MOLECULAR BIOLOGY

Enhancing the RNA engineering toolkit

The CRISPR-Cas13 system can be used to engineer RNA

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RNA plays important and diverse roles in biology, but tools to study and engineer it are limited. Abudayyeh *et al.* (*1*) and, on page 1019 of this issue, Cox *et al.* (*2*) have developed effective programmable tools using newly discovered CRISPR-associated 13 (Cas13) enzymes to achieve precise RNA targeting, cleavage, tracking, and editing in mammalian cells.

Recent advances in the ability to precisely edit genomic DNA by using the CRISPR-Cas9 system, which has evolved to recognize double-stranded DNA (dsDNA) as an adaptive immune response in bacteria, have revolutionized the field of genome engineering (*3*). In the CRISPR-Cas9 system, the Cas9 endonuclease can be targeted to specific DNA sequences containing a protospacer adjacent motif (PAM) by single-guide RNAs (sgRNAs) that are easily programmable. Yet, the development of simple and scalable tools to manipulate and measure RNA has lagged behind. Previous efforts repurposed the CRISPR-associated deoxyribonuclease (DNase) Cas9 (*4*). For example, biochemical work established that Cas9 could be specifically directed to bind and cut single-stranded RNA (ssRNA) targets by providing the PAM in trans as part of an oligonucleotide (PAMmer) that hybridizes to the target RNA (*4*). This approach has been used to track RNA localization (*5*) and to eliminate toxic RNA repeats (*6*) in living cells with well-designed RNA-targeting Cas9 (RCas9) systems. In such systems, effective RNA recognition by Cas9 DNase is achieved by the presence of a PAMmer DNA sequence that is composed of a mixture of 2′-O methylated RNA and DNA bases to avoid forming a substrate for ribonuclease (RNase) H and an adjacent spacer sequence complementary to the Cas9-associated sgRNA within a modified scaffold vector (*5, 6*). Although designing CRISPR-Cas9 DNase to target RNA has highlighted the potential power of RNA manipulation, its requirements for recognition of a dsDNA region and for multiple components to generate an active RCas9 have constrained its broad applicability for RNA engineering.

Most prokaryotic adaptive immune systems generally target DNA substrates, but type III and VI CRISPR systems can be directed against ssRNA substrates (*7–11*). Recently, it has been demonstrated that the class 2 type VI CRISPR-Cas effectors, Cas13a (previously known as C2c2) and Cas13b, are single-component programmable RNA-guided RNA-targeting RNases that have both RNA processing and programmed RNA cleavage activities (see the figure) (*7, 8*). Bacterial Cas13a enzymes from different species have RNase activity responsible for CRISPR RNA (crRNA) maturation that is distinct from their RNA-programmed RNA cleavage activity (*8*). Programmed cleavage is mediated by catalytic residues in the two conserved higher eukaryote and prokaryote nucleotide-binding (HEPN) domains (*7, 8*). Owing to these distinct properties, Cas13 can be directed to cleave ssRNA targets carrying complementary protospacers by a single crRNA to knock down specific messenger RNAs (mRNAs) in bacteria (*7, 11*). In biochemical assays, Cas13 engages in promiscuous RNA cleavage upon target recognition, a behavior termed the "collateral effect" (*7, 8*). The collateral effect has been co-opted for Cas13a-based detection of pathogenic and cellular nucleic acids with high sensitivity and specificity (*12*).

