Genome Engineering

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CRISPR Craze Conquers the RNA World: Precise Manipulation of DNA and RNA Based on a Bacterial Defense System**

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Cas9 · CRISPR · RNA · RNA manipulation · RNA targeting

Genome engineering—the targeted, specific modification of the genome of an organism—is invaluable for fundamental research and also affects our daily lives. It is used to identify the function of genes, to engineer microbes as well as food crops, and to correct disease-associated mutations in cell cultures, animal models, and maybe soon in humans. Methods to insert or delete genes into a number of organisms have been known for a long time, but this transgenesis did not allow control over the specific site of insertion within the genome. To avoid unforeseen and unwanted effects, however, precise manipulation of individual genes in cells and organisms is absolutely required.

Over the last few decades, different proteins have been engineered for precise targeting with great success. Notable examples include zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs), which are reviewed in Ref. [1]. However, the CRISPR system, which relies on RNA-guided targeting, revolutionized the field within a few years because it performs as well as protein-based methods but is faster and easier to implement.[2]

CRISPR stands for *c*lustered, *r*egularly *i*nterspaced, *s*hort, *p*alindromic *r*epeats and is an adaptive defense system in bacteria for protection against foreign nucleic acids.[3] Sequences derived from foreign DNA are incorporated into the bacterial host genome and stored as a memory of previous infections. Transcripts from these regions in complex with one or more the protein components (a single endonuclease Cas9 in the simplest case) identify and cleave complementary target DNA.

Based on the simple type II CRISPR system, the CRISPR technology was developed and proved efficient for the programmed cutting of dsDNA in vitro and in a variety of cells and organisms (Figure 1). It requires only two components to perform genome editing: the Cas9 nuclease and a dual-guide RNA (gRNA) composed of a CRISPR RNA tailored to the target sequence and a fixed trans-activating CRISPR RNA. The choice of target sequences is only limited by the requirement for a short motif called PAM (protospacer adjacent motif) next to the target sequence. The PAM is species-specific and the most widely used type II CRISPR–Cas9 from *Streptococcus pyogenes* recognizes 5′-NGG-3′. Target DNA without an adjacent PAM (like the cell's own genome) will not be cleaved by CRISPR–Cas9 (Figure 1).

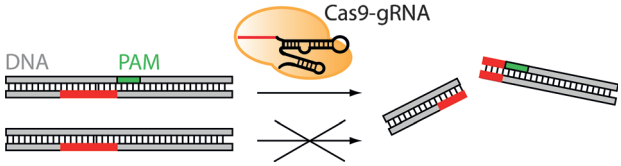


Figure 1. Sequence-specific cleavage of DNA by Cas9–gRNA. Cas9 can target and cleave dsDNA matching the part of the dual gRNA shown in red. Adjacent to the target site, a short PAM (shown in green) in the counterstrand is required for recognition and cleavage by Cas9.

Since the CRISPR technology is beautifully simple and efficient at targeting DNA in a sequence-specific manner within whole genomes, many researchers were hoping for a similar system for sequence-specific RNA manipulation. However, although RNA targeting by a few CRISPR/Cas systems has been demonstrated,[4] the popular *S. pyogenes* Cas9–gRNA neither binds to ssRNA nor cleaves it.

By looking closely at the molecular and mechanistic details of Cas9 target recognition and cleavage, Jennifer Doudna and co-workers have now discovered a trick to make *S. pyogenes* CRISPR/Cas9 applicable to RNA.[5] The PAM (which in dsDNA is located next to the target site but on the opposite strand: Figure 1) is important for recruiting Cas9–gRNA and triggering cleavage.[6] PAM recognition may trigger catalysis in an allosteric manner because if it is added as a separate PAM-presenting oligonucleotide (PAMmer) "in trans", ssDNA targets can suddenly be cleaved.[6a] Indeed, the addition of a DNA PAMmer in trans to a ssRNA target triggered Cas9 binding and cleavage of the target RNA (Figure 2).[5] In this way, RNA can be cleaved in a sequence-specific and easily programmable fashion by using Cas9–gRNA and an additional PAMmer.

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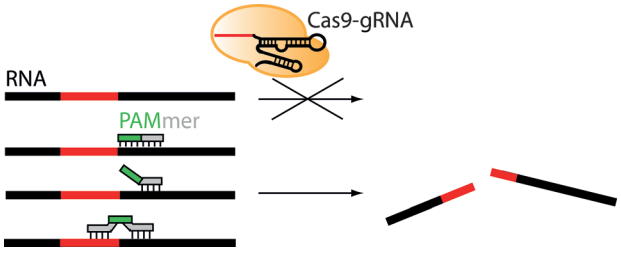


Figure 2. Sequence-specific cleavage of RNA by Cas9–gRNA. Normally, ssRNA is not cleaved by *S. pyogenes* Cas9–gRNA. The addition of a short DNA oligonucleotide (PAMmer) in trans that contains the PAM triggers sequence-specific RNA recognition and cleavage by Cas9–gRNA. The PAM does not have to form base pairs with the RNA but can be added as an overhang or bridge to the PAMmer. This allows the cleavage of RNA without cleavage of the corresponding DNA. A bridge PAMmer ranging into the target sequence increases the specificity of Cas9–gRNA binding to ssRNA.

