CRISPR-Cas9: Engineering a Revolution in Gene Editing

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Humans are an exceptionally adaptive species—a characteristic that has enabled us to flourish all over the planet. We have adapted genetically and epigenetically to many different climates and habitats, and these adaptive mutations have been passed down to subsequent generations. However, these are not the only mechanisms at play; we also have thrived by modifying our surroundings, passing this information on to the next generation so that it can be built upon, refined, and improved. Now, technological advances in genomic engineering hold the potential to give us the key to not only modifying our external environment, but also engineering genetic adaptations for ourselves as well as other species.

Starting with the discovery of mysterious palindromic, repeated DNA sequences in E. coli in 1987, scientists began investigating the function of this seemingly odd phenomenon. Out of this natural curiosity grew an entirely new way to modify DNA: CRISPR-Cas9. The system evolved as a self-defense mechanism for bacteria—essentially, a way to self-vaccinate against invading viruses and plasmids (Science 23 March 2007, p. 1709, sciencemag.org). This “adaptive immunity,” has enabled bacteria to continuously arm and arm themselves against invaders. However, now that scientists have taken the reins, the CRISPR-Cas9 system has been retooled into a more globally viable technology, whereby the genetic code of virtually any species can be modified, and for more than simply self-protection.

Named as a runner up for Science Magazine’s “Breakthroughs of the Year” in 2013 (Science 20 December 2013, p. 1434; sciencemag.org), the CRISPR-Cas9 system is revolutionizing genomic engineering and equipping scientists with the ability to precisely modify the DNA of essentially any organism. This gene editing could potentially confer genetic advantages that previously took large amounts of evolutionary time (and perhaps a bit of luck), taxing genetic breeding strategies, or bulkier and more complex genomic editing tools to acquire. Speculations about what’s on the horizon seem to be limitless at this point.

How powerful is this technique? The ability for precision genome engineering comes with the potential to enhance food production, medicinal discoveries, and energy solutions, to name a few. Studies over the past several years have shown how subsets of genes in the human genome are easier to use, modifying fuel/energy sources, and even elucidating the multiple genetic contributions underlying human diseases—from heart disease to mental illnesses.

Science has been at the forefront of publishing some of the groundbreaking work as scientists have begun to unravel the CRISPR-Cas9 system and invent novel ways to use this tool. Two such papers were the seminal work of Feng Zhang and George Church, which were simultaneously published and the first studies to show that the CRISPR-Cas9 system can alter mammalian genomes, including humans (see pages 20, 24). Since that time, a “CRISPR Craze” has begun (see page 17), whereby scientists have begun exploring more and more ingenious ways to use this technology.

Researchers have already progressed from studies on individual DNA alterations into studies using CRISPR-Cas9 as an “efficient, large-scale, loss-of-function screening method in mammalian cells,” in lieu of using RNAi screens (see page 24). Of course, it is hoped that these types of studies are just the tip of the iceberg for this new era of precision genetic engineering. In this booklet, we invite the reader to explore a selection of articles that highlight both the history of this technique and how it has grown into one of the most powerful and precise genomic engineering tools to date.

Tianna Hicklin, Ph.D.
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CRISPR: The democratization of gene editing
The elegance and simplicity of Cas9 have sparked the imagination of scientists across many scientific disciplines.

Recent development of genome editing technologies based on the RNA-guided CRISPR-associated endonuclease Cas9 has generated excitement across many fields, including biological research, biotechnологии, and medicine. For the first time, researchers have gained the ability to achieve targeted genomic modifications with efficiency and ease. This is particularly true when combined with the rapidly increasing amount of information available from genomic sequencing efforts available as well as innovative nucleic acid synthesis and delivery systems.

Unlike previous generations of genome editing tools based on zinc finger and transcription activator-like effector proteins, which achieve sequence recognition via protein-DNA interactions, Cas9 can be targeted to specific genomic loci with a guide RNA (gRNA). Once the Cas9/gRNA complex finds the DNA target via Watson-Crick base pairing, Cas9 introduces a double-strand DNA break at the target site, which in turn catalyzes targeted genome editing via non-homologous end joining or homology directed repair. The ability to use nucleic-acid hybridization rules to reprogram Cas9 specificity significantly simplifies genome editing applications particularly given that gRNAs are easily synthesized and introduced into cells to facilitate targeted genome modifications.

Despite being a nascent technology, Cas9 has been successfully used to generate species that can be modified, and for more than simply self-protection. Cas9 has already been broadly applied in many species to generate transgenic models, including mouse, rat, zebrafish, fruit flies, C. elegans, primates, and a variety of plant species. For each species, gRNAs can be easily designed based on reference genome sequences to target virtually any locus of choice. Moreover, direct application of Cas9 in embryos can significantly accelerate transgenic manipulation of whole organisms, including many previously intractable species.

In addition to facilitating the editing of individual genes, Cas9 can also be used for high throughput genetic screening applications where many genes are perturbed in a multiplexed fashion. Large libraries of gRNAs capable of targeting wide ranges of reference for altering crop resistance to infection and disease, advancing drug discovery, modifying energy/energy sources, and even elucidating the multiple genetic contributions underlying human diseases—from heart disease to mental illnesses.

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CRISPR-Cas9: ENGINEERING A REVOLUTION IN GENE EDITING
The start of a new genomic era
Science has been at the forefront of publishing some of the groundbreaking work as scientists have begun to unravel the CRISPR-Cas9 system.
The power and possibilities of genome engineering

By Jeffrey M. Perkel

Ever since scientists understood that DNA carried heritable information, they have had the ability to bend this code to their will. Manipulating the fundamental code for life would mean the ability to correct defects and permanently cure genetic disorders. But the tools that have been available up until now, although workable, are crude at best—like trying to perform surgery while wearing mittens. These days, though, the metaphorical mittens are off. Over the past decade, researchers have devised a succession of strategies that can handle just about any genetic rewrite they can imagine. These “genome-editing” technologies aren’t perfect, and they are typically limited to one or just a few changes at a time. But they do fill a crucial hole in biologists’ toolboxes. In so doing, they are redefining the genetic frontier, and redefining it for the better.

Genome editing technologies exist in a multitude of forms, some of which is based on homologous recombination. In general, homologous recombination rates are too low in mammalian cells to make this approach practical (a key reason why mouse embryonic stem cells, which is why mouse transgenic technology has been so successful). By inserting the repair template into a single-stranded recombinant adenovirus (rAAV) backbone however, efficiency improves considerably to the point that the approach is workable to generate precise genomic modifications.

