Precise Genome Engineering and The CRISPR Revolution
(Boldly Going Where No Technology Has Gone Before)

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ABSTRACT

Precise genome engineering in human cells has two general applications of importance and wide interest. One is the inactivation of genes ("knockouts"), a process utilized to delineate the loss-of-function phenotype(s) of a particular gene. The second application ("knock-ins") is essentially the process of gene therapy, which involves correcting a pre-existing mutated allele(s) of a gene back to wild-type in order to ameliorate some pathological phenotype associated with the mutation. Both of these processes require a form of DNA double-strand break repair known as homologous recombination. Although bacteria and lower eukaryotes utilize homologous recombination almost exclusively, a competing process, known as non-homologous end joining, predominates in higher eukaryotes and was presumed to prevent the use of precise genome engineering in human somatic cells in culture. A series of molecular and technical advances developed in the 1990's disproved this notion, but still resulted in a process that was cumbersome, labor-intensive, highly inefficient and slow. In the early 2000s, the use of new gene delivery vectors such as recombinant adeno-associated virus and the identification of programmable nucleases such as zinc finger nucleases and transcription activator-like effector nucleases significantly brightened the outlook for this field and resulted in gene delivery systems that facilitated both gene knockouts and gene therapy modifications at reasonable levels. In 2013, however, a new programmable nuclease, clustered regularly-interspersed short palindromic repeats/CRISPR-associated 9 was described that has revolutionized the field and made precise genome engineering accessible to anyone with even a rudimentary knowledge of

molecular biology. Thus, precise genome engineering in a wide variety of model organisms, as well as human somatic cells in culture, has become not only feasible, but also effortless, and it harbingers a golden age for directed mutagenesis.

I. INTRODUCTION

All researchers, who have been in science for a long time are able to recall technological discoveries that substantially improved or simplified their day-to-day laboratory existence. Examples such as Southern blotting for detecting specific fragments of DNA (Southern, 2006) or the yeast two hybrid for the identification of protein:protein interactions would fall into this category (Fields and Song, 1989). Much less frequently, there are technological discoveries that result in advancements not just for some particular field(s), but literally change the way that science is being performed everywhere. The description in 1975 by Georges Kohler and Cesar Milstein of the production of hybridomas and their ability to produce monoclonal antibodies certainly ranks in this category (Kohler and Milstein, 1975). The sudden ability to identify any protein in any model organism was profoundly useful to researchers world-wide. As influential, and perhaps even more transformative, was the discovery and development in the mid- to late-1980s by Kary Mullis of the polymerase chain reaction (PCR) (Saiki et al., 1985). At that time most molecular biologists spent vast amounts of their day-to-day life isolating and grafting small fragments of DNA — usually grown up in bacteria or isolated out of polyacrylamide gels — to one another to create some useful recombinant DNA molecule. PCR seismically altered the way that almost all researchers did science such that it was now possible to perform cloning experiments at a rate that was exponentially faster and with a precision that was orders of magnitude better.

Despite these reoccurring breakthroughs that permeate and drive scientific advancements, it is still difficult to comprehend and appreciate the enormity of the metaphorical tsunami of excitement and change that has resulted from the application in 2013 of clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) to the field of

mammalian precise genome engineering. In the past 3 years, there has been a nearly unfathomable outpouring of publications (now averaging 6 per day), review articles (this review will certainly be just one of hundreds published this year on CRISPR/Cas9), major workshops and scientific conferences (1 to 2 per month in every corner of the globe), discussions in the general press (including multiple feature editorials in the New York Times), even an international summit organized by a bevy of Nobel laureates [to lobby in on the ethics of human gene editing (Baltimore et al., 2015; Liang et al., 2015)] and finally its designation by the journal Science as "Breakthrough of the Year" (Travis, 2015). If scientific discoveries were solar flares, CRISPR/Cas9 would be bigger than the 1859 Carrington Event.

