



Shooting the messenger: Editing RNA mutations

Educator guide

PAPER DETAILS

Original title: RNA editing with CRISPR-Cas13

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DISCUSSION QUESTIONS

1. What are some of the advantages and disadvantages of using an RNA editing system like REPAIR compared to a DNA editing system? What are the different applications for these two kinds of systems?
2. What are some of the reasonings that shRNA-mediated knockdown causes more off-target effects than Cas13-mediated knockdown?
3. What are some of the reasons for off-target activity with REPAIR? What data support this statement? How did the authors reduce off-target effects with REPAIR? Why is it important to minimize off-target effects?
4. When analyzing off-targets, the authors increased sequencing coverage from 12.5x to 125x and detected more hits. Why did the authors stop at this coverage and not increase it even further? What if they caught more off-targets with 200x coverage?
Check the Supplementary Figures for a hint.
5. The authors suggested several conditions that could be potential targets for REPAIR. What do these conditions have in common? What other, related conditions could REPAIR also target?
6. Could REPAIR be used to treat cancers? How?
Hint: Do some background research on the mechanisms that cause cancer.
7. What are examples of cellular processes that require the activity of deaminases? Can we use REPAIR to control these processes?

LEARNING STANDARDS

SEP7

Structure and function
SP7

SEP6

Structure and function
SP5

SEP4

SEP7

LS1.A

RST.9-10.1

RST.9-10.2

RST.11-12.1

RST.11-12.2

SP5

SEP4

Structure and function
SP5

SP7

VC1

SEP7

SP7

EK3.B.1

RST.11-12.7

RST.11-12.9

SP6

SP7

EK3.B.1

ACTIVITIES FOR INTERACTIVE ENGAGEMENT

Writing an abstract

Students write a new abstract for the article at a grade-appropriate reading level.

Locating this study in the larger field

Students use the annotated list of references to explain how this research builds on the published work of at least one other independent group of scientists. Students will evaluate whether data from this research supports or contradicts previous conclusions, and reflect on the statement that scientific knowledge is a “community effort.”

Science in the news

Students explore news stories in the Related Resources tab and evaluate the stories for tone, accuracy, missing information, etc. They may then write their own news stories on the article.

Diagramming

Sketch out all of the application and subsequent developments (iterations) of the REPAIR system mentioned in the article. Which examples are the easiest to implement/achieve and which ones would require additional work?

Results and conclusions

Students diagram each of the experiments presented in the study (divided up by figure, if appropriate). They then consider the results depicted in each figure, and how these results support the conclusions of the study.

The next steps

Students design a follow-on experiment to this study that either addresses flaws or unanswered questions in the research at hand, or builds on it to explore a new question.

LEARNING STANDARDS

RST.9-10.2
RST.11-12.2
VC1

RST.9-10.8
RST.11-12.8
NS3

RST.9-10.5
RST.11-12.5
RST.9-10.6
RST.11-12.6
RST.9-10.8
RST.11-12.8

Structure and function
SP7
RST.9-10.2
RST.11-12.2
NS2

SEP4
RST.11-12.4

SP6
VC6

ARTICLE OVERVIEW

Article summary (recommended for educator-use only)

Genome editing with programmable enzymes has become a common laboratory tool with the potential to revolutionize medicine. We have learned how to switch genes off, modify them, and regulate their expression. Editing mRNA has remained a challenge with gene editing, as mRNA can be less stable and manageable than other molecules that carry genetic material. The authors of this study created an RNA editing complex by fusing a distant relative of Cas9, the protein Cas13, to the adenosine deaminase, ADAR. Cas13, guided by a short RNA molecule, can recognize a specific sequence in mRNA, and ADAR can modify a target base. The authors found the complex was able to correct mistakes in transcripts but had high off-target activity. Using rational mutagenesis, the researchers were able to increase its specificity by almost three orders of magnitude, making the tool a promising alternative to genome editing techniques.

Importance of this research

Genome editing tools based on CRISPR/Cas9 are ideally suited for gene knockout. However, these tools are limited when a precise modification is needed, especially in non-dividing cells (which may not be rapidly replicating DNA). Further, gene editing tools that modify DNA cut genetic material out, removing it from the genome permanently. To overcome these issues, the authors of this study adapted DNA base-editors (which can introduce changes in individual bases of DNA) to target RNA instead of DNA. This work represents an attempt to correct disease-causing mutations from the other side, at the level of RNA rather than DNA. Due to the transient nature of mRNA, this system will have fewer off-targets.

