Precise transcript targeting by CRISPR-Csm complexes

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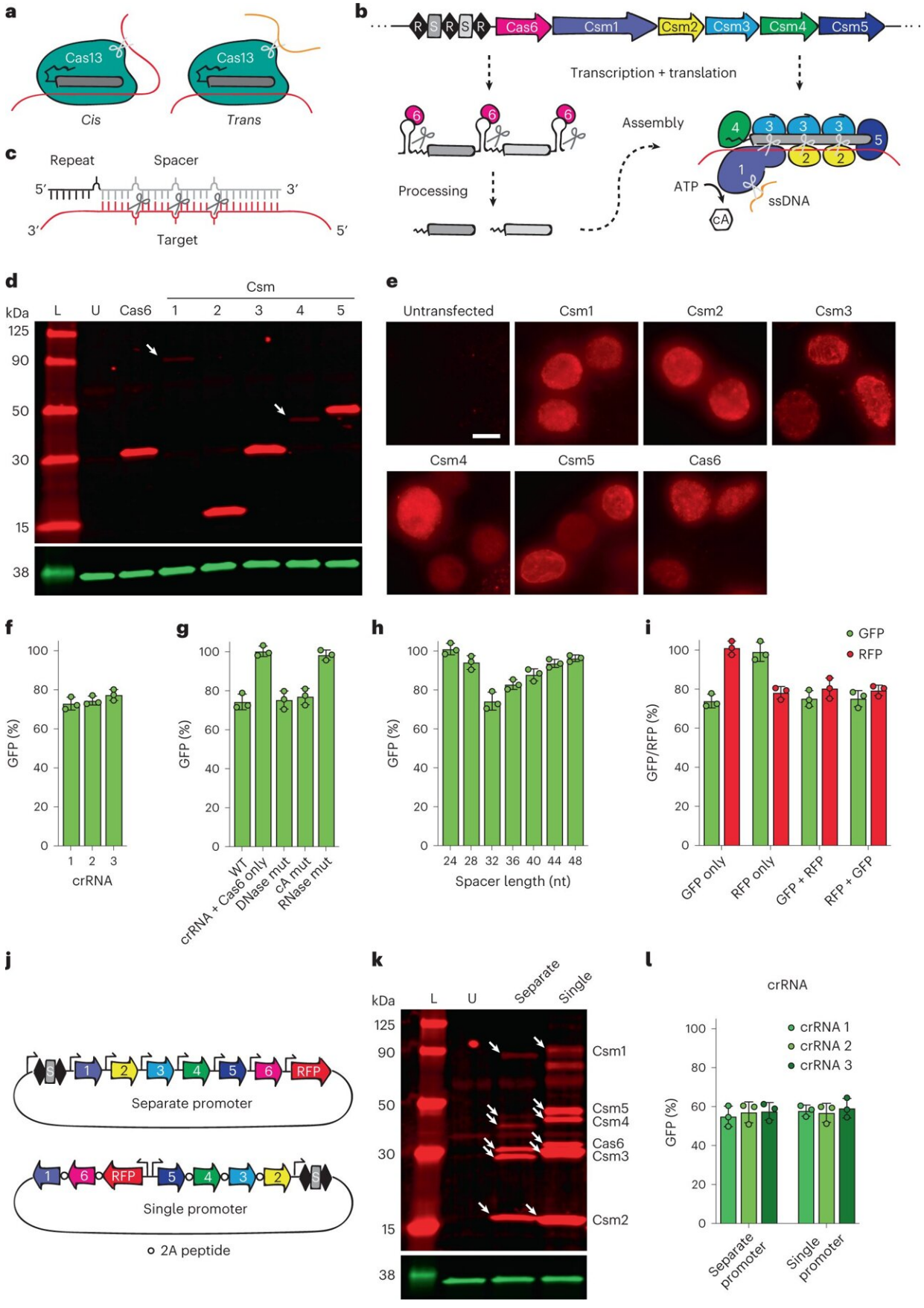

