

Repair of CRISPR-guided RNA breaks enables site-specific RNA excision in human cells

Anna Nemudraia, Artem Nemudryi, Blake Wiedenheft

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published in Science 384, 2024-04-25, doi: 10.1126/science.adk5518.

Title: Repair of CRISPR-guided RNA breaks enables site-specific RNA excision in human cells.

Authors: Anna Nemudraia^{1†}, Artem Nemudryi^{1†*}, and Blake Wiedenheft^{1*}

Affiliations:

¹ Department of Microbiology and Cell Biology, Montana State University; Bozeman, MT, 59717, USA

[†]These authors contributed equally to this work

*Corresponding authors. Email: artem.nemudryi@gmail.com and bwiedenheft@gmail.com

Abstract: Genome editing with CRISPR RNA-guided endonucleases generates DNA breaks that are resolved by cellular DNA repair machinery. However, analogous methods to manipulate RNA remain unavailable. Here, we show that site-specific RNA breaks generated with type III CRISPR complexes are repaired in human cells, and this repair can be used for programmable deletions in human transcripts to restore gene function. Collectively, this work establishes a technology for precise RNA manipulation with potential therapeutic applications.

One-Sentence Summary: CRISPR-guided RNA breaks are repaired in human cells, and this RNA repair can be used for programmable editing of human transcriptomes.

Main Text: CRISPR-guided endonucleases have enabled programmable DNA cleavage and the development of new therapeutics (1, 2). The first generation of CRISPR genome editing used Cas9 nuclease to make double-stranded DNA breaks that are repaired by the cell, leading to site-specific edits (3). These technologies have enriched our mechanistic understanding of DNA repair, and new insights into repair are used to improve methods for genome editing (4).

While DNA editing has the potential to cure genetic diseases, it can result in unintended changes to the genome (5-8) and toxic cellular stress responses (9, 10). In contrast, RNA editing can alter the cellular program without changing the DNA. However, options for editing RNA are limited. Cas13, a type VI CRISPR-guided endoribonuclease, has been used for RNA knockdown, but target recognition also activates collateral nuclease activity that degrades non-target RNAs (11, 12). Nuclease-inactivated mutants of Cas13 (dCas13) have been used for exon skipping (13), trans-splicing of synthetic RNA payloads (14), or sequence-specific delivery of base-editing enzymes for adenosine-to-inosine or cytosine-to-uracil conversions (15, 16). However, programmable deletions in RNA, analogous to those introduced by DNA editors, have not been described.

To address the need for versatile and facile RNA manipulation, we have developed a technology for sequence-specific RNA editing in living cells. This technology uses type III-A CRISPR complexes for programmable cleavage of target RNA. Unlike Cas13, type III complexes exclusively cleave the target RNA in six nucleotide increments (17, 18). We repurpose this cleavage activity to excise segments from human transcripts and show that resulting RNA fragments are repaired, which produces programmable deletions in target RNA. We apply this method to remove a non-sense mutation common in cystic fibrosis patients, which rescues protein expression and demonstrates the therapeutic potential of this technique. Overall, this work establishes a technology for RNA manipulation in living cells and provides a model for discovering new RNA repair pathways.

Programmable deletions in human transcripts

The programmable RNase activity of type III-A complexes has been used for RNA knockdown (19-21), but we hypothesized that cells repair CRISPR-guided RNA breaks, which could be leveraged for RNA editing (**Fig. 1A**). To test this hypothesis, we targeted two human transcripts, *PPIB* and *PARK7*, that are highly expressed in 293T cells (**fig. S1A**). Mutations in *PPIB* are associated with severe osteogenesis imperfecta, while mutations in *PARK7* are linked to Parkinson's disease (22). We transfected 293T cells with plasmids that express type III CRISPR-associated proteins from *Streptococcus thermophilus* (Csm2 – Csm5, Cas10, and Cas6) (19) fused to a nuclear localization signal (NLS) and RNA guides complementary to *PPIB* or *PARK7* mRNAs (SthCsm complex; **fig. S1**).

To quantify target transcripts, we designed RT-qPCR primers that flank target regions (**fig. S1**). Expression of the NLS-tagged SthCsm complex results in >90% reduction of target transcript levels compared to cells transfected with a non-complementary guide RNA (**Fig. 1B**), which is similar to the knockdown efficiencies reported for other guides (19). We hypothesized that the remaining target RNA was either not cleaved, cleaved and repaired without modification, or cleaved and repaired with indels at the target site (**Fig. 1C**). To test this hypothesis, we deep-sequenced *PPIB* and *PARK7* amplicons. We detected 6, 12, 18, or 24 nucleotide deletions in each of the two RNA targets, which are consistent with the six-nucleotide cleavage pattern of the SthCsm complex and not seen in controls transfected with a non-targeting guide (**Fig. 1D**). SthCsm-dependent deletions were detected in $4.4 \pm 0.6\%$ (guide 1) and $5.9 \pm 0.6\%$ (guide 2) of

PPIB amplicons, and $23.9 \pm 2.3\%$ (guide 1) and $65.8 \pm 3.6\%$ (guide 2) of *PARK7* amplicons (**Fig. 1D, fig. S1**). These data demonstrate that most of the target RNA is degraded, but some of the RNA is repaired in human cells.

To measure the kinetics of RNA repair, we extracted RNA from 293T cells at various time points after transfection with SthCsm plasmid targeting *PPIB* or *PARK7* transcripts. RT-qPCR and amplicon-seq demonstrated that transcripts with RNA deletions were detectable at 12 h post-transfection, accumulated over time, peaked at 48 h, and decreased with decreasing expression of the SthCsm (**Fig. 1, E and F; fig. S2**). The loss of SthCsm expression is likely due to plasmid loss with cell division. The RNA knockdown plateaued at 36 h, but the quantity of edited transcripts rose until 48 h, suggesting an accumulation of edited transcripts that cannot be re-cleaved. The decrease of edited RNA after 48 h suggests that the accumulation is capped by the RNA half-life, which was estimated as 11.4 h for *Ppib* and 7.8 h for *Park7* mouse transcripts (23).