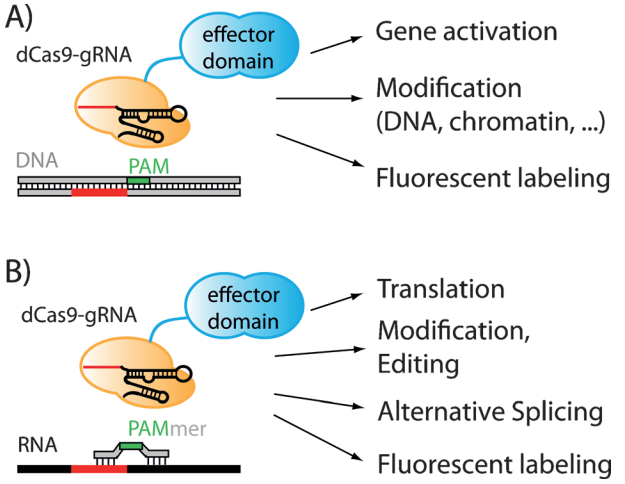


Figure 3. An inactive variant dCas9 provides a platform for engineering different protein functions with sequence specificity for nucleic acid targets. A) Effector domains with different functions fused to dCas9 have been successfully used for the sequence specific manipulation of dsDNA. B) Fusion of different effector domains to dCas9 in combination with PAMmers will provide a new way to manipulate RNAs in a sequence-specific manner.

Importantly, it is viable to target RNA without binding or cleaving the corresponding DNA when both DNA and RNA are present, which is a desirable feature for applications in cells. This is possible because the PAM itself does not have to form base pairs to the ssRNA. Consequently, a mismatched PAM can be introduced by the PAMmer, thus enabling cleavage of ssRNA without a reverse complementary PAM in the target strand, whereas dsDNA targets without an adjacent PAM remain intact (Figure 1), even in a mixture of dsDNA and ssRNA.

For dsDNA, the catalytically inactive double mutant dCas9 in complex with gRNA provides a versatile platform for delivering effector domains to a specific target region in the genome and has been used to modulate gene expression, to alter DNA methylation, and to fluorescently label gene loci by using fused fluorescent proteins (Figure 3 A, reviewed in Ref. [7]). It would be desirable to have an equally simple programmable and genetically encodable platform for sequence-specific RNA detection and manipulation. Such a system might allow sequence-specific RNA degradation (knock-down) in organisms that are not suitable for RNA interference and could complement existing approaches for sequence-specific RNA manipulation.

Indeed, ssRNA is also bound tightly by the catalytically inactive dCas9 if a PAMmer is present. However, high affinity binding of dCas9 to ssRNA in the presence of a PAMmer did not require correct base pairing between the gRNA and the ssRNA target. Such a lack of binding specificity is undesirable for targeting applications but can be eliminated by using bridge PAMmers that extend into the target region (Figure 2 and Figure 3 B). The PAMmer extension length has to be optimized to balance the tradeoff between ssRNA binding affinity and specificity. Nevertheless, bridge PAMmers in combination with dCas9–gRNA may provide a flexible platform for the sequence-specific targeting of ssRNA (Figure 3 B).

As a proof of concept, the authors used biotinylated dCas9 to isolate target mRNA from mammalian total RNA and cell lysate. While isolation from total RNA worked nicely, isolation from cells with DNA PAMmers led to serious degradation as a result of RNase H cleavage of the RNA–DNA heteroduplexes. Luckily, several modified PAMmers were also tolerated by Cas9 (including LNAs, 2′-OMe modifications, and 2′-F modifications at the ends and at several sites throughout the oligonucleotide). The combination of dCas9 with a highly 2′-OMe modified (ca. every second nucleotide) PAMmer allowed the isolation of target mRNA directly from HeLa cells without RNase H cleavage. While the use of dCas9–gRNA/PAMmer for the isolation of specific transcripts certainly represents an improvement on oligonucleotide-mediated RNA capture (because it works under physiological salt conditions and without crosslinking), it offers only a first glimpse at the potential applications that we may expect in the future (Figure 3 B).

Fusion of dCas9 to effector domains may enable stimulation of the translation of certain mRNAs, sequence-specific modification of RNAs (e.g. methylation, demethylation, A-to-I editing), enhancement or silencing of the use of alternative splice sites, or sequence-specific RNA labeling. To date, programmable RNA manipulation or labeling has been achieved through hybridization-based approaches (e.g., covalent fusion of a gRNA to an effector protein[8] or turn-on probes devoid of protein[9]) and genetic fusions with engineered RNA-binding proteins.[10] Both approaches have proven useful, and notable examples include transcript-specific translation enhancement,[10b] A-to-I editing,[8] and modulation of splicing,[11] as well as multiple approaches to RNA labeling.[9] However, both approaches also have limitations. Hybridization-based approaches typically require modified oligonucleotides, and cellular applications are mainly limited by the delivery. Protein-based approaches can be realized by taking advantage of the cellular machinery but considerable effort is required for designing new target specificities.[12]

The type II CRISPR/Cas9 system raises high hopes because it is simple and flexible. Its sequence specificity is

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based on complementary base pairs between the target and the gRNA, which can be easily designed, and it requires only one protein (Cas9), which can be genetically fused to different effector proteins (Figure 3). The gRNA is unmodified and can thus be produced by the cellular machinery, which is attractive for the manipulation of RNA inside cells. However, RNA targeting by the *S. pyogenes* CRISPR/Cas9 system is more complicated than dsDNA targeting. First, the PAMmer is composed of ssDNA or a mixture of ssDNA and modified nucleotides and cannot be produced by the cellular machinery. Therefore, as for hybridization-based RNA targeting approaches, efficient delivery of the PAMmer will be required. Second, there are multiple copies of a particular mRNA in a cell. It will be interesting to see how efficient effector proteins fused to dCas9 will be at performing multiple turnovers and whether prolonged application will cause off-target effects.

In summary, CRISPR/Cas9 undoubtedly represents one of the most significant advances in genome engineering. The extension of this technology to programmable RNA recognition, albeit with a new layer of complexity (PAMmers), will trigger a lot of exciting developments and may ultimately lead to a general approach for sequence-specific RNA manipulation.

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