a
Cas13
Cas13
Cis
Trans
b
R S R S R
Cas6
Csm1
Csm2
Csm3
Csm4
Csm5
Transcription + translation
Processing
Assembly
ATP
ssDNA
cA
c
Repeat
Spacer
5′
3′
3′
5′
Target
d
Csm
kDa
L
U
Cas6
1
2
3
4
5
125
90
50
30
15
38
e
Untransfected
Csm1
Csm2
Csm3
Csm4
Csm5
Cas6
f
GFP (%)
crRNA
g
GFP (%)
WT
crRNA + Cas6 only
DNase mut
cA mut
RNase mut
h
GFP (%)
Spacer length (nt)
i
GFP
RFP
GFP/RFP (%)
GFP only
RFP only
GFP + RFP
RFP + GFP
j
Separate promoter
Single promoter
2A peptide
k
kDa
L
U
Separate
Single
Csm1
Csm5
Csm4
Cas6
Csm3
Csm2
l
crRNA
crRNA 1
crRNA 2
crRNA 3
GFP (%)
Separate promoter
Single promoter

An all-in-one type III CRISPR-Cas system in human cells. (a) Diagram showing cis- and trans-cleavage of Cas13. (b) Diagram showing S. thermophilus type III-A CRISPR-Cas locus. crRNAs are transcribed from the CRISPR array, processed by Cas6 and assemble with Csm proteins. (c) Close-up of crRNA:target binding, showing the 6-nt cleavage pattern. (d) Western blot showing proper size and expression of Cas/Csm proteins (red) in HEK293T cells. Csm1 and Csm4 are less stable when expressed separately. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) shown as loading control (green). Arrows indicate faint bands. L, ladder; U, untransfected. One of two replicates with similar results is shown. (e) Immunofluorescence showing expression and nuclear localization of Cas/Csm proteins in HEK293T cells. Scale bar, 10 μm. One of two replicates with similar results is shown. (f) Relative GFP fluorescence (= MFI targeting crRNA/MFI nontargeting crRNA) of HEK293T-GFP cells transfected with plasmids expressing Cas6, Csm1-5 and the indicated GFP-targeting crRNA, measured by flow cytometry. Error bars indicate mean ± s.d. of three biological replicates. g, Same as f, but with the indicated Csm mutants (or crRNA + Cas6 only). GFP crRNA 1 was used to target GFP. Error bars indicate mean ± s.d. of three biological replicates. h, Same as f, but with GFP crRNA 1 adjusted to the indicated spacer length. Error bars indicate mean ± s.d. of three biological replicates. (i) Relative GFP and RFP fluorescence of HEK293T-GFP/RFP cells transfected with plasmids expressing Cas6, Csm1-5 and the indicated crRNAs (individual or multiplexed), measured by flow cytometry. GFP crRNA 1 was used to target GFP. RFP-targeting crRNA is listed in Supplementary Table 1 of the paper. Error bars indicate mean ± s.d. of three biological replicates. (j) Diagram showing all-in-one delivery vector designs. (k) Western blot showing proper size and expression of Cas/Csm proteins (red) in HEK293T cells. GAPDH is shown as loading control (green). Arrows indicate each subunit. One of two replicates with similar results is shown. l, Relative GFP fluorescence of HEK293T-GFP cells transfected with the indicated delivery vectors and expressing the indicated GFP-targeting crRNAs, measured by flow cytometry. Error bars indicate mean ± s.d. of three biological replicates. Credit: *Nature Biotechnology* (2023). DOI: 10.1038/s41587-022-01649-9