Most genome-editing strategies, however, rely on the concept of a customized DNA-cutting endonuclease. Several variants of this approach have been developed, but in all these cases, the idea is the same: To generate a double-stranded DNA break at a specified location in the genome of a live cell. In responding to that break, the cell may successfully stitch the two ends together (i.e., no change to the sequence occurs); it may inadvertently disable the gene by non-homologous end joining (NHEJ)-based frame-shifts; or it may repair the gene via homology-directed repair (HDR), a process that researchers can game by supplying their own templates to repair a point mutation or insert a missing gene.

That template molecule typically is supplied either in the form of a short oligonucleotide or double-stranded DNA plasmid. A newer strategy uses a AAV vector instead, which combines the enhanced nuclear uptake of a single-stranded molecule with the greater donor length of plasmids.

In its original implementation, genome editing was accomplished using meganucleases, a class of natural nucleases that targets relatively long DNA recognition sequences. But these enzymes proved difficult to tailor, requiring either mutagenesis or sophisticated protein engineering. Zinc finger nucleases (ZFNs) introduced the concept of programmability to genome editing. ZFNs fuse the DNA-binding domains of zinc finger transcription factors to a generic DNA endonuclease to induce a double-stranded DNA break at a defined position. Programming is achieved by selecting the identity and order of fingers used. In a zinc finger transcription factor, each “finger” recognizes a specific three- or four-base sequence. By simply stringing together an appropriate array of fingers, researchers can in theory target any sequence they desire, and researchers have been successful at doing precisely that, Sangamo BioSciences, for instance, has demonstrated it can safely use ZFN technology in human patients. In a study published earlier this year in the New England Journal of Medicine, the first ever to document genome engineering in the clinic, researchers knocked out the gene for the HIV co-receptor, CCR5, in T cells from HIV+ individuals, and then safely returned those cells to the patients, raising T cell counts overall and CD4 counts in particular (1).

Still, relatively few researchers have hitched their wagon to ZFN technology, as making high-quality ZFNs takes considerable skill. One key problem: Individual fingers don’t always function as expected in the context of an intact ZFN. Optimization—and therefore additional time and money—is often required.

Another class of artificial enzymes, the transcription activator-like (TAL) effector nucleases (TALENs), deliver similar benefits to ZFNs yet generally are easier to use. Like ZFNs, TALENs are modular transcription factors, with each so-called TAL module specifying a single base in the recognition sequence. As a general rule, HDR-mediated genome editing has targeted five (2). CRISPR-Cas9 is also relatively efficient, editing target sequences at surprisingly high rates. As a general rule, HDR-mediated genome editing typically occurs at much lower frequencies than NHEJ, requiring as it does a second piece of DNA containing the repair template. Similarly, it is easier to hit one allele than two. Yet Jaenisch found that 20% of mice embryonic stem cells tested using three sgRNAs simultaneously contained NHEJ-mediated mutations at all six alleles of those three genes, a 20% success rate (5). Church observed HDR-mediated repair rates in human cells of 3% and 8% using two separate CRISPRs, compared to about 0.5% with a TALEN directed at the same location (4). That’s not to say CRISPR-Cas9 is perfect. Multiple studies have documented off-target effects, and off-target effects using the system, for instance, at least in its original incarnation—something that could significantly limit potential clinical applications. Researchers have developed strategies to boost targeting specificity.

CRISPR-Cas9 is also relatively efficient, editing target sequences at surprisingly high rates. CRISPR-Cas9 offers several key benefits over competing endonuclease technologies. First, while meganucleases, ZFNs, and TALENs can be thought of as bespoke single-function machines, Cas9 is basically a programmable enzyme. All that is required is a construct expressing the generic Cas9 nuclease and a set of instructions in the form of a “single-guide RNA” (sgRNA) complementary to the desired target. The system is simple and inexpensive to implement and thus more attractive to researchers who might have been skittish of ZFNs and TALENs.

The second benefit is multiplexing. Since Cas9 is guided by its sgRNA, researchers can program it with multiple guide RNAs simultaneously. Feng Zhang and George Church, writing independently in Science, have both demonstrated the ability to target two sites simultaneously (2, 4), and Rudolf Jaenisch has targeted five (5). CRISPR-Cas9 is also relatively efficient, editing target sequences at surprisingly high rates. As a general rule, HDR-mediated genome editing typically occurs at much lower frequencies than NHEJ, requiring as it does a second piece of DNA containing the repair template. Similarly, it is easier to hit one allele than two. Yet Jaenisch found that 20% of mice embryonic stem cells tested using three sgRNAs simultaneously contained NHEJ-induced mutations at all six alleles of those three genes, a 20% success rate (5). Church observed HDR-mediated repair rates in human cells of 3% and 8% using two separate CRISPRs, compared to about 0.5% with a TALEN directed at the same location (4). That’s not to say CRISPR-Cas9 is perfect. Multiple studies have documented off-target effects, and off-target effects using the system, for instance, at least in its original incarnation—something that could significantly limit potential clinical applications. Researchers have developed strategies to boost targeting specificity.
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One particularly promising application of genome editing marries its power with induced pluripotent stem cell (iPS) technology. CRISPR has experimented with orthogonal Cas9 proteins to expand the technology’s reach even further, for instance by allowing combined genome editing and transcriptional regulation (6).

Indeed, researchers today are extending the CRISPR-Cas9 system on multiple fronts. Teams led independently by Feng Zhang and Eric Lander, writing in Science, recently demonstrated the utility of the system for large-scale screening.

The recent study, led by Daniel Anderson at Massachusetts Institute of Technology (MIT), illustrates the potential power of CRISPR-Cas9 in the clinic. Anderson’s team co-delivered an sgRNA, Cas9, and a 199-base-stranded donor template molecule into the tail vein of a mouse model of a genetic disease called hereditary tyrosinemia type I, caused by a single point mutation in the gene for fumarylacetoacetate hydrolase. Though such a strategy cannot directly be implemented in humans, the treatment did repair the mutation in one in every 250 liver cells in the treated animals, minimizing the liver damage and weight loss typically seen in this model (10).