To confirm that RNA deletions in *PPIB* and *PARK7* are produced by RNA cleavage, we used an inactive SthCsm complex with D33A amino acid substitution in the Csm3 ribonuclease (18). Recent work demonstrated robust knockdown of nuclear transcripts (i.e., *XIST*, *MALAT1*, and *NEAT1*) with the SthCsm complex. However, no knockdown is detected with inactive SthCsm complexes (19). To our surprise, targeting transcripts that are exported from the nucleus (i.e., *PPIB* and *PARK7*) with catalytically inactive NLS-tagged Csm complex (NLS-Csm^{dead}) resulted in a robust knockdown that was identical to the knockdown with nuclease-active Csm complexes (NLS-Csm^{wt}; $P = 0.88$) (**Fig. 1G; fig. S2**). Expression of the crRNA alone or crRNA together with crRNA-processing protein Cas6 resulted in far less knockdown, demonstrating that the Csm proteins are necessary for efficient knockdown. While knockdown efficiencies are similar for NLS-Csm^{dead} and NLS-Csm^{wt}, only the nuclease active complex produced programmable RNA excision (**Fig. 1H**). Sequencing target RNA after knockdown identified the characteristic deletions (increments of six) only in cells transfected with the NLS-Csm^{wt} plasmid (**fig. S2**).

RTCB ligase repairs type III CRISPR-mediated RNA breaks

RNA cleavage by type III CRISPR complexes leaves a 2',3'-cyclic phosphate (2',3'>P) and a 5'-hydroxyl (5'-OH) at each cut site (17). Mammalian RNA 2',3'-cyclic phosphate and 5'-OH ligase (RTCB) joins 2',3'>P and 5'-OH termini when introns are excised during tRNA splicing and non-conventional splicing of *XBP1* mRNA in the unfolded protein response (24-27) (**Fig. 2A**). To test if RTCB ligase repairs transcripts cleaved by SthCsm in human cells, we used Cas9 nuclease to knockdown RTCB in 293T cells (**fig. S3, see Supplementary Text for details**), and targeted *PARK7* transcript in cells with or without the RTCB depletion (**Fig. 2, B and C**). In wildtype 293T cells, $2.3 \pm 0.1\%$ of the target RNA was detected after transfection with SthCsm plasmid, while target RNA was ~ 1.5 -fold lower ($1.5 \pm 0.3\%$; $P = 0.029$) in RTCB-depleted cells.

Deep-sequencing of *PARK7* amplicons showed that the proportion of reads with deletions is significantly decreased in cells with depleted RTCB (**Fig. 2D**). In samples with wildtype levels of RTCB, deletions in target sequence were detected in $59.7 \pm 5.1\%$ of the reads, while the frequency of these deletions was $24.4 \pm 4.8\%$ ($P < 0.001$) in cells with depleted RTCB (**Fig. 2E**). Complementation of RTCB with a plasmid encoding RTCB cDNA increased the abundance of ligated *PARK7* transcript ~ 1.5 -fold compared to an untransfected control (**Fig. 2F**; $P = 0.02$). While the complementation of RNA repair was not complete ($\sim 76\%$), RTCB activity may be affected by the transfection efficiency and non-physiological expression levels. Overall, the

reduction of RNA repair with knockdown and complementation with *RTCB* overexpression support the role of RTCB in the repair of CRISPR-guided RNA breaks.

To determine if repair of CRISPR-guided RNA breaks happens in the nucleus or cytoplasm, we removed the NLS tags from the SthCsm complex and targeted nuclear (*XIST*) or exported (*PPIB* and *PARK7*) transcripts. SthCsm complexes without the NLS tag localized in the cytoplasm (**Fig. 2G**) and depleted *PPIB* ($31.2 \pm 6.2\%$ knockdown, $P = 0.023$) (**fig. S4**) and *PARK7* ($41.9 \pm 2.1\%$ knockdown, $P = 0.017$) (**Fig. 2H**). However, RNA retained in the nucleus (*i.e.*, *XIST*) was not depleted by the untagged Csm complex ($P = 0.47$) (**Fig. 2I**). Deep sequencing detected programmable deletions in $1.4 \pm 0.2\%$ of *PPIB* and $9.1 \pm 0.9\%$ of *PARK7* reads, demonstrating that transcripts cleaved in the cytoplasm are repaired in the cytoplasm (**fig. S4, C-E**). Knockdown of *PPIB* and *PARK7* with cytoplasmic Csm complex was less efficient (~ 30 - 40%) than with NLS-tagged Csm complex ($>90\%$). However, this does not mean that RNA cleavage in the cytoplasm is less efficient. In this experiment, we extracted total cellular RNA that contained both cytoplasmic RNA and nascent RNA from the nucleus. The latter is inaccessible to the cytoplasmic Csm complex and contributed to the qPCR signal.

NLS-tagged Csm complex localized in the nucleus (**Fig. 2G**) and knocked down $42.4 \pm 11.9\%$ of the nuclear *XIST* transcript ($P = 0.009$; **Fig. 2J**). Deep sequencing of the *XIST* amplicons identified site-specific deletions (**Fig. 3, A and B**), indicating that cleaved *XIST* RNA is repaired in the nucleus. Collectively, these results demonstrate that RNA repair happens both in the nucleus and in the cytoplasm of human cells.

We hypothesize that human transcripts bound by inactive SthCsm complex (~ 350 kDa) fail RNA quality control during nuclear export or that the bound complex blocks translation in the cytoplasm, which results in the decay of the target RNA (28). In agreement with the latter possibility, targeting *PPIB* and *PARK7* in the cytoplasm with nuclease-inactive SthCsm (Csm^{dead}) resulted in significant RNA knockdown (**Fig. 2H, fig. S4**), but targeting nuclear transcript *XIST* with NLS-Csm^{dead} did not change its relative quantity (**Fig. 2J**, $P = 0.26$).

Multiple RNA breaks produce large RNA excisions

Human *XIST* is a long non-coding RNA that contains eight copies of a repetitive sequence (repeat A) required for *XIST*-mediated inactivation of the X-chromosome (29, 30). Previous work has targeted repeat A with GFP-fused nuclease-inactive SthCsm complex for live imaging of *XIST* RNA (19). We hypothesized that targeting repeat A in *XIST* with catalytically active SthCsm complexes will result in simultaneous cleavage at multiple locations, and repair of these cleavages will generate large deletions. We tested four different guide RNAs that target repeats in *XIST* (**Fig. 3, A and B**). Sequencing of amplicons that span the repetitive region identified the characteristic 6, 12, and 18 nucleotide deletions but also large deletions consistent with multiple cleavages at the repeated sequence (**Fig. 3, C and D**). These data indicate that simultaneous cleavages at multiple locations within the same transcript can be used for large RNA deletions.