As will be discussed below, CRISPR/Cas9 is, in its simplest incarnation, just an RNA programmable restriction enzyme (Figure 1). At first reflection, this does not seem to be that earth shattering of an activity nor a particularly useful one. The limitation of restriction enzymes, however, is that their recognition sequence is relatively small: usually 4 or 6 base pairs. As a consequence of which, all classic restriction enzymes recognize so many sites in the human genome that their utility is extremely constrained. Cas9, however, is dependent upon a ~100 nt long (which includes a ~20 nt long targeting stretch) single guide RNA (sgRNA) molecule. Because of the stability of complementary RNA:DNA
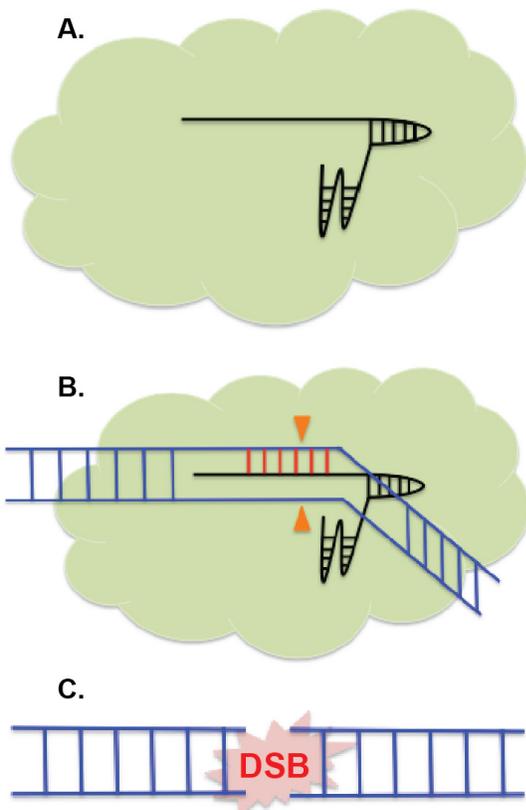

Figure 1. How CRISPR/Cas9 works. A. A cartoon of the Cas9 RNP, which consists of Cas9 (green cloud) protein and the sgRNA (black line). Short black lines represent RNA:RNA base pairing. B. Cas9 in action. dsDNA is shown as blue lines and short blue lines represent DNA:DNA base pairing. The short red lines represent DNA:RNA base pairing. The two orange triangles represent the two active sites of Cas9. C. The result of Cas9 action. A dsDNA molecule is shown with blunt ends because of the DSB.

hybridization and because of the length of the RNA molecule, each Cas9 is essentially directed

to one and only one (although see below for qualifications concerning this attribute) location in the human genome. It is this ability — the ability to do meaningful chemistry at a precisely defined locus in the human genome — that provides CRISPR/Cas9 with its unique usefulness. For the purposes of this review, I will provide a brief overview of the pathways relevant for precise genome engineering and then give a short history of the field. In the main part of the review, I will attempt to concisely summarize some of the areas where CRISPR/Cas9 has been put to relatively spectacular uses. Finally, I will end with a speculative section on the potential usefulness for space exploration. As mentioned above, there are literally hundreds of relevant and very current reviews [*e.g*., (Jiang and Marraffini, 2015; Ledford, 2016; Wright et al., 2016)] of either the CRISPR/Cas9 technology, its application or its ethical implications (Kuzma, 2016) and the reader is encouraged to seek those reviews out for further insight into this topic.

II. OVERVIEW

Precise genome engineering is the intentional modification of a genetic locus in a living cell [reviewed by (Hendrickson, 2008; Song et al., 2014)]. This technology has two general applications of importance and wide interest. One is the inactivation of genes ("**knockouts**"), a process in which the two wild-type alleles of a gene are mutated in order to delineate the loss-of-function phenotype(s) of that particular gene. The second application is the clinically more relevant process of **gene therapy**, which, in its strictest sense, involves correcting a pre-existing mutated allele(s) of a gene back to wild-type in order to ameliorate some pathological phenotype associated with the mutation [(Gammon, 2014; Ledford, 2015a); a "**knock-in**"]. Importantly, although these applications are — at the DNA level — reciprocal opposites of one another, they are mechanistically identical and utilize the same four basic steps: 1) a **search** for homologous sequences between the incoming donor DNA and the chromosomal DNA; 2) **breakage** (usually in the form of **double-stranded breaks; DSBs**) of the DNA at the site of targeting; 3) **exchange** of DNA/genetic information between the donor DNA and the chromosomal DNA; 4) **ligation** of

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the broken chromosome to restore its structural integrity. Altogether, these four steps define a process referred to as **homologous recombination (HR)** or alternatively, **homology directed repair (HDR)**, which is absolutely required for gene targeting to occur [reviewed by (Kowalczykowski, 2015; Prakash et al., 2015)]. Although HDR was known to predominate in bacteria and lower eukaryotes, the competing process of **non-homologous end joining (NHEJ)** [reviewed by (Fell and Schild-Poulter, 2015; Woodbine et al., 2014)], in which the incoming donor DNA is randomly integrated within the genome, predominates in higher eukaryotes and was presumed to prevent the use of gene targeting in human somatic cells in culture. A series of molecular and technical advances disproved this notion, but still resulted in a process that was cumbersome, labor-intensive, highly inefficient and slow (Sedivy et al., 1999). Within the past decade, the use recombinant adeno-associated virus [**rAAV**; reviewed by (Khan et al., 2011; Xiao et al., 2012)], zinc finger nucleases [**ZFNs**; (Carroll, 2011)] and transcription activator-like effector nucleases [**TALENs**; (Bogdanove and Voytas, 2011)] significantly brightened the outlook for this field and resulted in gene delivery systems that facilitated both gene knockouts and gene therapy modifications at reasonable and clinically-relevant levels. The discovery and application of **CRISPR/Cas9** technology, however, has resulted in a "go, at throttle up" moment that has resulted — not in a disastrous implosion of the field — but in a spectacular boost of the field into the stratosphere. Thus, gene targeting in human somatic cells in culture and in model organisms has become not only feasible, but also almost effortless, and it harbingers a golden age for directed evolution.