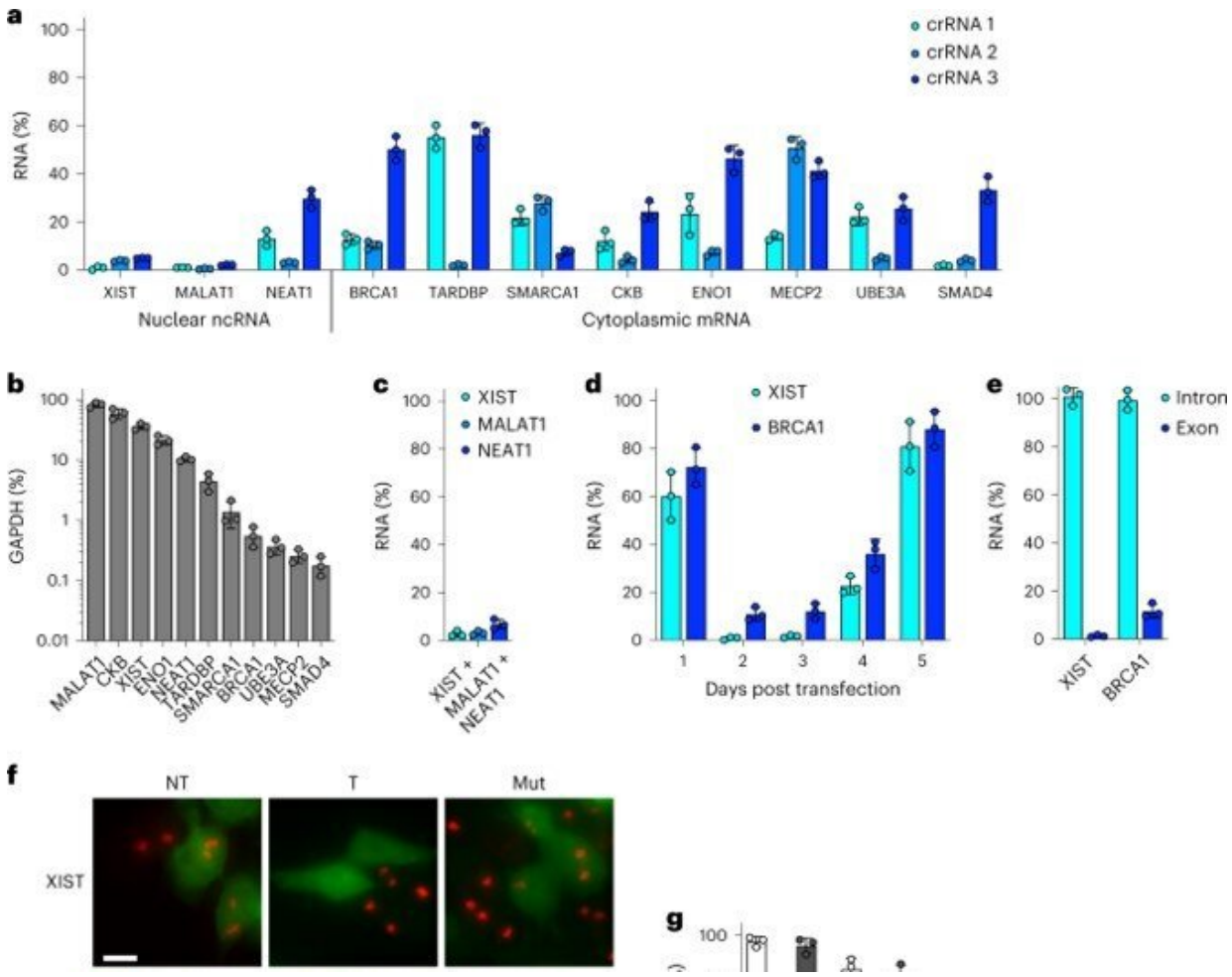
Mammalian cells are inherently complex due to subcellular compartments, thereby making the process of robust transcript targeting of nucleic acids somewhat challenging in the molecular biology lab. In a recent report now published in *Nature Biotechnology*, David Colognori and a research team headed by Chemistry Nobel Laureate Jennifer Doudna, who discovered and expanded on the CRISPR-Cas9 technology alongside Emmanuel Charpentier, in the year 2020, incorporated a new complex to the CRISPR complex during this study.

The team used the clustered regularly incorporated short palindrome repeats (CRISPR)-Csm complex; which included a protein known as Csm, a type III-A CRISPR-Cas interference complex found in the prokaryotic immune system. Thereafter, the molecular biologists accomplished surgical RNA ablation (deletion) of the nuclear and cytoplasmic transcripts via single-vector delivery. The vector-bound Steptococcus thermophilus Csm-complex provided high-efficiency RNA knockdown; a method to silence gene expression with minimal off-target impact in human cells and outperform existing genome editing technologies such as short-hairpin RNA and Cas13-mediated knockdown. By catalytically inactivating the Csm, the team achieved durable RNA binding for live-cell RNA imaging to establish the efficiency of the CRISPR-Cas effector system as RNA-targeting tools in eukaryotes.