Programmable excision of premature stop codons in RNA restores protein expression

The ClinVar database documents more than 40,000 non-sense mutations (*i.e.*, stop codons) in the human population (22). We hypothesized that break-and-repair RNA editing can be used to delete premature stop codons and restore the expression of the encoded proteins (**Fig. 4A**). To test this, we developed a reporter plasmid that encodes for GFP fused to an N-terminal flag-hemagglutinin (flag-HA) epitope. Between the epitope and the *gfp* sequence, we inserted a stop codon to terminate translation (stop-GFP; **Fig. 4B**). Transfection of the stop-GFP plasmid in

293T cells produced no detectable fluorescence. A plasmid with the same sequence but no stop codon produced a robust GFP signal 48 h post-transfection (**Fig. 4C**). To cut out the stop codon, we designed six guides for SthCsm complex that tile across the target RNA sequence in three-nucleotide increments (**Fig. 4B**). Transfection of the stop-GFP plasmid together with three of the six SthCsm complexes (crRNA 3, 4, and 6) partially restored expression of the GFP (**Fig. 4C**; **fig. S5A**). To confirm that transcripts edited with SthCsm produce functional GFP, we used site-directed mutagenesis to make the same mutations in the stop-GFP reporter plasmid (**fig. S5**). Transfection of plasmids containing the 12 or 18 nucleotide deletions resulted in the same levels of fluorescence as transfection of the positive control plasmid (**fig. S5D**).

To quantify the rescue of reporter expression, we developed a bicistronic plasmid that expresses GFP and firefly luciferase linked with a viral 2A peptide. At the 3'-end of the *gfp* gene, we inserted a stop codon, which permits GFP, but not luciferase expression (GFP-stop-Luc). To cut out the stop codon, we designed six SthCsm guide RNAs that tile across the stop codon (**Fig. 4D**). In cells expressing SthCsm complexes that target the stop codon, we detected luciferase activity with five of the six tested crRNAs, which ranged from $2.0 \pm 0.2\%$ with crRNA 3 to $5.3 \pm 0.5\%$ with crRNA 1 (**Fig. 4E**). Sequencing of the target RNA amplicons confirmed that samples with rescued luciferase activity contain RNA deletions eliminating the stop codon (**Fig. 4F**; **fig. S5**).

Type III-E CRISPR systems encode single polyprotein effectors that process CRISPR RNA and cleave the target RNA at two sites six nucleotides apart (31, 32). These effectors are simple and compact and have the potential to produce a single editing outcome, which makes them attractive for use in RNA editing. We expressed type III-E effector from *Desulfonema ishimotonii* fused to the NLS tag (DisCas7-11) in 293T cells with seven guide RNAs tiled across the stop codon of the GFP-stop-Luc reporter (**Fig. 4G**). Only crRNA 7 resulted in a significant increase of the luciferase signal ($0.9 \pm 0.2\%$; $P < 0.001$), which was less efficient than SthCsm (**fig. S5G**). In addition to editing, we quantified knockdown efficiencies of the target RNA using RT-qPCR. The RNA knockdown efficiency with SthCsm ranged from $36.8 \pm 7.2\%$ to $54.0 \pm 2.8\%$, while knockdowns with DisCas7-11 were less than half as efficient ($12.2 \pm 5.9\%$ with guide 6 and $16.5 \pm 11.2\%$ with guide 7) (**fig. S5I**). Analysis of sequenced amplicons identified six nucleotide deletions in $0.04 \pm 0.01\%$ of the reads at the site targeted with DisCas7-11 (**fig. S5H**).

An enhanced version of the DisCas7-11 complex (eDisCas7-11) has been recently engineered for CRISPR-assisted trans-splicing by adding positively charged amino acids to regions of the protein that are physically close to the bound target RNA (33). Targeting the GFP-stop-Luc reporter with eDisCas7-11 resulted in a more efficient knockdown ($52.3 \pm 3.8\%$ with guide 6 and $47.7 \pm 7.8\%$ with guide 7; **fig. S5I**), rescue of $0.9 \pm 0.1\%$ of luciferase activity ($P = 0.003$; **Fig. 4H**), and programmed RNA excisions with $0.29 \pm 0.03\%$ efficiency (**Fig. 4I**).

Programmed RNA excision with DisCas7-11 and eDisCas7-11 is less efficient compared to targeting with SthCsm. The low frequency of deletions with DisCas7-11 is consistent with the inefficiency of the DisCas7-11 nuclease in biochemical assays and the low frequency of two simultaneous breaks in the target RNA (31, 32). Collectively, these data demonstrate the potential of type III-E effectors to produce a single RNA editing outcome, but further engineering of the Cas7-11 nuclease will be important to repurpose this enzyme for RNA excision.

Programmable excision of non-sense mutation in the *CFTR* transcript

After testing fluorescent and luminescent reporters, we sought to determine the therapeutic potential of CRISPR-guided RNA excision by targeting a clinically relevant nonsense mutation (c.3846G>A; W1282X) in the *CFTR* mRNA. The truncated CFTR^{W1282X} protein is partially functional, but the premature stop codon triggers nonsense-mediated decay (NMD) of the *CFTR*^{W1282X} mRNA in the cytoplasm (34, 35). The decay of *CFTR* mRNA results in insufficient levels of CFTR protein, leading to the development of cystic fibrosis. We hypothesized that targeting the *CFTR*^{W1282X} transcript with NLS-tagged SthCsm would remove the W1282X stop codon in the nucleus, and the repaired RNA would escape NMD in the cytoplasm, rescuing protein expression and potentially rescuing disease phenotype.

To screen for crRNAs that guide efficient excision of W1282X in the *CFTR*, we created a luciferase-based NMD-reporter plasmid building off previously published designs (**Fig. 5A**) (36). We screened eight crRNAs and identified one (crRNA 5) that guides the most efficient removal of the premature termination codon in the fLuc-CFTR^{W1282X} reporter (**Fig. 5, B and C; fig. S6A**). The co-transfection of fLuc-CFTR^{W1282X} and a plasmid encoding for Csm complex with crRNA 5 restored the C-terminus of CFTR protein compared to the cells that received non-targeting Csm complex (**Fig. 5D, fig. S6B**). In addition, expression of the Csm complex and crRNA 5 increased luciferase activity by 11.1% ($P = 0.024$) compared to the non-targeting control, indicating escape from the NMD (**Fig. 5E**).

Finally, we used immortalized human bronchial epithelium cells with the *CFTR*^{W1282X} allele (16HBEge *CFTR*^{W1282X}) to test if CRISPR-guided RNA excision can remove the W1282X codon in the endogenous *CFTR* transcript. RT-qPCR and amplicon-sequencing identified editing of $3.9 \pm 0.8\%$ CFTR transcripts (**Fig. 5F, fig. S6C**). Future studies will be needed to measure the impact of this correction on cellular physiology and determine the RNA editing levels required for therapeutic outcomes.

Discussion

Precise DNA manipulation has been enabled by breaking DNA at sequence-specific locations and repairing the resulting fragments. In a previous study, we applied the concept of break-and-repair to engineer viral RNA genomes *in vitro* (37). Here, we extend this approach to make RNA deletions in human cells, which is facilitated by the repair of programmable RNA breaks made using type III CRISPR complexes.

