a specific location. What does one do with a metastatic cancer. Oftentimes you do not even know where the cells may be. Then again one also faces the issue of the stem cell and it special characteristic. This delivery most likely be difficult.

3. Is CRISPR-Cas9 a dose related system approach rather than an all-encompassing curative approach? Namely does it cut-and-paste a large set of genes but perhaps not all?

Is the delivery system akin to dosing in normal pharmacokinetics or is it a totally different mechanism.

4. How does CRISPR-Cas deal with metastatic cells wherein there are multiple sets of genetic alterations?

The multiplicity of gene breakdowns and the process in which this happens becomes a complex driver for applying this technology. Is there a single key to solving the problem or must one continue to track changes and chase the shadows of the genetic changes?

5. What are the potential deleterious sequellae possible from a CRISPR approach and how can they best be avoided?

The ultimate question will be what else this process can do. The unintended consequences may be significant. As was noted above:

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene.

What then are those mistakes which can occur, especially when targeting multiple genes?

7 APPENDIX: TERMS AND DEFINITIONS

Term	*Definition*
Cas9[10]	CRISPR-associated (Cas) proteins to direct degradation of complementary sequences present within invading viral and plasmid DNA. The *Streptococcus pyogenes* SF370 type II CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two noncoding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs)
CRISPR	Clustered regularly interspaced short palindromic repeats.
crRNA	CRISPR RNA
tracrRNA[11]	In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded*** (ds) NAspecific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein
sgRNA[12]	Type II CRISPR–Cas systems have been adapted as a genome-engineering tool. In this system, crRNA teams up with a second RNA, called trans-acting CRISPR RNA (tracrRNA), which is critical for crRNA maturation and recruiting the Cas9 nuclease to DNA. The RNA that guides Cas9 uses a short (20-nt) sequence to identify its genomic target. ***This three-component system was simplified by fusing together crRNA and tracrRNA, creating a single chimeric ‘‘guide’’ RNA abbreviated as sgRNA or simply gRNA.***
PAM[13]	The CRISPR locus is transcribed and processed into short CRISPR RNAs (crRNAs) that guide the Cas to the complementary genomic target sequence. There are at least eleven different CRISPR– Cas systems, which have been grouped into three major types (I–III). In the type I and II systems, nucleotides adjacent to the protospacer in the targeted genome comprise the protospacer adjacent motif (PAM). The PAM is essential for Cas to cleave its target DNA, enabling the CRISPR–Cas system to differentiate between the invading viral genome and the CRISPR locus in the host genome, which does not incorporate the PAM. For additional details on this fascinating prokaryotic adaptive immune response.
Homologous	Homologous chromosomes are a set of one maternal chromosome and one

[10] Jinek et al
[11] Jinek et al
[12] Harrison et al
[13] Harrison et al

Term	*Definition*
	paternal chromosome that pair up with each other inside a cell during meiosis.
RuvC[14]	RuvC is the resolvase, which cleaves the Holliday junction. It is thought to bind either on the open, DNA exposed face of a single RuvA tetramer, or to replace one of the two tetramers. Binding is proposed to be mediated by an unstructured loop on RuvC, which becomes structured on binding RuvA. RuvC can be bound to the complex in either orientation, therefore resolving Holliday junctions in either a horizontal or vertical manner. Cas9 contains domains homologous to both HNH and RuvC endonucleases.
HNH[15]	The domain HNHc (SMART ID: SM00507, SCOP nomenclature: HNH family) is associated with a range of DNAbinding proteins, performing a variety of binding and cutting functions. Several of the proteins are hypothetical or putative proteins of no well-defined function. The ones with known function are involved in a range of cellular processes including bacterial toxicity, homing functions in groups I and II introns and inteins, recombination, developmentally controlled DNA rearrangement, phage packaging, and restriction endonuclease activity Cas9 contains domains homologous to both HNH and RuvC endonucleases

[14] Bennett and West
[15] Mehta et al

8 REFERENCES

1. Baltimore, D., et al, A prudent path forward for genomic engineering and germline gene modification, Science Express 19 March 2015.
2. Bennett, R., S. West, RuvC protein resolves Holliday junctions via cleavage of the continuous (noncrossover) strands, Proc. Natl. Acad. Sci. USA, Vol. 92, pp. 5635-5639, June 1995, Biochemistry
3. Brouns, S., A Swiss Army Knife of Immunity, Science, 17 AUGUST 2012 VOL 337.
4. Cain, A., C. Boinett, A CRISPR view of genome sequences, Nature Reviews, APRIL 2013, VOLUME 11.
5. Cong, L., et al, Multiplex Genome Engineering Using CRISPR/Cas Systems, Science, VOL 339 15 FEBRUARY 2013.
6. Cox, Therapeutic genome editing: prospects and challenges, NATURE MEDICINE VOLUME 21 | NUMBER 2 | FEBRUARY 2015.
7. Doudna, J., E. Charpentier, The new frontier of genome engineering with CRISPR-Cas9, Science, 28 NOVEMBER 2014 • VOL 346 ISSUE 6213
8. Gantz, Bier, The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations, Science Express, 19 March 2015
9. Gilbert, L., et al, CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes, Cell 154, 442–451, July 18, 2013.
10. Harrison, M., et al, A CRISPR view of development, GENES & DEVELOPMENT 28:1859–1872, 2014.
11. Horvath, P.; Barrangou, R. (2010). "CRISPR/Cas, the Immune System of Bacteria and Archaea". Science 327 (5962): 167–170.
12. Hsu, P., et al, Development and Applications of CRISPR-Cas9 for Genome Engineering, Cell 157, June 5, 2014
13. http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html
14. http://www.sigmaaldrich.com/technical-documents/articles/biology/crispr-cas9-genome-editing.html?cm_mmc=Google_Biomolecules-_-search-_-CRISPR_CRISPR-_-cas9_Broad&cm_guid=1-_-10000000000000038482-_-42705054918
15. Jia, H, Wang N (2014) Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA. PLoS ONE 9(4): e93806. doi:10.1371/journal.pone.0093806 .
16. Jinek, M., et al, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity, Science 337, 816 (2012).
17. Jinek, M., et al, Structures of Cas9 Endonucleases Reveal RNA-Mediated Conformational Activation, Science, 343, (2014).