III. BACKGROUND

III A. NON-HOMOLOGOUS END JOINING

A double-stranded piece of linear DNA introduced into a mammalian cell can be incorporated into that cell's genome by either HDR or NHEJ [reviewed by (Schipler and Iliakis, 2013)]. Bacteria and lower eukaryotes utilize almost exclusively HDR for the uptake of foreign

DNA. In higher eukaryotes, however, integration proceeds more frequently by a process that does not require extended regions of homology. Specifically, mammalian cells — and humans in particular — have evolved a highly efficient ability to join nonhomologous DNA molecules together [(Roth and Wilson, 1985); reviewed by (Symington and Gautier, 2011)]. In their seminal work on gene targeting, Capecchi and co-workers showed that although somatic mammalian cells can integrate a linear duplex DNA into corresponding homologous chromosomal sequences using HDR, the frequency with which recombination into nonhomologous sequences occurred was at least 1000-fold greater (Thomas and Capecchi, 1987). This NHEJ pathway, often referred to as the "classical" or C-NHEJ pathway, appears to be predominately active during the G_1/early S phase of the cell cycle (Lee et al., 1997; Takata et al., 1998). In the last decade, it has been appreciated that there is at least one additional sub pathway of NHEJ [often referred to as "alternative" NHEJ or A-NHEJ; (Haber, 2008; Iliakis, 2009)] that is operational in mammalian cells. Given that NHEJ DSB repair (especially A-NHEJ) can be error-prone (Betermier et al., 2014), an attribute that bacteria and lower eukaryotes can ill-afford; the increased percentage of noncoding DNA in higher eukaryotes may have facilitated the evolution of this pathway. In summary, mammals are different from bacteria and lower eukaryotes in that DSB repair proceeds primarily through NHEJ recombinational pathways. Moreover, NHEJ must be overcome in order to facilitate gene targeting and this can occur only when the incoming DNA is shunted into the HDR pathway.

III B. HOMOLOGY DEPENDENT REPAIR

In HDR [reviewed by (Symington and Gautier, 2011)], the DNA ends of the incoming DNA are resected to yield 3'-single-stranded DNA overhangs. The resulting overhangs are then coated by **Rad51** (<u>rad</u>iation sensitive <u>51</u>). Rad51 is the key strand exchange protein in HR [reviewed by (Jasin and Rothstein, 2013)]. It is essential for the homology searches on the target DNA — *i.e*., the entire human genome — that are required to localize the incoming DNA to its

specific, cognate chromosomal counterpart. In humans, there are at least seven Rad51 family members and almost all of them have been implicated in some aspect of HDR and also in human disease (Thacker, 2005). Strand invasion into the homologous chromosomal sequence likely requires a bevy of accessory proteins and ultimately results in a cross-stranded intermediate first described by Robin Holliday (Holliday, 1964) and named accordingly in his honor. Gene targeting generates a complex structure with multiple (usually 2 to 4) **Holliday junctions** that is essentially identical to the linearized plasmid recombination intermediates that were extensively defined in yeast (Hastings et al., 1993; Langston and Symington, 2004). Ultimately, the resolution of this structure probably requires the participation of helicases of the RecQ (recombination defective Q) family [reviewed by (Bizard and Hickson, 2014)] and certainly requires the action of Holliday junction resolvase(s) to repeatedly nick the strands [reviewed by (Wyatt and West, 2014)]. The resolution of the cross-stranded intermediates with crossovers generates a modified chromosome in which the original chromosomal sequences have been replaced by sequences present on the incoming donor DNA. As is the case with NHEJ, there are also sub pathways of HDR that likely funnel intermediates into non-productive (in terms of gene targeting) products and the use of these pathways is likely highly cell cycle (Orthwein et al., 2015) and tissue (Jasin and Rothstein, 2013) variable. In summary, human somatic cells express all the gene products needed to carry out gene targeting. These events occur, however, at very low frequency due to the preferred usage of NHEJ and alternative pathways of HDR.