We anticipate that the deletion of toxic mutants, complemented by fractional repair, could be therapeutic. Alternatively, premature stop codons that trigger NMD result in low to no protein (e.g. CFTR^{W1282X} mutation), and excision of premature stop codons could rescue protein expression. Other potential applications of site-specific RNA excision include editing non-coding RNAs (38), removing disease-causing trinucleotide expansions (39), or correcting intronic mutations that dysregulate splicing (40).

Repair of RNAs is understudied, and the biological significance of this process is often dismissed due to the high turnover of RNA in the cell. We hypothesize that end-modifying enzymes, additional ligases, nucleases (e.g., exosome complex), and other RNA processing factors regulate this process, and tuning RNA processing has the potential to improve the efficiency of RNA editing. We have established reporter assays for RNA repair, which enable the discovery of new RNA repair pathways and rapid screening for conditions that favor RNA repair to improve the efficiency of RNA editing.

We show that Csm-mediated knockdown of cytoplasmic transcripts does not require target RNA cleavage (**Figs. 1G and 2H**). However, cleavage is required for site-specific RNA deletion (**Fig. 1H**). This observation suggests that the efficiency of RNA repair is an underestimate because most of the target RNA is degraded by RNA quality control pathways before it can be repaired. Single-molecule studies of enzymatic activities of the Csm complex from *Streptococcus thermophilus* (used in this work) demonstrate that cleavage happens within seconds, but then the complex retains cleavage products for over ~80 minutes (41). These data suggest that RNA release, rather than cleavage or ligation, may be the rate-limiting step in RNA editing.

The kinetics of Csm complex dissociation is on the same timescale as mRNA transport to the cytoplasm (10-30 min for β -actin mRNA) (42), and it is possible that the Csm complex stalls ribosomes and triggers RNA decay before it releases target RNA for repair (43). These observations suggest that programmable ribonucleases that cleave and release the target quickly or cleave RNA outside of the bound sequence (i.e., Cas13) will enable more efficient editing.

Orthologs of Cas9 and Cas12 cleave complementary RNA targets, enabling programmable RNA manipulation with single-subunit effectors (44-50). RNA cleavage by Cas9 or Cas12 likely produces a 5'-phosphate and a 3'-hydroxyl, similar to DNA cleavage (51, 52). These ends are not compatible with RTCB-mediated ligation, but other cellular RNA repair pathways may enable Cas9- and Cas12-mediated RNA editing (53).

Unlike DNA editing, which often depends on adjacent sequence motifs (i.e., PAM), type III CRISPR systems only require complementarity between the RNA guide and the RNA target (18), which improves target site versatility. The frequency and distribution of RNA repair outcomes varied across RNA targets that we tested, which may suggest that local sequence context, secondary structure, RNA modifications, or other factors may influence the efficiency of target binding, target cleavage, or repair. Screens like those performed for Cas13 (54, 55) will help establish predictive models to enhance the efficiencies of RNA editing and advance this technology for therapeutic applications.

The initial efficiency of editing with Cas9 was ~3-30% for indels and ~0.5-0.7% efficiency for homology-directed repair (56). A decade later, Cas9-based technologies are precise, efficient, versatile, and clinically relevant (57). Here, we demonstrate programmable break-and-repair editing of RNA, which reveals an important aspect of RNA biology that has not been explored. We anticipate that efficiencies will improve and that these technologies will lead to new insights about the RNA damage response and enable discoveries of previously unrecognized mechanisms of RNA repair.

References and Notes

1. M. Jinek *et al.*, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).
2. G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences* **109**, (2012).
3. F. A. Ran *et al.*, Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* **8**, 2281-2308 (2013).
4. M. van Overbeek *et al.*, DNA Repair Profiling Reveals Nonrandom Outcomes at Cas9-Mediated Breaks. *Mol Cell* **63**, 633-646 (2016).

5. M. Kosicki, K. Tomberg, A. Bradley, Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology* **36**, 765-771 (2018).
6. Y. Fu *et al.*, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**, 822-826 (2013).
7. P. D. Hsu *et al.*, DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827-832 (2013).
8. V. Pattanayak *et al.*, High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839-843 (2013).
9. E. Haapaniemi, S. Botla, J. Persson, B. Schmierer, J. Taipale, CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nature Medicine* **24**, 927-930 (2018).
10. R. J. Ihry *et al.*, p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells. *Nature Medicine* **24**, 939-946 (2018).
11. O. O. Abudayyeh *et al.*, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* **353**, aaf5573 (2016).
12. O. O. Abudayyeh *et al.*, RNA targeting with CRISPR-Cas13. *Nature* **550**, 280-284 (2017).
13. S. Konermann *et al.*, Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell* **173**, 665-676.e614 (2018).
14. J. Borrajo *et al.*, Programmable multi-kilobase RNA editing using CRISPR-mediated trans-splicing. *bioRxiv*, (2023).
15. D. B. T. Cox *et al.*, RNA editing with CRISPR-Cas13. *Science* **358**, 1019-1027 (2017).
16. O. O. Abudayyeh *et al.*, A cytosine deaminase for programmable single-base RNA editing. *Science* **365**, 382-386 (2019).
17. C. R. Hale *et al.*, RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139**, 945-956 (2009).
18. G. Tamulaitis *et al.*, Programmable RNA shredding by the type III-A CRISPR-Cas system of *Streptococcus thermophilus*. *Mol Cell* **56**, 506-517 (2014).
19. D. Colognori, M. Trinidad, J. A. Doudna, Precise transcript targeting by CRISPR-Csm complexes. *Nature Biotechnology*, (2023).
20. W. T. Woodside *et al.*, Type III-A CRISPR systems as a versatile gene knockdown technology. *Rna* **28**, 1074-1088 (2022).
21. T. Fricke *et al.*, Targeted RNA Knockdown by a Type III CRISPR-Cas Complex in Zebrafish. *The CRISPR Journal* **3**, 299-313 (2020).
22. <https://www.ncbi.nlm.nih.gov/clinvar>
23. L. V. Sharova *et al.*, Database for mRNA Half-Life of 19 977 Genes Obtained by DNA Microarray Analysis of Pluripotent and Differentiating Mouse Embryonic Stem Cells. *DNA Research* **16**, 45-58 (2009).
24. Y. Lu, F.-X. Liang, X. Wang, A Synthetic Biology Approach Identifies the Mammalian UPR RNA Ligase RtcB. *Molecular Cell* **55**, 758-770 (2014).
25. J. Jurkin *et al.*, The mammalian tRNA ligase complex mediates splicing of XBP1 mRNA and controls antibody secretion in plasma cells. *EMBO J* **33**, 2922-2936 (2014).
26. J. Popow *et al.*, HSPC117 is the essential subunit of a human tRNA splicing ligase complex. *Science* **331**, 760-764 (2011).
27. A. Kroupova *et al.*, Molecular architecture of the human tRNA ligase complex. *eLife* **10**, (2021).