One particularly promising application of genome editing marries its power with induced pluripotent stem cell (iPS) technology. iPS cells, in which researchers turn a somatic cell into an embryonic-like pluripotent stem cell using four transformation factors and/or small molecules, are opening the door not only to new studies of disease etiology, but corrective cellular therapies as well, as evidenced by a new iPS cell-based clinical study launched recently in Japan.

That study hopes to differentiate patient-specific iPS cells into retinal pigment epithelial cells for the treatment of age-related macular degeneration. But combined with genome-editing technology, iPS cells can do much, more–reconstituting a sickle cell anemia patient’s bone marrow with hematopoietic stem cells in which the mutant human hemoglobin gene has been repaired, for instance. Sangamo BioSciences demonstrated recently that it could use ZFNs to drive HDR-based repair of the IL2-Gamma gene in mammalian hematopoietic stem cells, but that work was not done in a clinical setting (11).

Where ZFNs, or any genome editing technology for that matter, will go from here is an open question. But given the pace of research and the steady stream of promising findings so far, it’s a good bet that it’s going to be an exciting ride.

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Genetic screens in human cells using the CRISPR-Cas9 system

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The bacterial clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system for genome editing has greatly expanded the toolbox for mammalian genetics, enabling the rapid generation of the transgenic mouse models required to fully understand the mechanisms of diseases (14). However, this approach has also been shown to carry off-target effects on other mRNAs, resulting in the suppression of target gene levels and can have deleterious effects on the phenotype (RNAi) (3). In this two-step process, a single-guide RNA (sgRNA) directs the Cas9 nuclease to cause a double-strand break (DSB) at a precise target site by a 20-base pair (bp) sequence at the 5′ end of the sgRNA, allowing for much greater ease of construction of knockout reagents. Mutant cells lines and mice bearing multiple modified alleles can be generated (4, 5).

To test the ability to simultaneously screen tens of thousands of sgRNAs, we designed a new screening strategy for mice, consisting of multiple sgRNA targeting 7114 genes in the murine genome (defined as sites differing by up to 3 bp from sgRNA targets) (fig. S1) (6). Minimal cleavage (<2%) was observed at all sites, with one exception, which was the only site that had been previously shown to be cleaved (<20%) by Cas9. The rest of the genome (defined as sites differing by up to 3 bp from sgRNA targets) (fig. S1) (6). Minimal cleavage (<2%) was observed at all sites, with one exception, which was the only site that had been previously shown to be cleaved (<20%) by Cas9.

We first tested the concept in the near-haploid human KEMT CML cell line by creating a doxycycline-inducible expressing the Cas9 nuclease with a FLAG-tag at its N terminus under a doxycycline-inducible promoter (Fig. 1B). Transduction of these cells at low multiplicity of infection (MOI) with a lentivirus expressing a CRISPR/Cas9 targeting the endogenous AAVS1 locus revealed substantial cleavage at the AAVS1 loci 1 day after infection (Fig. 2A). Moreover, because the sgRNA was stably expressed, genomic cleavage continued to increase over the time course of the experiment (fig. S1). One interesting finding of the loci revealed that repair of Cas9-induced double-strand breaks resulted in small deletions (<20 bp) in the target sequence, with tiny insertions or substitutions (<3 bp) occurring in a lower-frequency collection (Fig. 1B). We also found a lower-frequency collection, which was the majority of the lesions, occurring in a protein-coding context (Fig. 2A). The low-frequency collection, which was the majority of the lesions, occurring in a protein-coding context (Fig. 2A).

We also analyzed off-target activity of CRISPR-Cas9. Although the specificity of CRISPR-Cas9 has been extensively characterized in transfection-based settings (20–22), there remained a desire for off-targeting in our system, in which Cas9 and a sgRNA targeting AAVS1 (sgAAVS1) were stably expressed in the cell. We compared the level of cleavage observed at the target locus (9%) with levels of off-targeting in nontransgenic mice, finding that the Cas9–sgAAVS1 combination resulted in the complete destruction of the genomic loci (defined as sites differing by up to 3 bp from sgRNA targets) (fig. S1A). Minimal cleavage (<2%) was observed at all sites, with one exception, which was the only site that had been previously shown to be cleaved (<20%) by Cas9.

We first tested the concept in the near-haploid human KEMT CML cell line by creating a doxycycline-inducible expressing the Cas9 nuclease with a FLAG-tag at its N terminus under a doxycycline-inducible promoter (Fig. 1B). Transduction of these cells at low multiplicity of infection (MOI) with a lentivirus expressing a CRISPR/Cas9 targeting the endogenous AAVS1 locus revealed substantial cleavage at the AAVS1 loci 1 day after infection (Fig. 2A). Moreover, because the sgRNA was stably expressed, genomic cleavage continued to increase over the time course of the experiment (fig. S1). One interesting finding of the loci revealed that repair of Cas9-induced double-strand breaks resulted in small deletions (<20 bp) in the target sequence, with tiny insertions or substitutions (<3 bp) occurring in a lower-frequency collection (Fig. 1B). We also found a lower-frequency collection, which was the majority of the lesions, occurring in a protein-coding context (Fig. 2A). The low-frequency collection, which was the majority of the lesions, occurring in a protein-coding context (Fig. 2A).
The fact that few of the other 20 most abundant sgRNAs targeted one of these four genes. The corresponding to these genes made up >30% of sgRNA barcodes in the final population. At 24 hours post-infection (dpi), MMR-proficient cells are unable to repair 6-TG–induced lesions and arrest at the G2-M phase (fig. S3B and table S4). For the 20 highest scoring genes, we found independent evidence for essentiality, based primarily on data from large-scale functional studies in model organisms (fig. S3C). To evaluate the results at a global level, we tested 4227 gene sets to see whether they showed strong signatures of essentiality by using gene set enrichment analysis (29). Gene sets related to functions such as ribosomal biogenesis, DNA replication, transcription, and protein degradation—showed strong concordance between the sets of ribosomal protein genes, the data allowed us to search for factors that might explain this difference in efficacy of sgRNAs. Because the majority of ribosomal protein genes are essential, we hypothesized that the level of deletion of a given ribosomal protein-targeting sgRNA could serve as a proxy for its cleavage efficiency. Applying this approach, we found several trends related to sgRNA efficacy. (i) sgRNA sequences with very high or low GC content were less effective against their targets. (ii) sgRNAs targeting the last codon of a gene were more effective than those targeting earlier exons, which is consistent with the notion that disruption of the terminal exon would be expected to have less impact on gene function. (iii) sgRNAs targeting the last exon would be expected to have less impact than those targeting the nontranscribed strand, which is consistent with the notion that disruption of the terminal exon would be expected to have less impact on gene function. (iv) sgRNA efficacy depends on the sequence of the guide RNA. To test this, we developed a method to profile the sgRNA sequences targeted by each CRISPR-Cas9 protein. Diagram depicts cellular DNA repair processes. Only sgRNAs targeting complementary DNA strands to target are enriched. The diagram was modified and adapted from (32).
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Second, a large proportion of sgRNAs successfully generated genomic integrants, we found that the nucleotide composition of the spacer sequence was the most important determinant of Cas9 loading (Fig. 3F). Specifically, those sgRNAs containing purines in the last four nucleotides of the spacer had the highest Cas9 loading, while pyrimidines were disfavored. A similar pattern emerged when we examined deletion of ribosomal protein-targeting sgRNAs. As an independent test, we used a classifier to predict the efficacy of sgRNA targeting ribosomal protein genes in the absence of ribosomal protein expression. The top two thirds of our predictions exhibited threshold higher efficiency than that of the remaining fraction, confirming the accuracy of the algorithm.