18. Konermann, S., et al, Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex, 2 9 J A N U A RY 2 0 1 5 | VO L 5 1 7 | N AT U R E | 5 8 3
19. Makarova, K., et al, A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action, Biology Direct2006, 1:7 doi:10.1186/1745-6150-1-7.
20. Mali, P., et al, Cas9 as a versatile tool for engineering biology, nature methods, VOL.10 NO.10, OCTOBER 2013.
21. Mali, P., et al, RNA-Guided Human Genome Engineering via Cas9, SCIENCE VOL 339 15 FEBRUARY 2013
22. Marraffini L., E. Sontheimer, Self versus non-self discrimination during CRISPR RNA-directed immunity, Nature, Vol 463|28 January 2010
23. Marraffini L., E. Sontheimer, CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea, Nat Rev Genet. 2010 March ; 11(3).
24. McGarty, T., CRISPR and Cancer, Telmarc White Paper No 111, April 2014, http://www.telmarc.com/Documents/White%20Papers/111%20CRISPR%20and%20Cancer.pdf
25. McGarty, T., Prostate Cancer Genomics, DRAFT, http://www.telmarc.com/Documents/Books/Prostate%20Cancer%20Systems%20Approach%2003.pdf
26. McGarty, T., Melanoma Genomics, DRAFT, http://www.telmarc.com/Documents/Books/Melanoma%20Genomics%2007.pdf
27. Mehta, P., et al, HNH family subclassification leads to identification of commonality in the His-Me endonuclease superfamily, Protein Science (2004), 13:295–300.
28. Meijer, et al, Nucleotide sequence of the origin of replication of the Escherichia coli K-12 chromosome, Proc. Natl. Acad. Sci. USA, Vol. 76, No. 2, pp. 580-584, February 1979
29. Oost, J., New Tool for Genome Surgery, Science, Science 339, 768 (2013).
30. Port, F. et al, An optimized CRISPR/Cas toolbox for efficient germline and somatic genome engineering in Drosophila, bioRxiv posted online March 24, 2014, Access the most recent version at doi: 10.1101/003541
31. Qi, L. et al, Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression, Cell 152, 1173–1183, February 28, 2013.
32. Ran, F., et al, Genome engineering using the CRISPR-Cas9 system, Nature Protocols | VOL.8 NO.11 | 2013 | 2281
33. Ran, F., et al, In vivo genome editing using Staphylococcus aureus Cas9, Nature, 520, 186–191, (09 April 2015)
34. Randow et al, Cellular Self-Defense: How Cell-Autonomous Immunity Protects Against Pathogens, Science, VOL 340 10 MAY 2013.

35. Shalem, O., et al, Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells, Science Express, 12 December 2013.

36. Sontheimer, E., L. Marraffini, Slicer for DNA, 4 November 2010 VOL 468 Nature 4 5

37. Stephen, A., et al, Dragging Ras Back in the Ring, Cancer Cell 25, March 17, 2014.

38. Trevino, A., F. Zhang, Genome Editing Using Cas9 Nickases, Methods in Enzymology, Elsevier, Volume 546, 2014

39. \van der Oost, J., New Tool for Genome Surgery, Science 339, 768 (2013)

40. Villion, M., S.. Moineau, The double-edged sword of CRISPR-Cas systems, Cell Research (2013) 23:15-17.

41. Wang, T., et al, Genetic Screens in Human Cells Using the CRISPR/Cas9 System, Science Express, 12 December 2013.

42. Watson, J., et al, Molecular Biology of the Gene 5th Ed , Pearson, 2004.

43. Way, J., et al, Integrating Biological Redesign: Where Synthetic Biology Came From and Where It Needs to Go, Cell 157, March 27, 2014.

44. Yin, H., et al, Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype, Nature Biotechnology, (2014).

45. Zhang, F., et al., CRISPR/Cas9 for genome editing: progress, implications and challenges, Hum. Mol. Genet. (2014).

46. ZhangLab, MIT, http://zlab.mit.edu/publications.html