28. L. E. Maquat, G. G. Carmichael, Quality Control of mRNA Function. *Cell* **104**, 173-176 (2001).
29. A. Wutz, T. P. Rasmussen, R. Jaenisch, Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nature Genetics* **30**, 167-174 (2002).
- 5 30. J. Minks, S. E. L. Baldry, C. Yang, A. M. Cotton, C. J. Brown, XIST-induced silencing of flanking genes is achieved by additive action of repeat a monomers in human somatic cells. *Epigenetics & Chromatin* **6**, (2013).
31. S. P. B. van Beljouw *et al.*, The gRAMP CRISPR-Cas effector is an RNA endonuclease complexed with a caspase-like peptidase. *Science* **373**, 1349-1353 (2021).
- 10 32. A. Özcan *et al.*, Programmable RNA targeting with the single-protein CRISPR effector Cas7-11. *Nature* **597**, 720-725 (2021).
33. C. Schmitt-Ulms *et al.*, Programmable RNA writing with trans-splicing. *bioRxiv*, (2024).
34. S. M. Rowe *et al.*, Restoration of W1282X CFTR Activity by Enhanced Expression. *American Journal of Respiratory Cell and Molecular Biology* **37**, 347-356 (2007).
- 15 35. E. Nagy, L. E. Maquat, A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. *Trends in Biochemical Sciences* **23**, 198-199 (1998).
36. Y. J. Kim *et al.*, Gene-specific nonsense-mediated mRNA decay targeting for cystic fibrosis therapy. *Nature Communications* **13**, (2022).
- 20 37. A. Nemudryi *et al.*, CRISPR-based engineering of RNA viruses. *Science Advances* **9**, (2023).
38. K. Nemeth, R. Bayraktar, M. Ferracin, G. A. Calin, Non-coding RNAs in disease: from mechanisms to therapeutics. *Nature Reviews Genetics* **25**, 211-232 (2023).
39. R. Batra *et al.*, Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9. *Cell* **170**, 899-912.e810 (2017).
- 25 40. R. Vaz-Drago, N. Custódio, M. Carmo-Fonseca, Deep intronic mutations and human disease. *Human Genetics* **136**, 1093-1111 (2017).
41. P. Irmisch, I. Mogila, B. Samatanga, G. Tamulaitis, R. Seidel, Retention of the RNA ends provides the molecular memory for maintaining the activation of the Csm complex. *Nucleic Acids Research*, (2024).
- 30 42. Y. a. Ben-Ari *et al.*, The life of an mRNA in space and time. *Journal of Cell Science* **123**, 1761-1774 (2010).
43. K. N. D'Orazio, R. Green, Ribosome states signal RNA quality control. *Molecular Cell* **81**, 1372-1383 (2021).
- 35 44. G. Dugar *et al.*, CRISPR RNA-Dependent Binding and Cleavage of Endogenous RNAs by the *Campylobacter jejuni* Cas9. *Molecular Cell* **69**, 893-905.e897 (2018).
45. M. R. O'Connell *et al.*, Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* **516**, 263-266 (2014).
46. B. A. Rousseau, Z. Hou, M. J. Gramelspacher, Y. Zhang, Programmable RNA Cleavage and Recognition by a Natural CRISPR-Cas9 System from *Neisseria meningitidis*. *Molecular Cell* **69**, 906-914.e904 (2018).
- 40 47. S. C. Strutt, R. M. Torrez, E. Kaya, O. A. Negrete, J. A. Doudna, RNA-dependent RNA targeting by CRISPR-Cas9. *eLife* **7**, (2018).
48. J. P. K. Bravo *et al.*, RNA targeting unleashes indiscriminate nuclease activity of CRISPR-Cas12a2. *Nature* **613**, 582-587 (2023).
- 45 49. O. Dmytrenko *et al.*, Cas12a2 elicits abortive infection through RNA-triggered destruction of dsDNA. *Nature* **613**, 588-594 (2023).

50. W. X. Yan *et al.*, Functionally diverse type V CRISPR-Cas systems. *Science* **363**, 88-91 (2019).
51. M. Jinek *et al.*, Structures of Cas9 Endonucleases Reveal RNA-Mediated Conformational Activation. *Science* **343**, (2014).
52. T. Yamano *et al.*, Crystal Structure of Cpf1 in Complex with Guide RNA and Target DNA. *Cell* **165**, 949-962 (2016).
53. Y. Yuan *et al.*, Chemoproteomic discovery of a human RNA ligase. *Nature Communications* **14**, (2023).
54. H.-H. Wessels *et al.*, Massively parallel Cas13 screens reveal principles for guide RNA design. *Nature Biotechnology* **38**, 722-727 (2020).
55. H.-H. Wessels *et al.*, Prediction of on-target and off-target activity of CRISPR–Cas13d guide RNAs using deep learning. *Nature Biotechnology*, (2023).
56. L. Cong *et al.*, Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **339**, 819-823 (2013).
57. L. Villiger *et al.*, CRISPR technologies for genome, epigenome and transcriptome editing. *Nature Reviews Molecular Cell Biology*, (2024).
58. A. Nemudraia, A. Nemudryi, B. Wiedenheft, Amplicon sequencing of human mRNA and reporter mRNA targeted with type III-A CRISPR complex of *Streptococcus thermophilus*, NCBI (2024); <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1099688/>
59. A. Nemudraia, A. Nemudryi, B. Wiedenheft, Code for generating figures and analyzing amplicon sequencing of human mRNA and reporter mRNA targeted with type III-A CRISPR complex from *Streptococcus thermophilus*, Zenodo (2024); doi: 10.5281/zenodo.10966960
60. M. W. Shen *et al.*, Predictable and precise template-free CRISPR editing of pathogenic variants. *Nature* **563**, 646-651 (2018).
61. K. Kato *et al.*, Structure and engineering of the type III-E CRISPR-Cas7-11 effector complex. *Cell* **185**, 2324-2337.e2316 (2022).
62. D. Conant *et al.*, Inference of CRISPR Edits from Sanger Trace Data. *The CRISPR Journal* **5**, 123-130 (2022).
63. D. Kim *et al.*, The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914-921.e910 (2020).
64. Y.-C. Lin *et al.*, Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nature Communications* **5**, (2014).