III C. A Brief History Of Precise Genome Engineering

Bacteria and especially yeast owe their popularity as model systems to the ease with which researchers can carry out forward genetic screens <u>and</u> use reverse genetics to precisely alter their genomes. In higher organisms, forward genetic screens are logistically (or ethically) often impractical and thus reverse genetic approaches are the only functional way to carry out the search for new genes and/or genome modifications. The first published report of gene targeting

in human cells occurred just over three decades ago when Smithies *et al*. attempted to disrupt the β-globin locus in the human EJ bladder carcinoma cell line (Smithies et al., 1985). No correctly targeted cell lines were obtained, but the same authors were subsequently able to successfully target the human β-globin locus in a human:mouse hybrid cell line. Several years later, Jasin *et al*. reported the first isogenic targeting of an endogenous locus (the CD4 gene) in a completely human (the T-lymphocyte JM) cell line (Jasin et al., 1990). Although valuable as proof-of-principle experiments, the reported frequency of 10^{-7} to 10^{-8} made this approach beyond daunting from a practical point of view except for the most masochistic (or stubborn) of researchers. The development of viral vectors (such as rAAV) to facilitate gene targeting greatly improved on these frequencies but were not widely adopted because they required some knowledge of virology and were still quite cumbersome in their design [reviewed by (Khan et al., 2011; Xiao et al., 2012)]. Taking a cue from studies done earlier in yeast, the laboratory of Maria Jasin subsequently made the important demonstration that the introduction of a DSB at the chromosomal locus of interest increased the frequency of gene targeting to a now useful 10^{-3} to 10^{-4} (Rouet et al., 1994; Smih et al., 1995). Again, while valuable as proof-of-principle, the technical problem with these experiments is that they were carried out with a fungal meganuclease that had no naturally occurring sites in the human genome. To be of utility for precise genome engineering, a researcher needs to be able to direct his DSB precisely (often to the nucleotide) to his/her chromosomal locus of interest. Thus, the field scrambled to devise programmable nucleases, which ultimately resulted in the early 2000's in the development and use of ZFNs (Carroll, 2011). While some ZFNs proved quite useful, many were difficult to produce and then showed only marginal and/or promiscuous cleavage activity. The field received in 2011 what looked like a remarkable breakthrough with the identification and use of TALENs (Bogdanove and Voytas, 2011). TALENs were easier to produce than ZFNs and appeared to have improved cleavage specificity. Unfortunately, TALEN production was still rather laborious and compounded by the fact that a unique TALEN needed to be manufactured for every site that a researcher wanted to engineer. Thus, when it was demonstrated in 2013 that
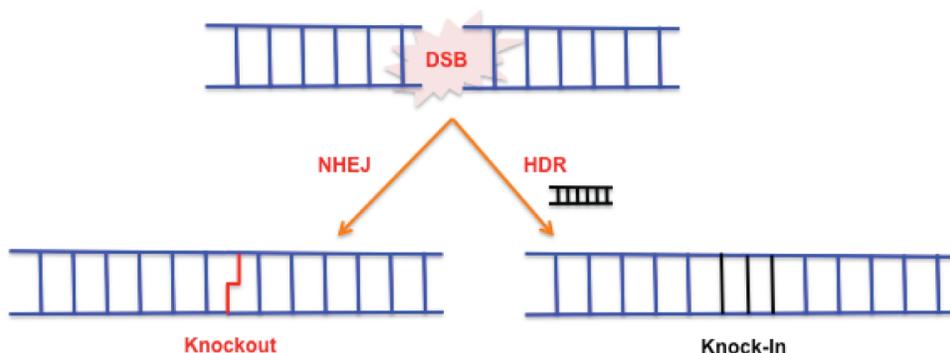
CRISPR/Cas9, which had been well-documented to be a RNA-programmable nuclease that bacteria used as a primitive adaptive immune system (Wright et al., 2016), could be re-programmed to work in mammalian cells the last major barrier to efficient human cell precise genome engineering was hurdled (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). In the intervening 3 years CRISPR/Cas9 has been utilized by thousands of laboratories to carry out a myriad of interesting biology and it is clearly the preferred molecular tool to carry out just about any reverse genetic experiment that can be envisioned.

IV. THE MANY USES OF CRISPR/CAS9

IV A. KNOCKOUTS

In the three decades of precise genome engineering preceding the development of CRISPR/Cas9 as a gene editing tool approximately 150 genes in the human genome had been modified by combination of simple DNA transfections, rAAV infections or through ZFN- or TALEN-mediated gene targeting (Hendrickson, 2008). In just three short years, the development of CRISPR/Cas9 has enlarged this number to essentially every gene in the human genome (~20,000). Needless to say, characterization of all of these modifications has lagged behind their generation, but it is only a matter of time before a phenotypic characterization of these modifications occurs.