Gene editing: From RNA interference methods to CRISPR-Cas complexes

Molecular biologists seek to alter RNA and protein levels without permanently affecting DNA; however, the task is nontrivial in basic research and in therapeutic applications. In the past, scientists had developed targeted RNA knockdown techniques in eukaryotes by using RNA interference or RNAi, whereby small interfering RNA-directed

argonaute nucleases (the active part of the RNA-induced silencing complex) cleaved complementary target RNAs. However, this method led to unintended cleavage of targets carrying partially similar or complementary sequences, especially when the complementarity occurred in the nucleating "seed" region of the siRNA, while being incompatible with eukaryotic model systems.



Robust KD of endogenous nuclear and cytoplasmic RNAs. (a) Relative RNA abundance (normalized to nontargeting crRNA) of the indicated targets in HEK293T cells transfected with all-in-one plasmid expressing Cas/Csm proteins and the indicated crRNAs, measured by RT-qPCR. Error bars indicate mean ± s.d. of three biological replicates. (b) Relative RNA abundance (normalized to

GAPDH) of the indicated targets in untransfected HEK293T cells, measured by RT-qPCR. Error bars indicate mean ± s.d. of three biological replicates. (c) Relative RNA abundance (normalized to nontargeting crRNA) of the indicated targets in HEK293T cells transfected with all-in-one plasmid expressing Cas/Csm proteins and the indicated crRNAs (multiplexed), measured by RT-qPCR. XIST crRNA 1, MALAT1 crRNA 1 and NEAT1 crRNA 2 were used to target XIST, MALAT1 and NEAT1, respectively. Error bars indicate mean ± s.d. of three biological replicates. (d) Relative RNA abundance (normalized to nontargeting crRNA) of XIST and BRCA1 in HEK293T cells at the indicated times post transfection with all-in-one plasmid, measured by RT-qPCR. XIST crRNA 1 and BRCA1 crRNA 2 were used to target XIST and BRCA1, respectively. Error bars indicate mean ± s.d. of three biological replicates. (e) Relative RNA abundance (normalized to nontargeting crRNA) of XIST and BRCA1 in HEK293T cells transfected with all-in-one plasmid expressing Cas/Csm proteins and intron- or exon-targeting crRNAs, measured by RT-qPCR. XIST crRNA 1 and BRCA1 crRNA 2 were used to target XIST and BRCA1 exons, respectively. Intron-targeting crRNAs are listed in Supplementary Table 1. Error bars indicate mean ± s.d. of three biological replicates. (f), RNA FISH (red) for the indicated targets in HEK293T cells transfected with all-in-one plasmid expressing targeting (T) or nontargeting (NT) crRNA and RNase-active or -inactive (Mut) Cas/Csm proteins. Untransfected cells serve as internal control for transfected (green) cells. XIST crRNA 1, MALAT1 crRNA 1 and NEAT1 crRNA 2 were used to target XIST, MALAT1 and NEAT1, respectively. Scale bar, 10 μm. g, Quantification of f. One hundred transfected cells were counted for each condition. Error bars indicate mean ± s.d. of three biological replicates. Credit: *Nature Biotechnology* (2023). DOI: 10.1038/s41587-022-01649-9