Experimental methods

- Protein engineering (molecular biology techniques, plasmid construction)
 - Design of fusion proteins: Optimization of codons, choice of localization sequence, testing multiple fusion variants.
 - Structure-guided mutagenesis: Generation of protein variants with mutations at amino acids important for the function.
- *Gluc/Cluc* luciferase assay
 - To monitor the effect of Cas13 nuclease activity, the researchers constructed a vector that contained genes for two different luciferases. These luciferases use different substrates for the generation of light. This enabled the researchers to measure signals from both of them without interference. In control cells with irrelevant gRNA, both luciferases should produce maximal signal. If a gRNA targets the *Gluc* luciferase gene and a nuclease is active or has favorable conditions, the corresponding *Gluc* signal should decrease. To correct for transfection level, the authors normalized the *Gluc* signal to the *Cluc* signal.
- Ampicillin interference assay
 - To determine the targeting preference for Cas13 orthologs, the researchers transformed bacterial cells with two plasmids. One contained a Cas13b ortholog while the second plasmid included an ampicillin resistance gene and a gRNA against this gene with randomly generated 5' and 3' PFS around the target

sequence. If targeting were successful due to favorable PFS, bacterial cells died because they lost ampicillin resistance (it was removed by the gene editor). The surviving cells were collected and analyzed by deep sequencing, which provided PFS motifs irrelevant for targeting (those that the gene editor did not have any effect on). By subtracting these PFSs from the starting pool, the researchers obtain preferred PFSs.

- *Cluc* luciferase assay
 - The researchers “broke” the gene for the *Cluc* luciferase by introducing a mutation in the UGG codon with the resultant UAG stop codon. As this codon was positioned in the beginning of the *Cluc* transcript, no luciferase was synthesized in the cell. If the Cas13b-mediated RNA recognition and the ADAR-mediated editing were able to fix the mutation, *Cluc* expression was restored.
- Library luciferase screening
 - This method was used to determine whether the Cas13 nucleases would cleave an RNA molecule if the target sequence contained mutations. The authors generated a library, i.e., a collection, of plasmids with the *Gluc* gene where each plasmid had a mutation in the gRNA target site. This was a big library with all possible single- and double-nucleotide substitutions in the 36-nt target site. The authors then transfected cells with a nuclease, a gRNA for the non-mutated target site, and a whole library with all possible plasmid variants. After 48 hours, the researchers collected cells, extracted RNA, and determined which of the mutated sequences from the library were left uncleaved. The transcript with the unmodified sequence was depleted most efficiently so that its level was the lowest after the cleavage. The levels of all other sequences with substitutions decreased to a lesser extent or did not decrease at all. The better Cas13 cut the sequence, the higher the depletion of this sequence was.
- RNA sequencing
 - Determining the sequences and abundance of all possible mRNA molecules in cells. The researchers used this method to determine targeting preferences for Cas13 and REPAIR, to measure the editing rate for exogenous and endogenous transcripts, as well as to identify off-target events.
- shRNA assay
 - This method was directly compared with the REPAIR system. The shRNA molecules for RNAi and the gRNA molecules for REPAIR were position-matched to account for the varying sequence properties along the transcript.

Conclusions

- The Cas13b protein demonstrated a high level of knockdown and produced minimal off-target effects. Moreover, Cas13-mediated knockdown was much more efficient and specific than shRNA-mediated knockdown.
- The fusion between catalytically dead Cas13b and ADAR with enhanced deamination capability (the REPAIR system) could successfully bind and edit exogenous and endogenous RNA targets. Its off-target activity could be reduced by choosing a gRNA with a specific length and with a certain positioning relative to the target site.
- The REPAIR system has no significant preferences at either PFS or the nucleotides flanking the target. This implies that REPAIR can correct adenosine in any sequence context and can potentially target all G>A disease-causing mutations.
- Rational mutagenesis of the ADAR2 domain allowed the researchers to increase the specificity of the REPAIR system and reduce levels of off-target editing by almost three orders of magnitude.

LEARNING STANDARDS ALIGNMENT

The following tables provide an overview of the learning standards covered by this article, including the A Framework for K-12 Science Education (Framework), Common Core State Standards English Language Arts-Literacy (CCSS ELA), Common Core State Standards Statistics and Probability (CCSS HSS), AP Science Practices, and Vision and Change for Undergraduate Education. Where applicable, activities and information will be marked with specific standards to which they are linked.