Using this algorithm, we designed a whole-genome sgRNA library consisting of sequences predicted to have higher efficacy than those paired with the sgRNA pool used for our screens, this new collection was also filtered for potential off-target matches. This reference set of sgRNAs may be useful both for confirming the accuracy of the algorithm.

First, CRISPR-Cas9 inactivates genes at the DNA level. In the ten experiments described in this manuscript, CRISPR-Cas9 successfully generated mutations at their target sites. Although this parameter is difficult to directly assess in pooled screens, we can obtain an estimate in situ by analyzing genomic integrants at known genes. Applying a score analysis of our positive selection screens, we found that over 75% (46 of 60) of sgRNAs score at a significance threshold that perfectly separates true and false positives on a gene level (Fig. 4A to D). Together, these results show that the effective coverage is very high and that the rate of false negatives should be low, even in a large-scale screen. The threshold to identify sgRNAs that do not appear to significantly hamper our screens, according to several lines of evidence. Direct sequencing of potential off-target loci detected minimal cleavage at secondary sites, which typically reside in noncoding regions and do not affect gene function. Moreover, in the 6-TG screen the threshold was based on the fraction of the four members of the MMR pathway. In total, they represented over 30% of the final pool, which is a requirement for a significant number of sgRNAs combined. In the episomal screen, the two top genes scored far above background levels (τ values 100-fold smaller than that of the next best gene), enabling clear discrimination between true and false positive hits. Last, new versions of the CRISPR-Cas9 system have recently been developed that substantially decrease off-target effects by 1000-fold compared with earlier versions.

Although we limited our investigation to proliferation phenotypes, the sgRNA library approach can be applied to a much wider range of biological phenomena. With appropriate sgRNA libraries, the method should enable gene analyses of mammalian cells to be conducted with confidence and precision currently possible only in the study of microorganisms.

REFERENCES AND NOTES
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CRISPR-Cas9 knockout screening in human cells

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The simplicity of programming the CRISPR (clustered regularly interspaced short palindromic repeats)–associated nuclease Cas9 to modify genomic loci suggests a new way to interrogate genome function on a genome-wide scale. We show that lentiviral delivery of a genome-scale CRISPR-Cas9 guide RNA (sgRNA) library targeting genes with 64,751 unique guide sequences enables both negative and positive selection of genome-scale functional screening in human cells. For instance, we used the DCE screen to identify genes that target cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, we screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic RAF inhibitor. Our highest-ranking candidates include previously validated genes NF1 and MED22, as well as novel hits NF2, CUL3, TADA2B, and TADA1. We observe a high level of functional consistency between independent gene hits targeting the same gene and a high rate of hit confirmation, demonstrating the promise of genome-scale screening with Cas9.

A major goal since the completion of the Human Genome Project is the functional characterization of all annotated genetic elements in normal biological processes and disease (3). Genome-scale loss-of-function analyses have provided a wealth of information in diverse model systems (2–5). In mammalian cells, RNA interference (RNAi) is the predominant method for genome-wide loss-of-function screening (2, 3), but it is limited by the inherent unspecificity of protein depletion by RNAi and confounding off-target effects. The RNA-guided CRISPR (clustered regularly interspaced short palindromic repeats)–associated nuclease Cas9 provides an effective means of introducing targeted loss-of-function mutations at specific sites in the genome (8). Cas9 can be programmed to introduce DNA double-strand breaks (DSBs) at specific genomic sites (4). Here, we describe a genome-scale guide RNA (gRNA) that, when targeted to coding regions of genes can create frame shift mutations (deletion) mutations that result in a loss-of-function allele. Because the targeting specificity of Cas9 is conferred by short guide sequences, which can be easily engineered by DNA backbone lentiviral vectors and the genome-scale sgRNA library, this method can be used in a variety of contexts. The method is currently limited by cost and execution of high-throughput systems, but is promisingly scalable.

Fig. 3. Negative selection screens using CRISPR-Cas9 reveal rules governing sgRNA efficacy. (A) Selective depletion of sgRNAs targeting exons and introns present in the transgene (Intr) or individual sgRNAs targeting the same genomic region (Intr). Cumulative distribution functions of log fold change in sgRNA abundance before and after KRAS and WT KMB7 cells. (B) Depletion of sgRNAs targeting the same genomic region (Intr) and the x axis denote members of the gene set analyzed. (C) Depleted sgRNA target genes involved in fundamental biological processes. Gene set enrichment analysis was performed on genes ranked by their combined depletion scores from screens in H646 and H4. The association between gene function and depletion score is depicted in the x axis denote members of the gene set analyzed. (D) Features influencing sgRNA efficacy. Depletion (log fold change) of sgRNAs targeting ribosomal protein genes was used as an indicator of sgRNA efficacy. Correlation between log fold change and spacer NGC (centric left, even position targeted) and strand targeted (right) are depicted (τ = 0.05). (F) sgRNA target sequence preferences for Cas9 loading and cleavage. As indicated by the boxplot, sgRNA cleavage at specific nucleotide preferences for Cas9 loading are determined by counting sgRNAs bound to Cas9 normalized to the number of corresponding genomic integrations. Heatmaps depict sequence dependent variation in Cas9 loading (top) and ribosomal protein depletion (bottom). The red color scale represents the median value ofCas9 affinity or log2 fold change for all sgRNAs with the specified nucleotide at the specified position. (G) sgRNA efficacy prediction. Ribosomal protein target sgRNAs were designated as “weak” or “strong” on the basis of their log fold change and used to assign a support-vector machine (SVM) classifier. As an independent test, the SVM was used to predict the efficacy of sgRNA targeting 400 essential nonsilencing genomic targets (P = 0.05).
To determine the efficacy of gene knockout by lentiCRISPR transduction, we tested six sgRNAs targeting enhanced green fluorescent protein (EGFP) in a human embryonic kidney (HEK) 293T cell line containing a single copy of EGFP (Fig. S1). After transduction at a low multiplicity of infection (MOI = 0.1) followed by selection with puromycin, lentiCRISPR abolished EGFP fluorescence in 93 ± 6% of cells after 11 days (Fig. 1B). Deep sequencing of the EGFP locus revealed a 92 ± 6% indel frequency (i.e., a 10−3 sequencing reads per condition) (Fig. S2). In contrast, transduction of cells with lentiviral vectors expressing EGFP-targeting shRNA led to incomplete knockdown of EGFP fluorescence (Fig. S3).