Acknowledgments: Base-calling of Nanopore sequencing data was performed with the Tempest High Performance Computing System, which is maintained by University Information Technology Research Cyberinfrastructure at Montana State University. We thank Dr. Matthew Taylor for the generous use of the fluorescent microscope. Some diagrams and schematics were created with BioRender.com.

Funding:

National Institutes of Health grant 5K99AI171893-02 (to A. Nemudryi)

National Institutes of Health grant R35GM134867 (to B.W.)

National Science Foundation grant 2331325 (to B.W.)

Author contributions:

Conceptualization: A. Nemudryi, A. Nemudraia, and B.W.

Methodology: A. Nemudryi and A. Nemudraia.

Investigation: A. Nemudryi and A. Nemudraia.

Formal analysis: A. Nemudryi and A. Nemudraia.

5 Visualization: A. Nemudryi and A. Nemudraia.

Supervision: A. Nemudryi, A. Nemudraia, and B.W.

Funding acquisition: B.W. and A. Nemudryi.

Writing - Original Draft: A. Nemudryi and A. Nemudraia.

Writing - Review & Editing: B.W., A. Nemudryi, and A. Nemudraia.

10 **Competing interests:** B.W. is the founder of SurGene LLC. B.W., A. Nemudryi, and A. Nemudraia are inventors of the patent applications US 63/523,592 and US 63/534,305 pertaining to use of type III CRISPR-Cas system for sequence-specific editing of RNA filed by Montana State University.

15 **Data and materials availability:** Raw sequencing data were deposited to the NCBI Sequence Read Archive under accession number PRJNA1099688 (58). The code used to analyze sequencing data and generate figures has been deposited to Zenodo (59). Plasmids generated in this study are available through Addgene and by request from the authors.

Supplementary Materials

20 Materials and Methods

Supplementary Text

Figs. S1 to S6

Tables S1 to S7

Data S1

25 References (60-64)

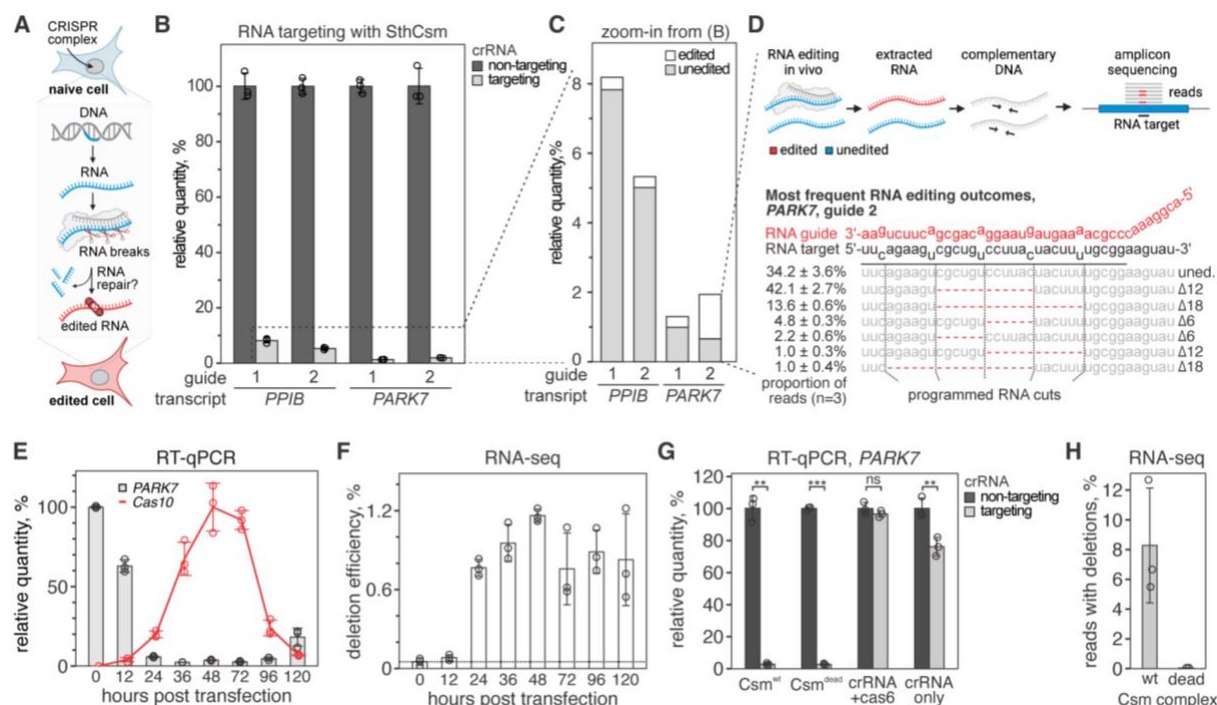


Fig. 1. Programmable deletions of RNA with RNA-guided type III-A CRISPR complexes. (A) Diagram of RNA editing in eukaryotic cells through sequence-specific RNA cleavage and RNA repair. (B) Human cells (293T) were transfected with plasmids encoding for NLS-tagged type III CRISPR complex of *Streptococcus thermophilus* (SthCsm) and RNA guides targeting *PPIB* or *PARK7* messenger RNAs. Target transcripts were quantified with RT-qPCR, and the qPCR signal was normalized to *ACTB* and a non-targeting guide RNA control. Data is shown as mean ± SD of three biological replicates. (C) Zoom-in from panel B. Deep sequencing was used to quantify the proportion of signal that is derived from edited RNA. (D) *Top*: schematics of deep sequencing approach used to quantify RNA editing. *Bottom*: top five most frequent RNA editing outcomes in *PARK7* transcript (guide 2). Dotted lines indicate the positions of RNA breaks by the SthCsm complex. Data is shown as mean ± SD. (E) Kinetics of *PARK7* mRNA knockdown (guide 2) vs. NLS-tagged SthCsm complex expression. *PARK7* qPCR signal was normalized to *ACTB* and 0 h time point. Cas10 expression was normalized to *ACTB* and maximum expression level at 48 h. Data is shown as mean ± SD of three biological replicates. (F) *PARK7* qPCR products in (E) were sequenced, and deletion efficiency was calculated as [relative quantity] × [fraction of reads with deletions]. (G) *PARK7* knockdown efficiencies with the “wildtype” NLS-tagged SthCsm complex (Csm^{wt}), catalytically inactive NLS-tagged SthCsm complex (Csm^{dead}), crRNA expressed only with Cas6 gene (no *csm* genes), and crRNA alone were measured using RT-qPCR. Welch’s t-test was used to compare samples expressing targeting and non-targeting crRNA. ** - $p < 0.01$, *** - $p < 0.001$, ns – non-significant. (H) RT-qPCR products in (G) were sequenced, and a fraction of reads with programmable deletions was quantified.