> ***"With Cas13 now on the scene, RNA engineering is poised to take off."***

In contrast to Cas9 DNase, Cas13 evolved as an RNA-targeted RNase, which does not require a PAMmer for targeted RNA recognition, providing a simple platform for RNA engineering in live cells. Abudayyeh *et al.* identified Cas13a from *Leptotrichia wadei* (LwaCas13a) as the most active in mediating RNA knockdown (achieved through RNA cleavage) in *Escherichia coli* after evaluating a panel of 15 Cas13a orthologues. The corresponding mammalian codon-optimized LwaCas13a fused with monomeric superfolder green fluorescent protein (msfGFP) and a nuclear localization sequence (NLS) (LwaCas13a-msfGFP-NLS) resulted in comparable levels of knockdown with that of position-matched RNA interference with short hairpin RNA (shRNA) on reporter and endogenous mRNAs in mammalian cells; knockdown was also efficient in plant cells. Consistent with biochemical assays, Cas13a-mediated knockdown was abolished by mutating the HEPN catalytic domains of LwaCas13a. In addition, activated Cas13a exhibited no detectable nonspecific or collateral RNA degradation in mammalian cells, as shown by the lack of off-target transcriptomic changes, normal cell growth, and RNA size distribution in cells treated with activated LwaCas13a (*1*). Cas13a-mediated RNA silencing in living cells was most efficient, with a crRNA encoding a 28-nucleotide spacer bearing a 3′ H nucleotide (meaning, any nucleotide but G) protospacer flanking site (PFS) preference; was sensitive to mismatches in the central seed region (the sequence that hybridizes with target RNAs and thus mediates silencing) of the guide-target duplex; efficiently cut nuclear-retained RNAs such as long noncoding RNAs; and could target multiple mRNAs through streamlined multiplexed delivery of LwaCas13a guides. In addition, the catalytically dead LwaCas13a (dLwaCas13a) could bind and track abundant transcripts to manipulate and visualize RNAs over time. These functions of LwaCas13a in mammalian cells provide an unprecedented, flexible platform for the development of new RNA operating tools without genetically encoded RNA aptamers and exogenously expressed aptamer-associated proteins in live cells.

Knockdown with LwaCas13a is not consistently efficient (*1*). To identify a more robust RNA-targeting CRISPR system, Cox *et al.* evaluated 43 mammalian codon-optimized versions of Cas13a, Cas13b, and Cas13c in silencing mRNAs in a reporter gene expression assay in human embryonic kidney cells with PFS-compatible sgRNAs. This effort identified several new Cas13b proteins with high levels of interference activity on reporter mRNAs. Among them, the Cas13b from *Prevotella sp. P5-125* (PspCas13b) had the highest level of RNA interference. PspCas13b achieved consistent, robust, and specific knockdown of mRNA (see the figure) in mammalian cells, showing no requirement of additional protein for its stabilization in live cells; high specificity and consistently increased levels of knockdown relative to LwaCas13a (*1*) and position-matched shRNAs on examined mRNAs; no PFS constraint; and

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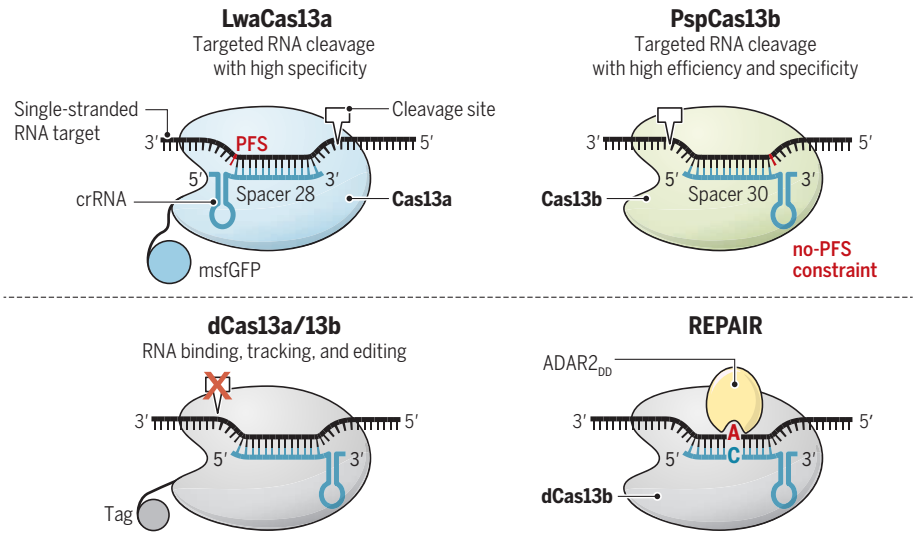
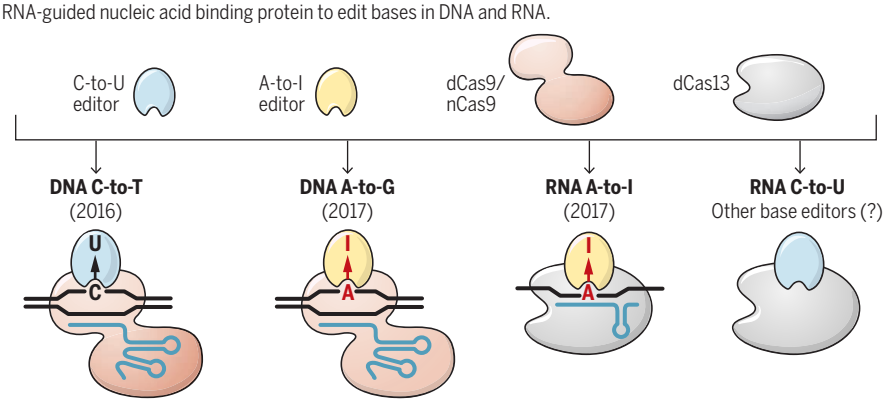
RNA engineering with the RNA-guided RNase Cas13