Given the high efficacy of gene knockout by lentiCRISPR, we tested the feasibility of conducting genome-scale CRISPR-Cas9 knockout (GeCKO) screening with a pooled lentiCRISPR library. We designed a library of sgRNAs targeting 42,597 human genes (encompassing the human genome with an average coverage of 3 to 4 sgRNAs per gene [table S1]), and each target site was selected to minimize off-target modification (14) (see supplementary text).

To test the efficacy of the full GeCKO library at achieving knockout of endogenous gene targets, we conducted a negative selection screen by profiling the depletion of sgRNAs targeting essential genes (Fig. 2A). We transduced the human melanoma cell line A375 and the human stem cell line NHEK2 with the GeCKO library at a MOI of 0.3. As expected, deep sequencing (figs. S3 and S4) revealed a significant reduction in the diversity of sgRNAs in the surviving A375 and NHEK2 cells (Fig. 2, B and C), compared with vehicle-treated cells (Fig. 2, D and E), which harbor the V600E gain-of-function protein kinase inhibitor vemurafenib (PLX) resistance. Given the high efficacy of gene knockout by CRISPR-Cas9, we used several methods to evaluate the degree of consistency among the sgRNAs targeting each gene.

For a subset of genes, we found enrichment of 3 to 5 sgRNAs that target each gene 14 days after PLX treatment (Fig. 3E), suggesting that loss of these individual genes contributes to PLX resistance. We used the RNAi Gene Enrichment Ranking (RIGER) algorithm to rank screening hits by the consistent enrichment among multiple sgRNAs targeting the same gene (Fig. 3F and table S4) (22). Our highest-ranking genes included previously reported candidate genes (27, 28) and several genes not previously implicated in PLX resistance, including neurofibromin 2 (NF2), and also several genes unrelated in sequence, are each mutated in similar but distinct forms of neurofibromatosis (29). In addition, epigenetic dysregulation resulting from mutations in the mechanistically related STAGA and Mediator complexes (25) may have a role in acquired drug resistance. All of these hits were also identified as PLX targets in an independent library transduction (figs. S7 and S8 and tables S5 and S6).

A similar screen to identify PLX drug resistance in A375 cells was previously conducted using a pooled library of 90,000 shRNAs (29). To compare the efficacy and reliability of genome-scale shRNA screening with GeCKO, we used several methods to evaluate the degree of consistency among the sgRNAs or shRNAs targeting the same candidate gene. First, we plotted the P values for the top 100 hits using either RIGER (fig. 4A) or redundant gene activity (RGA) (fig. 4B) scoring. Lower P values for the GeCKO versus shRNA indicate better scoring consistency among sgRNAs. Second, for the top 10 RIGER hits genes, 78 ± 27% of sgRNAs targeting each gene ranked among the top 5% of enriched sgRNAs, whereas 20 ± 12% of shRNAs targeting each gene ranked among the top 5% of enriched shRNAs (Fig. 4B). We validated top-ranking genes from the GeCKO screen individually using 3 to 5 sgRNAs (Fig. 4, C to E, and figs. S10 and S11).
transduced with individual NF2-targeting lentCRISPR or shRNA vectors. Controls were GFP-targeting lentCRISPR or null harbouring shRNA vectors. Cells transduced with NF2-targeting lentCRISPR show a significant increase (F(3,0,3,0.003, n = 4 replicates) in the half-maximal effective concentration (EC50), whereas cells transduced with NF2-targeting shRNA vectors do not (F(3,0,4,0.51, n = 4 replicates)).

For NF2, we found that 4 out of 5 sgRNAs resulted in >98% allele modification 7 days after transduction, and all 5 sgRNAs showed >96% allele modification 14 days after transduction (Fig. 4C). We compared sgRNA and snRNA-mediated protein depletion and PLX resistance using Western blot (Fig. 4D) and cell growth assays (Fig. 4E). Interestingly, although all five sgRNAs conferred resistance to PLX, only the best shRNA achieved significant perturbation modalities, including genome-engineering.org) in the half-maximal effective concentration (EC50), whereas cells transduced with NF2-targeting shRNA vectors do not (F(3,0,4,0.51, n = 4 replicates)).

Fig. 4. Comparison of GeCKO and shRNA screens and validation of neurofibromin 2 (NF2). (A) EC50 values for the shRNA screen (n = 40) vs. the CRISPR-Cas9 screen (n = 20). The CRISPR-Cas9 screen results in PLX resistance. Analysis using the RSA algorithm shows a similar trend (fig. S9). (B) For the screen (n = 40) vs. the CRISPR-Cas9 screen (n = 20). The CRISPR-Cas9 screen results in PLX resistance. Analysis using the RSA algorithm shows a similar trend (fig. S9).

REFERENCES AND NOTES

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REFERENCES AND NOTES
DNA surgeon. With just a guide RNA and a protein called Cas9, researchers first showed that the CRISPR system can home in on and cut specific DNA, knocking out a gene or enabling part of it to be replaced by substitute DNA. Barrangou says. “The only limitation today is to think of creative ways to engineer combinations of technologies.”