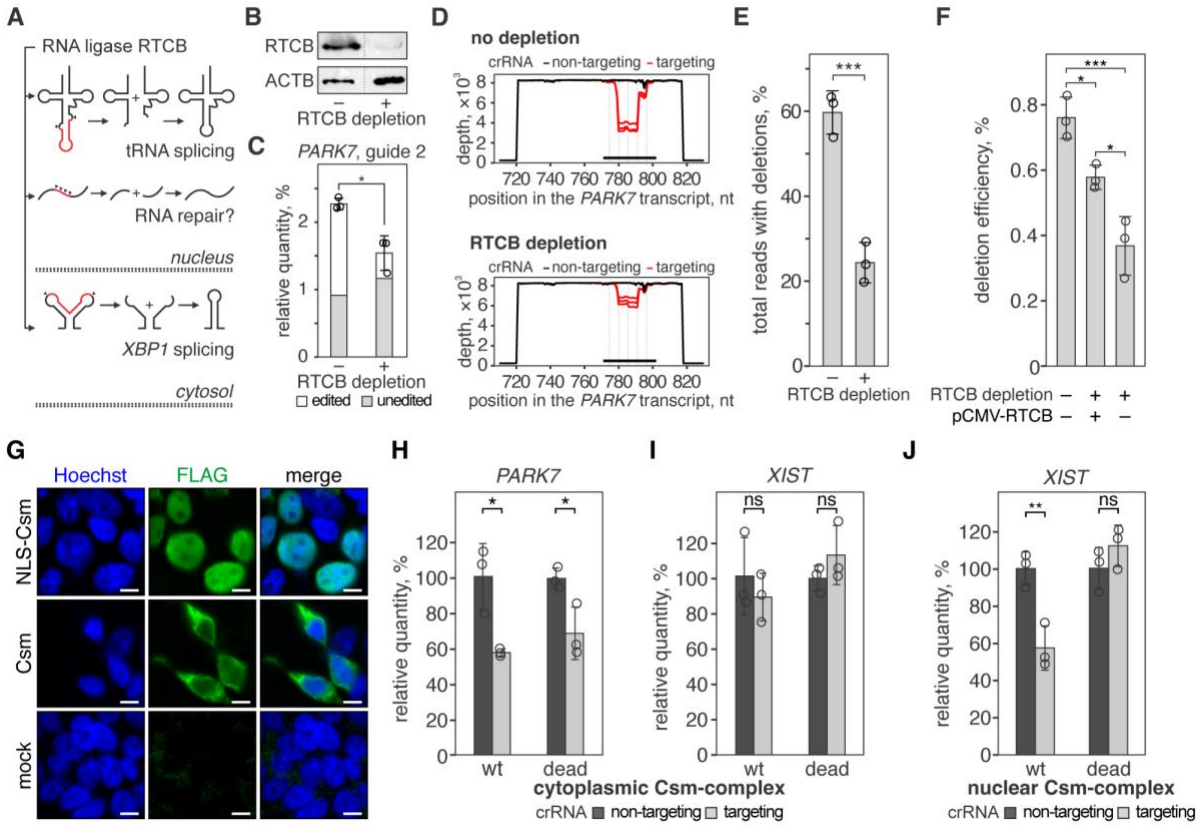


Fig. 2. RTCB ligase repairs RNA cleaved by the type III CRISPR complex. (A) Human RNA ligase RTCB joins RNA ends that are produced in tRNA splicing (top) and non-canonical *XBP1* mRNA splicing (bottom) during the unfolded protein response. We hypothesized that the ligase activity of RTCB is involved in the RNA repair of CRISPR-guided RNA breaks (middle). (B) Western blot with anti-RTCB or anti-ACTB (loading control) antibodies was performed with lysates of 293T cells with (+) or without (-) RTCB depletion. See the uncropped images in fig. S3. (C) The *PARK7* transcript was targeted with SthCsm (guide 2) in 293T cells with (+) or without (-) RTCB depletion. The *PARK7* transcript was quantified with RT-qPCR and normalized to *ACTB* and non-targeting guide RNA. Data are shown as the mean $\pm$ standard deviation of three biological replicates. **P* < 0.05, ****P* < 0.001; one-way analysis of variance (ANOVA) with Tukey HSD post-hoc comparisons. (D) qPCR products in (C) were sequenced, and resulting reads were aligned to the reference sequence of the *PARK7* transcript (NM_007262, GenBank). Graphs show sequencing depth (y-axes) at the amplified region of the transcript (x-axes). Every line shows a biological replicate (n = 3). The horizontal black bar indicates a region complementary to the guide RNA of the SthCsm complex. Vertical dotted lines mark predicted positions of RNA breaks. (E) Quantification of deletions in the target region of the *PARK7* transcript. Data is shown as the mean $\pm$ standard deviation of three biological replicates. Welch's t-test was used to compare mean values. *** p < 0.001. (F) RTCB deficient cells were transfected with a plasmid expressing RTCB (pCMV-RTCB), and the efficiency of CRISPR RNA-guided programmable deletions in *PARK7* was quantified as in panels C-E. * p < 0.05; OneWelch's t-test. (G) Immunostaining of cells expressing Flag-tagged Csm complex with (NLS-Csm) or without (Csm) NLS-tag. Scale bars are 10 μ m. (H-J) Knockdown of *PARK7* and *XIST* transcripts with cytoplasmic SthCsm (no NLS) (H, I) and knockdown of *XIST* with nuclear Csm (NLS-tagged) (J) was quantified with RT-qPCR and normalized to *ACTB* and non-targeting control. "wt" – nuclease-active SthCsm, "dead" – catalytically inactivated SthCsm (Csm3^{D33A} mutation). **P* < 0.05, ns – non-significant; Welch's t-test. Data is shown as mean $\pm$ SD (n = 3).