The simplest, but often most important, modification that a researcher can make is a knockout of a gene of interest. This type of mutation allows a researcher to assess the loss-of-function phenotype of a gene. CRISPR/Cas9 accomplishes this exactly the way that ZFN- and TALEN-mediated modification did: by relying on the abundant

Figure 2. DSB processing in human cells. When a chromosome suffers a DSB, it is most frequently repaired by NHEJ. Because NHEJ can be imprecise, nucleotide changes in the form of indels (red jagged line) are often incorporated at the site of joining, causing frameshift mutations if the site of the break is within an exon, *i.e.* a "knockout". At a much lower frequency, the DSB can be repaired by HDR. In this process, a donor DNA is required, which in the case of precise genome engineering can be a piece of DNA of the investigator's choosing. Some of the genetic information on the donor DNA will be incorporated into the repaired chromosome in what is referred to as a "knock-in".

cellular NHEJ systems to inaccurately repair the resulting DSB (Figure 2). Thus, the empirical observation is that nucleotide alterations (insertions and deletions) occur at high frequency at the sites of CRISPR/Cas9 cleavage (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). If the locus of editing is an exon, these alterations invariably cause frameshift mutations that inactive the gene of interest. The great improvement that CRISPR/Cas9 provides over the earlier technologies is two-fold. First, it is so efficient that often biallelic modification occurs. Thus, rAAV-, ZFN- or TALEN-mediated strategies invariably resulted in only one of two homologous chromosomes in a diploid mammalian cell becoming modified. This required isolation and characterization of the intermediate heterozygous cell line, before an entirely new round of transfection/infection could be done to obtain null cell lines. In contrast, CRISPR/Cas9 is so efficient that modification of both chromosomes occurs simultaneously at high frequency (Cong and Zhang, 2015).

The fact that CRISPR/Cas9 can make biallelic mutations at high frequency has an additional advantage: it has allowed for the construction of whole genome knockout libraries. Thus, researchers over a decade ago constructed knockout libraries for every non-essential gene in the yeast genome. These knockout libraries allowed yeast researchers to ask global questions about cellular regulation that were simply not possibly when single mutants were analyzed one at a time (Myers et al., 2011; Piotrowski et al., 2015; Szappanos et al., 2011). The construction of the yeast knockout libraries was, however, vastly simplified by the haploid nature of yeast, where disruption of only a single allele was needed to obtain a null phenotype. Similar libraries for higher eukaryotic model systems (*e.g.*, zebrafish, mice, human somatic cells) were deemed impractical because the diploid nature of these organisms would require biallelic disruption to reveal phenotypically recessive traits. In 2014, taking advantage of the efficacy of CRISPR/Cas9, CRISPR-based whole genome knockout libraries for human somatic cells were described by two groups (Shalem et al., 2014; Wang et al., 2014). These groups constructed lentiviral vectors that permitted the expression of ~70,000 different sgRNAs capable of inactivating 18,000 human genes. The libraries and technology have been used successfully to

identify novel genes involved in oncogene inhibition (Shalem et al., 2014), mismatch DNA repair and topoisomerase poison sensitivities (Wang et al., 2014) and TNF signaling (Parnas et al., 2015). In addition, smaller versions of the libraries have been used to elucidate the cellular responses to anthrax and diphtheria toxin (Zhou et al., 2014). In conceptually parallel work, the ~2,000 <u>essential</u> genes in the human genome were identified by using CRISPR/Cas9 to inactivate every human gene and then characterize the genes that were depleted in the screen (Boone and Andrews, 2015; Wang et al., 2015). These types of unbiased, whole-genome screens are completely poised to revolutionize human somatic cell genetics.

The second big improvement is that CRISPR/Cas9 can be multiplexed. Since Cas9 is regulated at the level of a guide RNA and each sgRNA is only ~100 nt long, multiple sgRNAs to multiple targets can be expressed at the same time facilitating simultaneous, biallelic conversions. Thus, the construction of doubly- or triply- or multiply-modified genetically modified cells/organisms, which previously would have taken years to painstakingly construct, can now be done in a single, quick experiment. Some of the reported successes have been rather spectacular. In one instance, 5 different genes in mouse embryonic stem cells were simultaneously modified (Wang et al., 2013), most of them biallelically. More recently all 62 (!!) endogenous retroviruses in a porcine cell were coordinately inactivated (Yang et al., 2015) in an effort to derive pigs more suitable for human xenotransplantation.