CRISPed rice. Earlier this month, researchers showed CRISPR works in plants, such as rice, where the knocked-out gene resulted in dwarf alleles individuals.

CRISPR technique, a new mouse model could be ready for testing in a matter of weeks. And Zhang thinks the approach is not limited to mice. “The technology is so simple that it can be ready for testing in a matter of weeks. And Zhang thinks the approach is not limited to mice,” he says.

CRISPR in action: Engineering a Revolution in Gene Editing

one site for each strand of the DNA’s double helix. And in the past week, several papers have detailed how the CRISPR system can be modified to target specific DNA sequences. This precision targeting drives the growing excitement that CRISPR has generated, as more and more people begin to think about delivering CRISPR systems as treatments for human diseases.

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Multiple genome engineering using CRISPR/Cas systems

Le Cong,1,3,4 F. Ann Ran,1,3,4 David Cox,1 Shuailiang Lin,1,2 Robert Barretto,1 Naomi Halbli,1 Patrice D. Hsu,1 Xuebing Wu,2 Wenyuan Jiang,1 Luissana A. Marraffini,1 Feng Zhang1†

CRISPR–Cas9: ENGINEERING A REVOLUTION IN GENE EDITING

ARTICLES   REPORT

To harness this prokaryotic RNA-programmable nuclease system to introduce targeted double-stranded breaks (DSBs) in mammalian chromosomes through homologous expression of the key components. It has been previously shown that expression of tracrRNA, pre-crRNA, host factor ribonuclease (RNase) III, and Cas9 nuclease is necessary and suf-}

sient for cleavage of DNA in vitro (22, 23) and in prokaryotic cells (20, 22). We con-}

structed a type II CRISPR/Cas system, we expressed an 89-nu-
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125x71 to 565x407

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Figure 1. The type II CRISPR locus from S. pyogenes SF370 is functional in mammalian cells to facilitate targeted DSBs of DNA. (A) Engineering of SpCas9 and SpNFase III with NLSS enables import into the mammalian nuclear compartmentalization in mammalian cells. (B) Mammalian expression of human codon-optimized SpCas9 (SpCas9) and SpNFase III (SpNFase III) genes were driven by the elongation fac-

tor 1α (EF1α) promoter, whereas tracrRNA and pre-crRNA (tracrRNA and pre-crRNA array) genes were driven by the EF1α promoter. (C) Schematic representation of base pairing between target locus and EMX2 targeting crRNA. Red arrow indicates putative cleavage site. (D) SURVEYOR assay for SpCas9-mediated DSBs as well as representative sequences of mutated alleles identified from 187 clonal ampiclons. Red dashes, deleted bases; red bases, insertions or mutations.

to harness this prokaryotic RNA-programmable nuclease system to introduce targeted double-stranded breaks (DSBs) in mammalian chromosomes through homologous expression of the key components. It has been previously shown that expression of tracrRNA, pre-crRNA, host factor ribonuclease (RNase) III, and Cas9 nuclease is necessary and sufficient for cleavage of DNA in vitro (22, 23) and in prokaryotic cells (20, 22). We codon-optimized the S. pyogenes Cas9 (SpCas9) and in prokaryotic cells (20, 22). We con-}

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Figure 4. Applications of Cas9 for homologous recombination and multiplex genome engineering. (A) Mutation of the RxR1 domain converts Cas9 into a nicking enzyme (SpCas9). HDI, Valdinopirine-arginine histidine endonuclease domain. (B) Coexpression of EMX1 targeting chimeric RNA with SpCas9 leads to indels, whereas SpCas9 alone does not (N = 3). (C) Schematic representation of the recombinase strategy. A homology repair construct is designed to insert a restriction site into EMX1 locus. Primers used to amplify the modified region are shown as red arrows. (D) Restriction fragment length polymorphism gel analysis. Arrows indicate fragments generated by SpCas9-directed intermediate restriction-site megachromatogram showing successful recombinant. (E) SpCas9 can facilitate multiplex genomic modification by using a crRNA array that contains two spacers targeting EMX1 and PVRL2. Schematic showing the design of the crRNA array (top). Both spacers mediate efficient protospacer cleavage (bottom). (F) SpCas9 can be used to achieve precise genomic deletion. Two spacers targeting EMX1 (top) mediated a 228-bp genomic deletion (bottom).
RNA-guided human genome engineering via Cas9

Prashant Mali,1,2 Luhan Yang,3*, Kevin M. Esselt,4 John Aach,† Marc Glell,2 James E. DiCarlo,4 Julie E. Norville,1 George M. Church,1,2†

CRISPR-CAS9: ENGINEERING A REVOLUTION IN GENE EDITING

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CRISPR-CAS9: ENGINEERING A REVOLUTION IN GENE EDITING

A bacterial and archaeal clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system provides a straightforward and efficient way to introduce targeted mutations into the genomes of a broad range of organisms. Cas9, one such Cas protein, can target DNA sequences using a guide RNA (gRNA) that is complementary to the target DNA sequence. The fully defined nature of the CRISPR/Cas9 system, its simplicity to execute, and its potential for multiplexed genome editing has raised considerable interest in the scientific community as a platform technology. Moreover, CRISPR/Cas9 is now being explored for safe genome modification and perhaps for therapeutic intervention in human patients. Thus, the CRISPR/Cas9 system potentially offers new opportunities to alter the human genome in a precise and predictable manner.

Bacterial and archaeal clustered regularly interspaced short palindromic repeats (CRISPR) systems rely on CRISPR RNA (crRNA) and CRISPR-associated (Cas) proteins to direct degradative or transcriptional silencing of foreign nucleic acids. Here, we engineered the type II bacterial CRISPR system to function with custom guide RNA (gRNA) in human cells. For the endogenous AAVS1 locus, we obtained targeting rates of 10 to 25% in 293T cells, 3 to 8% in K562 cells, and 3 to 6% in induced pluripotent stem cells. We show that this process relies on CRISPR components; it is sequence-specific and, upon introduction of multiple gRNAs, can mediate multiplexed editing across the genome. We also computationally generate a genome-wide resource of ~190 K unique gRNA target sites at 40.5% of human exons. Our results establish a RNA-guided editing tool for facile, robust, and multiplexed human genome engineering.