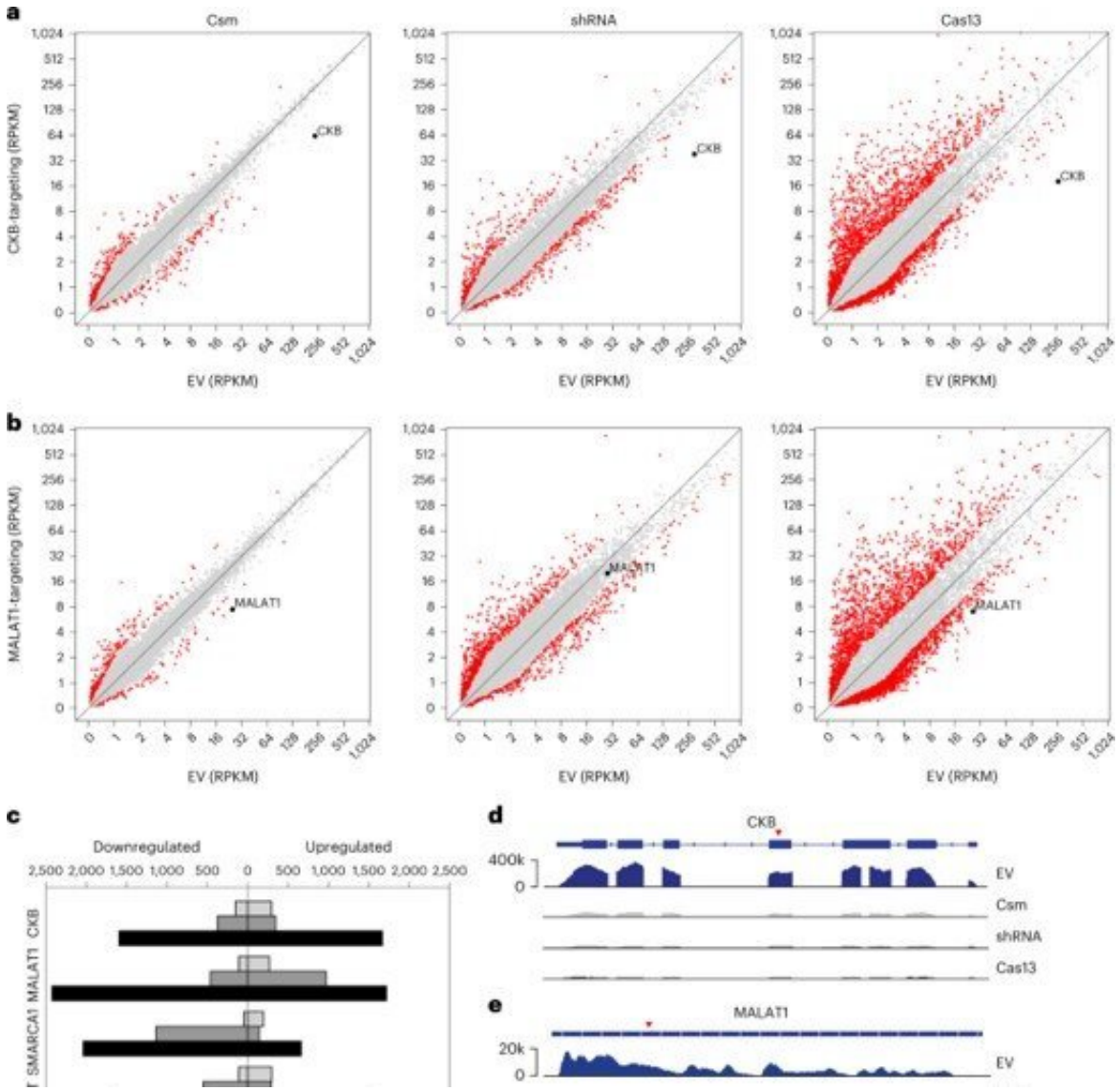
Meanwhile, the now-widely popular clustered regularly interspaced short palindrome repeats (CRISPR) and CRISPR associated proteins (CRISPR-Cas) that form adaptive defense systems against infectious agents in prokaryotes can function as DNA or RNA nucleases that can be regulated for gene editing applications. Much like small interfering RNA, the Cas nuclease is composed of small RNAs to recognize nucleic

acid targets through complementary base-pairing. In this work, Colognori and colleagues used the Csm protein as an attractive tool for RNA knockdown or gene silencing. The protein is exclusively found in prokaryotes and can be introduced to eukaryotes without the use of intersecting host regulatory pathways, allowing the research team to demonstrate the Csm system as a versatile RNA knockdown method in eukaryotes.

The all-in-one type III CRISPR-Cas system

The scientists chose the type III-A Csm complex from Streptococcus thermophilus based on a few features, such as—its existing biochemical and structural characterization in bacteria, optimal functionality, and preceding work conducted in Zebrafish embryos, and in other eukaryotic cells, including human cell cultures. The team verified the expression of individual protein components in immortalized human embryonic kidney cells and simplified the delivery of the Csm system by incorporating all components into a single vector. They used Csm to knockdown highly overexpressed and heterologous transgenes and studied the knockdown at the protein level.