IV B. KNOCK-INS

The efficacy of generating knockout mutations using CRISPR/Cas9 is exceptionally high. This outcome is the direct result of two factors: the high efficiency of cleaving genomic DNA by CRISPR/Cas9 and the predominant use of error-prone NHEJ in higher eukaryotes. In contrast, the ability to engage in precise genome engineering, where a genome is altered to exactly the researcher's choosing, is generally referred to as a knock-in mutation. This type of modification is extremely attractive to gene therapists and to anyone trying to precisely alter a genome with

the goal of achieving specific phenotypic outcomes. Knock-in mutations, however, rely not on NHEJ, but HDR (Figure 2). As a consequence, achieving knock-in mutations in higher eukaryotes is a low frequency event. Indeed, the frequency of generating knock-in mutations using rAAV (Hurley et al., 2007), ZFNs (Cui et al., 2011), TALENs (Miller et al., 2011) and even CRISPR/Cas9 (Wang et al., 2013; Yang et al., 2013) is generally in the range of only a few percent and suggests that the rate-limiting step is not nuclease-dependent genomic cleavage, but the low frequency of HDR. As a consequence there have been recent reports in which investigators have used either small molecule inhibitors of NHEJ (Chu et al., 2015; Maruyama et al., 2015) or activators of HDR (Song et al., 2016) to enhance CRISPR/Cas9-mediated knock-ins. Because of the anticipated utility of precise genome engineering, a concerted effort is on-going in many laboratories to improve these preliminary modifications and develop novel approaches focusing either on delivery of the donor DNA or modulation of the cellular DSB repair systems.

An additional important application of knock-in technology is not in altering the primary sequence of a gene, but in appending onto the gene (generally at the N- or C-terminus) a useful epitope (*aka*, **epitope-tagging**). Epitope-tagging (using a bevy of either fluorescent tags or epitopes readily recognized by commercially available antibodies) has a myriad of uses including, but certainly not limited to, 1) determining the cellular location or the cell cycle regulated expression of a protein, 2) permitting immunological detection of a protein that is inherently non-immunogenic, 3) generating a protein that can be used in chromatin immunoprecipitation or similar assays and 4) generating proteins whose expression can be tightly and reversibly regulated (Brizzard, 2008; Chung et al., 2015; Vandemoortele et al., 2016). Again, the utility of CRISPR/Cas9 to make precise DSBs in the human genome where cassettes containing these useful epitopes can be inserted in-frame, has greatly enhanced this technology (Lackner et al., 2015).

IV C. EVEN A "DEAD" CAS9 CAN BE EXCEPTIONALLY USEFUL — CRISPRA AND CRISPRI

In its native conformation, Cas9 is a double-stranded nuclease that makes a blunt DSB at a target locus. It does so through the concerted action of two catalytic cores that coordinately make juxtaposed single-stranded breaks on opposing strands (Sternberg et al., 2015). Molecular biologists have taken advantage of this fact to produce Cas9 variants in which either one of the active sites has been mutated (referred to as "Cas9 nickase"; see below) or both active sites have been mutated (referred to as "dead Cas9" or "dCas9"). dCas9 has no DNA cleavage activity, an activity that this review has been extolling from the introductory sentence. In spite of this — or, actually, more accurately, because of this — dCas9 has proven exceptionally useful as a genome-engineering tool as well. Whereas dCas9 cannot cleave DNA, it still can localize (via the information encoded in the sgRNA) with pinpoint accuracy to any location within a genome. Researchers have taken advantage of this feature to engineer and then express chimeric versions of dCas9 that are fused to either transcriptional activation domains (CRISPR activation or **CRISPRa**) or transcriptional repression domains (CRISPR inhibition or **CRISPRi**) to selectively turn individual genes on or off, respectively [(Larson et al., 2013; Qi et al., 2013); reviewed in (Dominguez et al., 2016; La Russa and Qi, 2015)] (Figure 3). The elegance of these systems allows for complicated manipulation of a transcriptional process without physically altering the genomic DNA sequence. An additional attraction is that the systems are often fully reversible by using either light or small molecules to regulate the transcriptional activation/inhibition (Dominguez et al., 2016). Much of this technology is still being developed, but essentially any epigenetic modification (*e.g.*, histone methylation, histone acetylation or protein ubiquitination) for which the enzymatic activity has been biologically defined can be recapitulated with the CRISPRi/CRISPRa systems.

IV D. IS CRISPR/ CAS9 SAFE TO USE?

In its native conformation, CRISPR/Cas9 efficiently makes a DSB at a target locus. However, it is perhaps one of the few inviolate rules of biology that no biological process works perfectly all the time. Thus, almost simultaneously with the joyous hullabaloo that accompanied the initial successes with CRISPR/Cas9 came equally vocal, seasoned rejoinders of caution. In particular, it is well appreciated by radiation biologists everywhere that even a single mis-repaired or unrepaired DSB is either mutagenic or lethal, respectively. Thus, if CRISPR/Cas9 makes DSBs at even a low frequency at off-target sites, the ris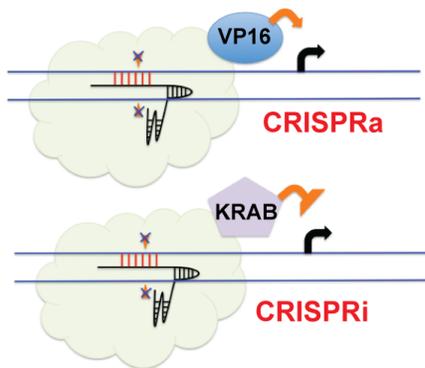k for unwanted genome alterations and/or toxicity is great. In particular, for basic research applications such off-target effects could certainly confound data interpretations and/or lead to spurious phenotypes whereas in clinical research applications such off-target effects could be outright lethal. Indeed, some early publications suggested that ancillary DSBs could not only occur but that in rare instances they could occur so often as to negate the utility of the system (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). Most of these critical reports, however, proved not to be generalizable.