In 2012, the CRISPR/Cas9 system was found to be a simple, fast, and versatile method for genome editing in a variety of organisms, including mammals (1). Since then, the CRISPR/Cas9 system has been successfully used in gene targeting in human cells (2, 3) and in yeast (4). It offers potentially safe genome modification and may provide a platform technology for many applications, such as safe genome modification and perhaps for therapeutic intervention in human patients. Therefore, CRISPR/Cas9 has the potential to offer new opportunities to alter the human genome in a precise and predictable manner. However, the mechanism of action of CRISPR/Cas9 is still not fully understood (5).

In this study, we report that CRISPR/Cas9 can be adapted to mediate human genome editing (Fig. 1). We designed gRNAs to target any genetic site of the human genome, including transgenic sequences present in the genome. We leveraged the CRISPR/Cas9 system in human cells to demonstrate effective targeted genome editing (Fig. 1A). Two gRNAs (T1 and T2) were designed to target the AAVS1 locus (Fig. 1B). To confirm our observations, we also tested a Cas9D10A mutant that is defective for nuclease activity but retains DNA-binding function (6). The targeted sites were chosen from human genes that are likely to have low fitness costs (7). We also performed targeted experiments that were compatible with multiplexed editing of the human genome using the CRISPR/Cas9 system (8).

CRISPR/Cas9-mediated editing has a number of advantages over other genome editing technologies, including a simpler workflow, faster genome editing, and a more straightforward mechanism of action (9, 10). However, it has also been shown to have some limitations, such as the requirement for a perfect match of the guide RNA to the target site (11, 12). Another limitation is that CRISPR/Cas9 is currently limited to DNA sequences and does not have the capability to target RNA (13). The CRISPR/Cas9 system can be expanded through the use of homologs with different PAM requirements (14, 15), whereas inactivating both nuclease domains increases the ratio of HR to NHEJ (16, 17). However, the CRISPR/Cas9 system also has some limitations, such as the requirement for a perfect match of the guide RNA to the target site (18). Additionally, the CRISPR/Cas9 system is limited to DNA sequences and does not have the capability to target RNA (19).

In conclusion, we demonstrated that CRISPR/Cas9 can be adapted to mediate human genome editing (Fig. 1). We designed gRNAs to target any genetic site of the human genome, including transgenic sequences present in the genome. We leveraged the CRISPR/Cas9 system in human cells to demonstrate effective targeted genome editing (Fig. 1A). Two gRNAs (T1 and T2) were designed to target the AAVS1 locus (Fig. 1B). To confirm our observations, we also tested a Cas9D10A mutant that is defective for nuclease activity but retains DNA-binding function (6). The targeted sites were chosen from human genes that are likely to have low fitness costs (7). We also performed targeted experiments that were compatible with multiplexed editing of the human genome using the CRISPR/Cas9 system (8).

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Cas9
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addicted to
plasmid-encoded
antibiotic factors, thus
preventing
their disposal. Bacteria and
archaea defend themselves
against these invasive
elements using an
adaptive
immunity
system
hereby
clustered
regularly
interspaced
short
palindromic
repeats
(CRISPRs).

On page 30 [in this booklet], Jinek
et al
(2012)
could show
that
Cas9
from
bacteria is directed not by
one, but two small
RNAs to
cleave invader DNA.

CRISPR-Cas9 system
integrates short DNA
fragments from viruses and
plasmids into a specific
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locus of the cell's host
gene
function
as a
memory of past
invasions. This locus of the
‘‘cell’s most wanted’’ is then
transcribed into RNA,
whereby CRISPR RNA
(CR RNA)
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is cleaved in each
repeat to yield individual
CR RNA
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as a
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complexes).

Cascade-like
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(see the
Fig. 1).

The CRISPR
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CRISPR-Cas9
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al
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Target
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This
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CRISPR
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Cas9
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likely
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of
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CRISPR/Cas9: ENGINEERING A REVOLUTION IN GENE EDITING

Philippe Horvath* and Rodolphe Barrangou

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 invading 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, building species trees, and for intervention against undesirable genetic elements, and enhancing viral resistance in domesticated microbes.

I nterestingly, although outpopulated 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 has forced microbes to establish an array of defense systems that allow the cell to recognize and distinguish incoming “foreign” DNA, to “call” it, and to defend itself against it, thereby providing acquired immunity against viruses and plasmids.

CRISPR, a family of DNA repeats found in most archaeal (~96%) and bacterial (~4%) genomes (1–3). The initial discovery of a CRISPR structure was made fortuitously in Echerichia coli in 1987 (4). Later, similar structures were observed in genomes of various bacteria and archaea (5). CRISPR loci typically consist of several noncontiguous direct repeats separated by stretches of variable length (17). The CRISPR repeat-spacer unit forms the CRISPR defense systems present in Streptococcus thermophilus, Streptococcus cas, and Pyrococcus sp. The CRISPR effector complex, a cascade of CRISPR-associated (Cas) proteins, is responsible for the recognition and adaptation of target nucleic acid sequences.

The CRISPR loci have highly diverse and hypervariable spacer sequences, even between closely related strains (14–40), which were initially exploited for typing purposes. A variety of potential roles for CRISPR sequences have been suggested, including chromosomal rearrangement, moduli and eradication of stress-induced DNA damage, restriction, and invasion of novel DNA, as well as modification of inserted DNA (17). The CRISPR loci are composed of CRISPR arrays, which can be found in the chromosome and plasmids (14, 14–16). This led to the hypothesis that CRISPR may provide adaptive immunity against foreign genetic elements (6). A vast spectrum of immunity

In 2007, it was shown in Streptococcus thermophilus and Staphylococcus aureus that CRISPRs have been identified that provide acquired immunity against viruses and plasmids.

CRISPR, a name derived from cluster regularly interspaced short palindromic repeats, has been shown to confer immunity against viruses and plasmids. CRISPR systems are homologous to the CRISPR systems of Streptococcus pyogenes, Streptococcus thermophilus, and Pyrococcus furiosus. The CRISPR-Cas systems employ a RNase III enzyme called Cas9, which cleaves DNA with high specificity and efficiency. The CRISPR-Cas systems are homologous to the CRISPR systems of various species, including bacteria and archaea. They are composed of CRISPR arrays, which are composed of spacers from invading elements and spacers from mobile genetic elements. The CRISPR-Cas systems can be used to target specific DNA sequences, making them useful for gene editing and gene therapy applications.