During the study, the team transfected the kidney cells with an all-in-one vector to achieve more than 90% knockdown for all RNAs of interest. The results highlighted the highly robust nature of Csm as an efficient RNA knockdown tool. The researchers verified the outcomes by performing RNA fluorescent in situ hybridization (FISH) to visualize the characteristic morphologies. The work showed how the active Csm complexes yielded similarly robust knockdown via both molecular and microbiology-related methods.



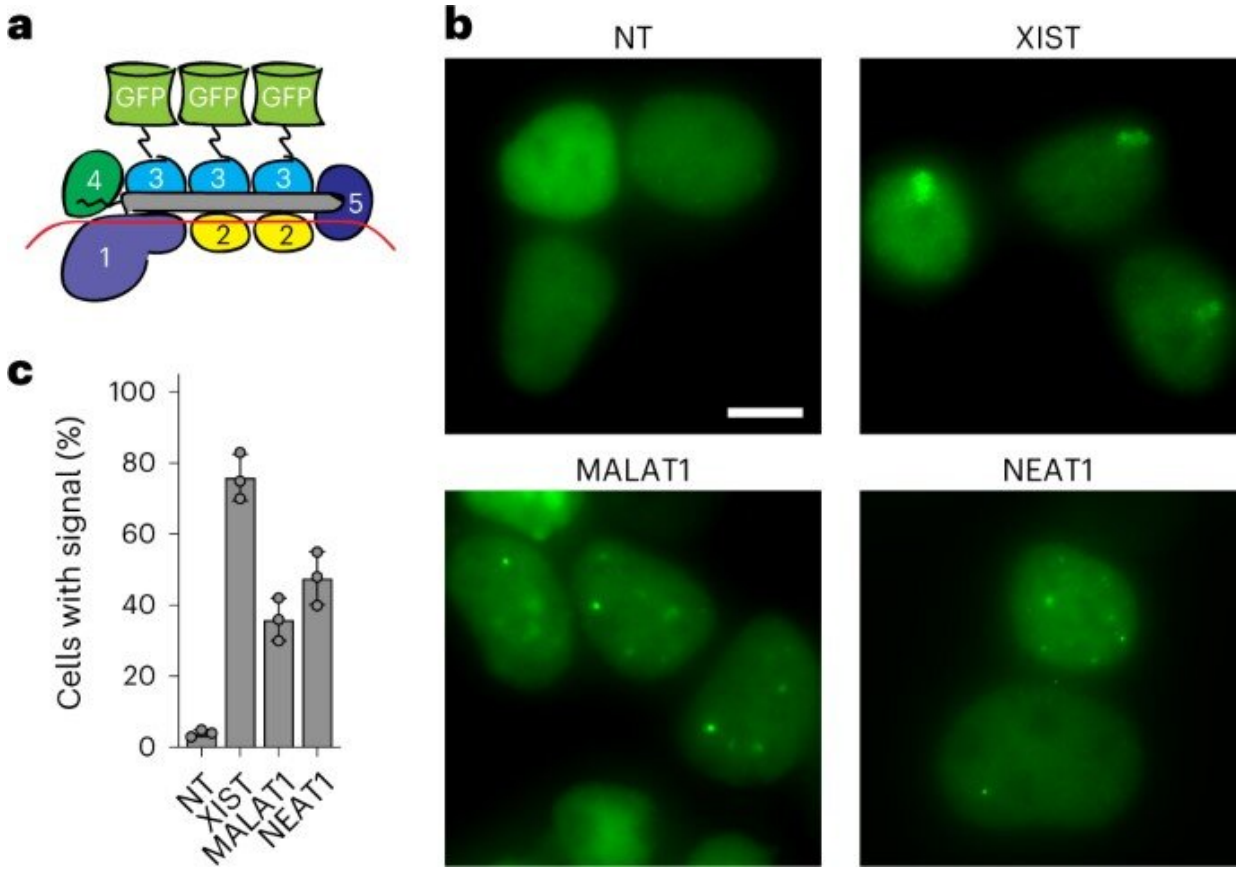
RNA KD with minimal off-targets or cytotoxicity. (a,b,) Scatterplots showing differential transcript levels between HEK293T cells transfected with plasmid expressing Csm, Cas13 or shRNA targeting CKB (a) or MALAT1 (b) versus EV control. Target transcript indicated in black; off-targets (≥2-fold change) indicated in red. (c), Quantification of upregulated or downregulated transcripts (≥2-fold change) for each sample. CKB crRNA 1, MALAT1 crRNA 2, SMARCA1 crRNA 1 and XIST crRNA 1 were used to target CKB, MALAT1, SMARCA1 and XIST, respectively. (d,e), RNA-seq read coverage across target transcripts CKB (d) or MALAT1 (e). Red arrow indicates location of crRNA/shRNA target site. (f), Relative cell viability and proliferation (normalized to EV control) of HEK293T cells at the indicated times post transfection with the indicated targeting (T) or nontargeting (NT) plasmids,