Figure 3. Uses for dCas9. The small blue "x's" over the orange triangles indicate that the catalytic cores of Cas9 have been inactivated and the enzyme can no longer cleave DNA — but very importantly — it can still bind DNA with high specificity. Cas9 can be fused with a variety of transcriptional activators (VP16 as a blue oval is cartooned). In this configuration, when Cas9:VP16 binds it will augment the transcription of nearby genes (CRISPRa). Alternatively, Cas9 can be fused with a variety of transcriptional repressors (KRAB as a purple pentagon is cartooned). In this configuration, when Cas9:KRAB binds it will silence the transcription of nearby genes (CRISPRi).

Indeed, the empirical observation that live mice could be generated at very high frequency from CRISPR/Cas9-modified mouse embryonic stem cells demonstrated that if off-target modifications were occurring, they were happening at such a low level as to not impede the very sensitive process of development (Wang et al., 2013; Yang et al., 2013). Subsequently, better computational programs for designing sgRNAs (Doench et al., 2014), a better understanding of the specificity attributes of the targeting sequence within the sgRNA (Knight et al., 2015) and how the sgRNA interacts with the target sequence (Sternberg et al., 2014) greatly improved the

efficacy for cleavage at the desired locus although extremely sensitive whole genome sequencing applications could still detect rare off-target activity (Tsai et al., 2015). Most recently, however, two groups independently reported the development of molecularly-evolved variants of Cas9 with enhanced binding and cleavage specificities such that these second-generation Cas9s had virtually no detectable (even by sensitive whole genome sequencing protocols) off-target effects (Kleinstiver et al., 2016; Slaymaker et al., 2016; Urnov, 2016). While the re-introduction of genetically-modified cells into human patients will always demand technology that is as perfect as can be humanely obtained, it appears as if CRISPR/Cas9 will be up to that rigorous challenge.

An additional noteworthy variation that came out of attempts to limit the off-target effects of CRISPR/Cas9 was the development of Cas9 variants that only nick genomic DNA (nickase Cas9, **nCas9**) (Ran et al., 2013). Thus, if only one of the two active nuclease sites is mutated, the resulting Cas9 still retains the ability to nick one of the two strands. Because nicks are not DSBs (and are likely not all that frequently converted to DSBs) there is no possibility of end joining acting on the broken DNA ends. This in principle [and practice (Ran et al., 2013)] greatly decreases the possibility of generating mutations at off-target sites. Needless to say, however, nicks, while surprisingly recombinogenic (Lee et al., 2004), are not nearly as recombinogenic as DSBs. Thus, the improvement observed for ancillary deleterious mutations is offset by the reduced frequencies of precise genome engineering. However, concerted effort is currently being expended trying to utilize and optimize nick-induced recombination (Davis and Maizels, 2014; Richardson et al., 2016) and if this can indeed be improved it certainly is likely to gain wide utilization especially in clinical settings.

V. IS THERE A USE FOR CRISPR/CAS9 IN SPACE?

While CRISPR/Cas9 may be the niftiest invention since sliced bread, it will, by its very nature, likely only have modest impact on most facets of space technology and travel. For example, it is difficult to envision how CRISPR/Cas9 might help improve, say, rocket design or planet colonization strategies. With that said, there is one area where CRISPR/Cas9 could have

a significant (and readily immediate) impact: space health and medicine. Thus, space is such an extreme environment that it is, in almost all instances, not particularly conducive to human life. Two major threats (and there are certainly more) humans face during long space flights are weightlessness and the exposure to cosmic radiation. Microgravity can cause humans to undergo muscle atrophy, bone density loss and suppression of immune function. Some of these effects can, at least in the short term, be counteracted by physical exercise, but it is unclear if exercise in and of itself would be sufficient to maintain the health of astronauts during long space voyages. Similarly, the very nature of being in space will perforce expose astronauts to gamma rays and high-energy nuclei (protons and heavier nuclei; **HZE particles**) of galactic cosmic rays (**GCR**) that would only worsen if the ship were exposed to solar flares (solar particle events; **SPE**) (Badhwar et al., 1992; Barcellos-Hoff et al., 2015). Radiation exposure increases the risk of getting cancer and, again, generally decreases immune function. The shielding built into most modern spacecraft is able to ameliorate most of the constitutive radiation effects, but provides little protection from (and may even augment the toxicity of) high-energy heavy nuclei GCR (Sridharan et al., 2015). In summary, long-term weightlessness and radiation exposure are just two biomedical parameters that will almost certainly pose health problems for astronauts and as such these (and other) processes need to be better understood before astronauts are put in harm's way.