The CRISPR-Cas systems have been shown to be effective in a wide range of organisms, including bacteria, archaea, and protists. The CRISPR-Cas systems are composed of a series of repeats and spacers, which are complementary to invading DNA or RNA sequences. The CRISPR-Cas systems are activated by the invasion of foreign DNA or RNA, which leads to the activation of the CRISPR-Cas system, resulting in the cleavage of the invading sequence at specific sites.

The CRISPR-Cas systems have several advantages over existing gene editing technologies, including increased efficiency, reduced off-target effects, and the ability to target non-dense regions of the genome. CRISPR-Cas systems have been successfully used in a variety of applications, including gene editing, gene therapy, and the study of gene function.

This is in agreement with the lack of congruence between the phylogeny of various CRISPR loci and the presence of CRISPR in different species. The presence of CRISPR in different species suggests that CRISPR is not present in all species, but rather evolved independently in different lineages. In some species, CRISPR may have evolved to confer immunity against specific viruses, while in other species, CRISPR may have evolved to confer immunity against mobile genetic elements. This suggests that CRISPR may have evolved for a variety of different purposes in different lineages.

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Currently, CRISPR-Cas9 is the most widely used system for gene editing, and it is being used in a variety of applications, including gene therapy, drug discovery, and basic research. The CRISPR-Cas9 system is composed of a single-guide RNA (sgRNA) and a Cas9 nuclease. The sgRNA is designed to bind to a specific DNA sequence, and Cas9 cleaves the DNA at the site of the sgRNA binding site. This allows for the insertion, deletion, or modification of DNA, which can be used to study gene function, generate mutations, or engineer new traits.

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CRISPR-CAS9: ENGINEERING A REVOLUTION IN GENE EDITING

A CRISPR-Cas system may target either DNA or RNA in viruses with viruses, plasmids, prophages, or other chromosomally encoded sequences.

Fig. 2. Overview of the CRISPR/Cas mechanism of action. (A) Immunoassay process. After insertion of exogenous DNA from viruses or plasmids, a Cas complex recognizes foreign DNA and integrates a novel repeat spacer unit at the leading end of the CRISPR locus. (B) Immunity process. The CRISPR repeat spacer array is transcribed into a pre-crRNA that is processed into mature crRNAs, which are subsequently used by a Cas complex to interact with the corresponding invading nucleic acid. The Cas9 proteins are represented as diamonds, spacers as rectangles, and the CRISPR leader is labeled L.

increase the selective pressure on invading elements and, consequently, could increase the chances of host survival by using multiple hurdles.

Because CRISPR-Cas systems may serve as a powerful tool for prophylactic and therapeutic applications, they have been shown to be the most hypervariable and dynamic systems in bacteria, such as CRISPR-based immunity have provided avenues for industrial applications, including exploiting hypervariability for typing purposes, driving viral evolution, providing resistance against recombinase-based transposons and integrating hypervariable microbial elements in bacteria. Indeed, in many instances, CRISPR-based immunity has provided avenues for industrial applications, includ-

REFERENCES AND NOTES

A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity

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Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNA (crRNA) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to the trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA guide sequence, the Cas9 DHN nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

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It has been demonstrated that the efficiency of incorporation of a targeted alteration is highly dependent on the distance from the nuclease-induced cut site to the site of the desired genomic alteration. The closer these two are to each other, the higher the efficiency, so it would make sense to use a guide RNA (gRNA) that lies as close to the site of the desired alteration as possible. However, the best gRNA to drive incorporation may not be the most specific, leading to off-target effects, and so a difficult choice needs to be made as to the right balance to strike between these two factors.

It has also been demonstrated that not all gRNAs are equal in activity. Therefore, the lack of sufficient activity by a given gRNA might also require one to use a gRNA which lies somewhat farther away from the target site. It is even possible, in some cases, that there simply is no suitable gRNA available that lies close enough (within 40bp) to the target site.

To address the donor side of the equation, researchers have mostly been using either single-stranded oligos or double-stranded plasmids to provide a donor template. Because of Horizon’s long history of using rAAV (recombinant adeno-associated virus) to edit and create hundreds of isogenic lines, we were interested to see whether the combination of rAAV and CRISPR might offer advantages not necessarily realized when using an oligo or plasmid alone.

It has been known for some time that rAAV is able to drive homologous recombination levels that are up to 1,000-fold higher than those seen when using a simple plasmid. Despite this vast improvement over plasmids, the levels of recombination seen are still relatively modest, making rAAV optimal for single allele alterations, but less so when looking to affect multiple alleles simultaneously. The efficiency of using rAAV is boosted significantly however, when a double-strand break is introduced in the vicinity of the homology region. In addition, rAAV has an extremely wide tropism making it very effective for delivery of a single-stranded piece of long DNA (approx. 4.8kb) directly to the nucleus of many cell types, particularly those which may be difficult to transfect.

To test the potential for a combination approach, we set up an experiment to look at more ‘real-world’ situations where the gRNA cut wasn’t necessarily located directly over the desired change and used a common cell line (HCT116) that is not particularly easy to transfect. We used CRISPR to introduce double strands breaks at various points in the genome of a line carrying a disabled copy of the GFP gene on one chromosome. The figure below shows the organization of the artificial GFP gene split into three exons, with the third exon carrying a mutation which prematurely terminates the GFP protein rendering it non-functional. Conversion of the adenosine (A) residue to a cysteine (C) restores GFP functionality. The location and relative distance of five different gRNA targets sites from the point mutation are shown below the expanded Exon 3 region.
We used both a plasmid and rAAV derived from that same plasmid as a donor and measured the ability of these donors to stimulate a genetic modification at the range of distances from the induced cut site. Remarkably the rAAV donor performs markedly better than the corresponding plasmid with an average 10-fold improvement at most distances. We are currently working to extend this research and recent results comparing rAAV with oligo and plasmid donors at different concentrations have supported this trend.

We acknowledge that the data is representative of only one cell line which does not transfect particularly well, but we feel it does fairly represent a real-world situation which often arises as experiments are designed in an ever widening variety of cellular backgrounds. We and other groups continue to look for technology combinations and other approaches that can be used to boost gene targeting efficiencies and lower the barriers to make even complex gene editing more routine.

References

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Figure 2: Plasmid and rAAV donors were compared for their ability to knock-in the activating mutation and restore GFP function through homology-directed repair. For each gRNA tested, rAAV donors provide higher frequencies of correct gene targeting than equivalent plasmids.

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