measured by WST-1 assay. CKB crRNA 1 was used for targeting. Error bars indicate mean ± s.d. of three biological replicates. (g), Relative abundance of RFP-positive (transfected) HEK293T cells at the indicated times post transfection with the indicated targeting (T) or nontargeting (NT) plasmids, measured by flow cytometry. CKB crRNA 1 was used for targeting. Error bars indicate mean ± s.d. of three biological replicates. Credit: *Nature Biotechnology* (2023). DOI: 10.1038/s41587-022-01649-9

Observing RNA knockdown activity with minimal off-targets and cytotoxicity

In order to assess the potential off-target effects of Csm-mediated knockdown in cells, the research team next performed RNA sequencing. They compared the efficiency of the complex with existing methods and performed knockdown for 48 hours. The team then completed fluorescence-activated cell sorting and sequencing methods with the transfected cells.

The results showed that the Csm expression did not disturb the cell environment. The researchers removed DNAse activity without affecting RNAse activity for minimal off-target effects of Csm-mediated RNA knockdown in human cells. When compared to severely cytotoxic Cas13 RNA-targeting CRISPR-Cas systems, the type III system did not display trans-cleavage activity for robust RNA knockdown and were without toxicity.



Live-cell RNA imaging without genetic manipulation. (a) Diagram showing Csm3-GFP fusion complex used for live-cell imaging. (b) Live-cell fluorescence imaging of HEK293T cells transfected with plasmid expressing Csm3-GFP fusion complex and the indicated crRNAs (Supplementary Table 1). NT, nontargeting. Scale bar, 10 μm. (c), Quantification of b. One hundred transfected cells were counted for each condition. Error bars indicate mean ± s.d. of three biological replicates. Credit: *Nature Biotechnology* (2023). DOI: 10.1038/s41587-022-01649-9

Live-cell RNA imaging without genetic manipulation

The team next sought to understand the process of tracking RNA in cells. While the process of tracking RNA in live cells is nontrivial,

molecular biologists had previously accomplished the task with RNA-binding proteins such as the catalytically inactive Cas13. The researchers explored the possibility of using the Csm-complex to track RNA target in live cells by fusing green fluorescent protein (GFP) to the catalytically inactive Csm protein. Next, they transfected the kidney cell line with the Csm-GFP plasmid and assayed live-cell fluorescence microscopy after 48 hours to show that the protein could easily visualize RNA in living cells.

Outlook

In this way, David Colognori and colleagues at the Doudna Lab showed the possibility of integrating the type III-A Csm complex derived from the prokaryote S. thermophilus as a powerful tool to accomplish eukaryotic RNA knockdown and gene silencing. The team conducted RNA knockdown experiments with high efficiency and specificity when compared to existing methods and outperformed existing competition. Most notably, the process was not accompanied by detectable cytotoxicity unlike other Cas13-based methods.

The researchers facilitated competent transfection of the multicomponent CRISPR-Cas systems in to a single deliverable plasmid. The capacity to effectively bring type III systems into eukaryotes for the first time in this way has significant implications across RNA diagnostics, health screens and for synthetic biology circuits in vivo.

More information: David Colognori et al, Precise transcript targeting by CRISPR-Csm complexes, *Nature Biotechnology* (2023). DOI: 10.1038/s41587-022-01649-9

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