A Framework for K-12 Science Education		
Science and Engineering Practices	Disciplinary Core Ideas	Crosscutting Concepts
<p>Analyzing and Interpreting Data (SEP4) Analyze data to identify design features or characteristics of the components of a proposed process or system to optimize it relative to criteria for success.</p> <p>Constructing Explanations and Designing Solutions (SEP6) Apply scientific reasoning, theory, and/or models to link evidence to the claims to assess the extent to which the reasoning and data support the explanation or conclusion.</p> <p>Engaging in Argument from Evidence (SEP7) Make and defend a claim based on evidence about the natural world or the effectiveness of a design solution that reflects scientific knowledge and student-generated evidence.</p>	<p>LS1.A: Structure and Function All cells contain genetic information in the form of DNA molecules. Genes are regions in the DNA that contain the instructions that code for the formation of proteins, which carry out most of the work of cells.</p> <p>LS3.A: Inheritance of Traits Each chromosome consists of a single very long DNA molecule, and each gene on the chromosome is a particular segment of that DNA. The instructions for forming species' characteristics are carried in DNA. All cells in an organism have the same genetic content, but the genes used (expressed) by the cell may be regulated in different ways. Not all DNA codes for a protein; some segments of DNA are involved in regulatory or structural functions, and some have no as-yet known function.</p>	<p>Structure and function The ways in which an object or living thing is shaped and its substructure determine many of its properties and functions</p>

Common Core State Standards English Language Arts-Literacy

Key Ideas and Details	Craft and Structure	Integration of Knowledge and Ideas
<p>RST.9-10.1 Cite specific textual evidence to support analysis of science and technical texts, attending to the precise details of explanations or descriptions.</p> <p>RST.9-10.2 Determine the central ideas or conclusions of a text; trace the text’s explanation or depiction of a complex process, phenomenon, or concept; provide an accurate summary of the text.</p> <p>RST.11-12.1 Cite specific textual evidence to support analysis of science and technical texts, attending to important distinctions the author makes and to any gaps or inconsistencies in the account.</p> <p>RST.11-12.2 Determine the central ideas or conclusions of a text; summarize complex concepts, processes, or information presented in a text by paraphrasing them in simpler but still accurate terms.</p>	<p>RST.9-10.4 Determine the meaning of symbols, key terms, and other domain-specific words and phrases as they are used in a specific scientific or technical context relevant to grades 9-10 texts and topics.</p> <p>RST.9-10.5 Analyze the structure of the relationships among concepts in a text, including relationships among key terms (e.g., force, friction, reaction force, energy).</p> <p>RST.9-10.6 Analyze the author’s purpose in providing an explanation, describing a procedure, or discussing an experiment in a text, defining the question the author seeks to address.</p> <p>RST.11-12.4 Determine the meaning of symbols, key terms, and other domain-specific words and phrases as they are used in a specific scientific or technical context relevant to grades 11-12 texts and topics.</p> <p>RST.11-12.5 Analyze how the text structures information or ideas into categories or hierarchies, demonstrating understanding of the information or ideas.</p> <p>RST.11-12.6 Analyze the author’s purpose in providing an explanation, describing a procedure, or discussing an experiment in a text, identifying important issues that remain unresolved.</p>	<p>RST.9-10.8 Assess the extent to which the reasoning and evidence in a text support the author’s claim or a recommendation for solving a scientific or technical problem.</p> <p>RST.9-10.9 Compare and contrast findings presented in a text to those from other sources (including their own experiments), noting when the findings support or contradict previous explanations or accounts.</p> <p>RST.11-12.8 Evaluate the hypotheses, data, analyses, and conclusions in a science or technical text, verifying the data when possible and corroborating or challenging conclusions with other sources of information.</p> <p>RST.11-12.9 Synthesize information from a range of sources (e.g., texts, experiments, simulations) into a coherent understanding of a process, phenomenon, or concept, resolving conflicting information when possible.</p>

AP Science Standards	
AP Science Practices	AP Biology Content Standards
<p>Science Practice 5 (SP5) The student can perform data analysis and evaluation of evidence.</p> <p>Science Practice 6 (SP6) The student can work with scientific explanations and theories.</p> <p>Science Practice 7 (SP7) The student is able to connect and relate knowledge across various scales, concepts and representations in and across domains.</p>	<p>Essential knowledge 3.A.1 (EK3.A.1) DNA, and in some cases RNA, is the primary source of heritable information.</p> <p>Essential knowledge 3.C.1 (EK3.C.1) Changes in genotype can result in changes in phenotype.</p> <p>Essential knowledge 4.A.1 (EK4.A.1) The subcomponents of biological molecules and their sequence determine the properties of that molecule.</p>

Connections to the Nature of Science	
Vision and Change for Undergraduate Biology Education Core Competencies and Disciplinary Practices	A Framework for K-12 Science Education Understandings About the Nature of Science
<p>Ability to apply the process of science (VC1) Use observational strategies and formulate hypotheses. Design experiments and evaluate accuracy and reliability of experimental evidence.</p> <p>Ability to understand the relationship between science and society (VC6) Understand how experimental results could affect society and evaluate ethical implications of biological research.</p>	<p>Scientific knowledge is based on empirical evidence (NS2) Science includes the process of coordinating patterns of evidence with current theory.</p> <p>Scientific knowledge is open to revision in light of new evidence (NS3) Most scientific knowledge is quite durable but is, in principle, subject to change based on new evidence and/or reinterpretation of existing evidence.</p>