Thus, an immediate, feasible and practical use of CRISPR/Cas9 would be to use the technology to perform precise genome engineering in animal models. This is an area where CRISPR/Cas has, in just a brief couple of years, already had significant impact — whereby a number of organisms have had their genomes "reimagined" and then re-engineered (Reardon, 2016). Specifically, there is now almost no restriction to engineering appropriate model organisms (mice certainly, but also more "human-like" animals such as pigs or monkeys). These genetically engineered animals could then be subjected to a myriad of weightlessness and radiation exposure studies to assess the impact of these conditions on bone density, cognitive function, cancer incidence, *etc*. While extrapolation of animal studies to human subjects is

always fraught with some uncertainty, these studies would at least give space biologists some indication of the physiological impact of the potential deleterious consequences of long-term space travel.

On a more futuristic note, the advent of CRISPR/Cas9 technology means that it is no longer science fantasy to realistically envision applying **gene enhancement** to astronauts. Thus, if an astronaut were genetically engineered to have stronger bones, more muscle mass, an improved immune system and higher levels of circulating radioprotectant molecules they would certainly be in a much better position to survive a long-term space voyage. Such a radical proposition, however, lands space exploration precisely in the middle of a very slippery ethical slope. Thus, germline therapy is, in almost all parts of the world, either legally banned or ethically frowned upon (Isasi et al., 2016; Kuzma, 2016; Ledford, 2015b). The furor surrounding the announcement of germline modification of even non-viable human embryos (Baltimore et al., 2015; Liang et al., 2015) is indicative of the current opinion on this topic. Nonetheless, CRISPR/Cas9 provides the technology to engineer the human genome/germline efficiently and history has repeatedly shown that mankind will generally do what it has the ability to do. Consequently, most ethicists and policy strategists believe that it is only a matter of time before a "CRISPR baby" is born (Kuzma, 2016; Ledford, 2015b). Again, the current bias is that when such gene modification is allowed, it would be done so only if therapeutically beneficial (*i.e.*, permitting alterations that are disease-preventing and/or to ameliorate on-going pain and suffering) and not for non-medical enhancements (*i.e.*, simply to make someone run faster or jump higher). Alas, gene enhancement for astronauts would likely ethically fall into some nebulous spot on that slippery slope: astronauts are voluntarily putting themselves in harm's way. It is obvious that the ethics in this situation get very complicated, very quickly. If astronauts can be deigned an exemption because of "occupational hazards" one can certainly think of other occupations where the participants might want to undergo genetic modification to allow them to perform their job better. However, unequal access to such treatments could lead to genetic classism where — at its extreme — people might try to become astronauts simply to

obtain a better body. Because even today many gene alterations can be made conditional one could envision a scenario where an astronaut would only be "genetically enhanced" while they were in space and once they returned to earth the enhancements could be (by-and-large) reversed. However, it is likely that even this compromise would be morally offensive to (many?) individuals. Thus, CRISPR/Cas9 has opened up a large ethical Pandora's box and the consequences of that need to be rigorously addressed.

Although the preceding paragraph may sound utterly fanciful, it is important to note that technically it is not. Thus, CRISPR/Cas9 has advanced the technology of gene editing from the *Le Voyage dans La Lune* realm to the Apollo era.   Hence, there is little reason to doubt that many of the speculative alterations described above could actually be accomplished within the next generation (say 20 to 30 years). In summary, it is clear that CRISPR/Cas9 could have a major impact on the biomedical parameters of space exploration, but if it is to do so, extremely precise, well-thought-out policy and ethical regulations needs to be in place to guide scientists as they apply this technology.

VI. SUMMARY

In the ~3 decades that have elapsed since the first report of a gene targeting study in human somatic cells [Smithies, 1985 #54], many endogenous genes have been disrupted or modified. Progress in this area, as is often true in many areas of science, was painfully slow such that after the first two decades only a handful of genes had been modified (Hendrickson, 2008; Sedivy et al., 1999). In the past decade, however, the field has seen spectacular technological progress, with the development of CRISPR/Cas9 being a slam-dunk exclamation point. Thus, as the promise of the CRISPR/Cas9 system comes to fruition, researchers working on human cells in culture will have a powerful and facile weapon at their scientific disposal. The application of this technology to precise human genome engineering is already possible and

it will likely only be a matter of time (and thoughtful ethical considerations) before this technology can be applied in a meaningful way to space exploration.

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