Two new systems, using LwaCas13a and PspCas13b, have been developed to engineer RNA. PspCas13b has been further developed to edit RNA by combining it with $ADAR2_{DD}$.



Different base-editing platforms

A nucleic acid modification enzyme, such as APOBEC/AID or ADAR1/2, can be combined with an RNA-guided nucleic acid binding protein to edit bases in DNA and RNA.



relative intolerance to single mismatches from base pairs 12 to 26 in 30-nucleotide-long spacers, and even less tolerance for double mismatches. Thus, Cas13b offers several advantages for more precise programmable RNA manipulation over Cas13a (*2*).

Cox *et al.* expand the applications of PspCas13b for RNA engineering to programmable base editing in RNA by fusing catalytically inactive PspCas13b (dCas13b) with the catalytic domain of adenosine (A) to inosine (I) deaminase 2 (ADAR2), a system referred to as RNA Editing for Programmable A to I Replacement (REPAIR). REPAIR directly converts A's to I's on mRNA and so could be used to engineer gene expression without disturbing the coding DNA. Depending on the length and quality of the RNA duplex that usually forms via the intramolecular hybridization of inverted repeat sequences within RNAs, ADAR editing can be either site-selective or promiscuous, and the resulting Is are functionally equivalent to guanosines (Gs) in translation and splicing (*13*). To engineer REPAIR, dCas13b was fused with the deaminase domain of ADAR2 ($ADAR2_{DD}$), which harbors the E488Q mutation to improve editing efficiency and the T375G mutation to decrease off-target editing through destabilization of $ADAR2_{DD}$-RNA binding. The dCas13b-$ADAR2_{DD}$ fusion was recruited to target A's by a 50-nucleotide sgRNA with an A-C mismatch at the target A, with the hybridized RNA creating the required duplex substrate for ADAR activity. This optimized REPAIR system could effectively replace the targeted A with an I at an efficiency of 27% on endogenous mRNAs, with either no detectable or only a handful of off-target edits (*2*). The relatively low A-to-I RNA base-editing efficiency could be due to the naturally low ADAR editing activity because only up to 50% editing was observed, even on long perfectly matched duplexes (*13*). Alternatively, endogenous RNA targets may harbor structures that impede editing or may be coated with proteins, hampering recognition by the sgRNA for dCas13-ADAR binding and subsequent deamination. Nevertheless, the fact that REPAIR has no strict sequence constraints, can edit transcripts containing pathogenic mutations, and can be minimized to fit within the packaging limit of adeno-associated virus (AAV) vectors for viral delivery (*2*) make it a promising tool for RNA editing in research and therapeutics.

Programmable dCas9 or nickase Cas9 (nCas9) fused to members of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family of cytidine deaminases or activation-induced cytidine deaminase (AID) can generate targeted C-to-T edits in DNA (*14*). Also, nCas9 fused to an enzyme with similar function to ADAR can lead to A-to-G base editing in genomic DNA (*15*). Compared with DNA base editing, the RNA base-editing platform described by Cox *et al.* directly converts A's to I's on RNA, independent of endogenous DNA repair pathways that are required for DNA base editing; it also does not have sequence constraints, such as the requirement of a nearby PAM for DNA base editors, and can specify a single A for editing. Importantly, engineering RNA lowers the risk of permanent and inherited off-target edits to DNA, although the diversity of RNA copy numbers and structures could pose challenges for the achievement of efficient base editing in abundant RNAs. Thus, both DNA and RNA bases, including unwanted human disease-associated sites, can be readily edited or corrected by using different base-editing platforms (see the figure).

The development of programmable Cas13 has great potential to promote new and powerful tools for a spectrum of RNA manipulations, from efficient gene knockdown, to high-throughput screening, to visualization and RNA tracking, and to recruitment of RNA-binding proteins to modulate RNA activities and for precise RNA base editing. One thing is certain: With Cas13 now on the scene, RNA engineering is poised to take off. ■

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