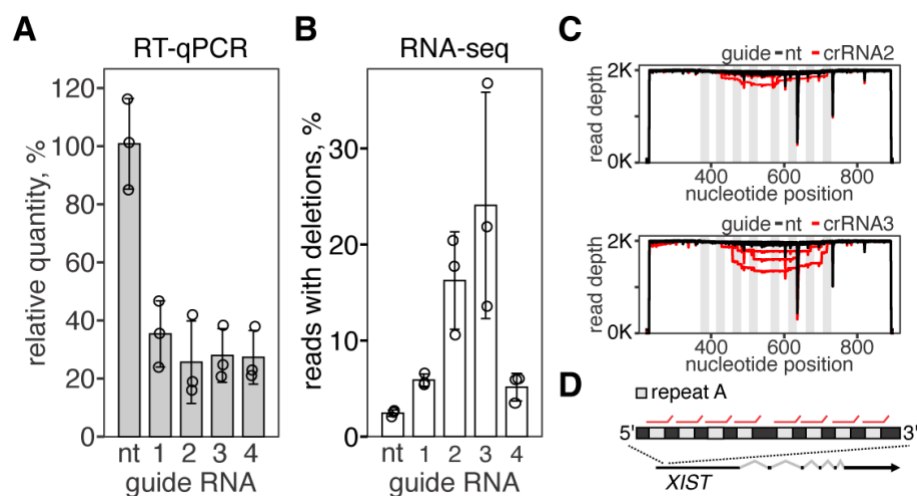


Fig. 3. Repair of concurrent RNA breaks results in large RNA excisions. (A) Repetitive region (repeat A) in *XIST* transcript was targeted with SthCsm complex with four different guide RNAs. Knockdown of *XIST* was quantified with RT-qPCR. Data is shown as the mean of three biological replicates $\pm$ SD. (B) Amplicon-seq was used to quantify programmed RNA deletions in *XIST*. (C) Repeat A was amplified and deep-sequenced. Reads were aligned to the reference sequence (NR_001564.2, GenBank). Graphs show sequencing depth (y-axes) at the amplified region of the transcript (x-axes). Every line shows a biological replicate (n = 3). Vertical light gray rectangles indicate the position of repeats targeted by Csm complexes. (D) Repeat A architecture in *XIST* lncRNA. Red lines show binding sites for Csm complexes.

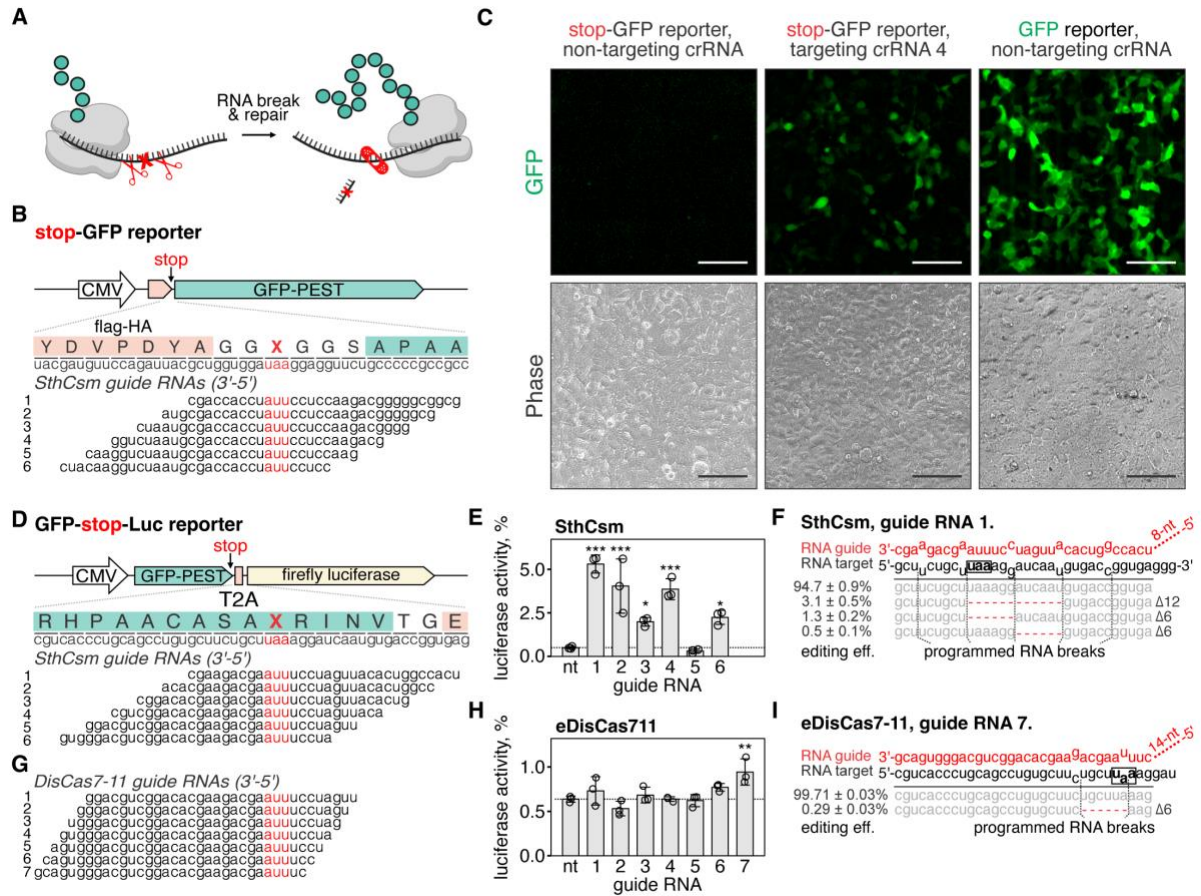


Fig. 4. Programmable excision of stop codons restores protein expression. (A) Schematic representation of the proposed approach for deleting premature stop codons in human transcripts. (B) *Top*: schematic diagram of the stop-GFP reporter plasmid. *Bottom*: Six guide RNAs for SthCsm complex were designed to excise the stop codon in the *gfp* transcript. Underlined (red) sections of target RNA are expected to be deleted. Vertical red ticks indicate predicted sites for RNA breaks. (C) Cells were transfected with plasmids for the stop-GFP reporter and SthCsm with a non-targeting guide (left), stop-GFP reporter and SthCsm with a targeting guide (middle), or GFP reporter and SthCsm with the non-targeting guide (right). Fluorescence microscopy was used to image cells 48 h post-transfection. Scale bars – 50 μ m. (D) *Top*: schematic diagram of the GFP-stop-Luc reporter plasmid. *Bottom*: Six crRNAs for SthCsm complex were designed to delete the stop codon at the 3'-end of the *gfp* gene. (E) Luciferase activity was measured in cell lysates 48 hours after transfection with GFP-stop-Luc and SthCsm plasmids. Luciferase activity is normalized to a control transfected with a reporter plasmid without the stop codon. Data are shown as mean $\pm$ SD of three replicates. Means were compared using one-way ANOVA, and samples with targeting guide RNAs were compared to the non-targeting control using one-tailed Dunnett's test. * P < 0.5, ** P < 0.1, *** P < 0.001. (F) Most frequent RNA editing outcomes in the sample with the most efficient rescue of luciferase activity in (B) (guide 1). Editing efficiency was quantified as mean $\pm$ SD of three biological replicates. The black box shows the stop codon that was targeted by type III CRISPR complexes. (G, H, I). The same as (D-F), but with eDisCas7-11.

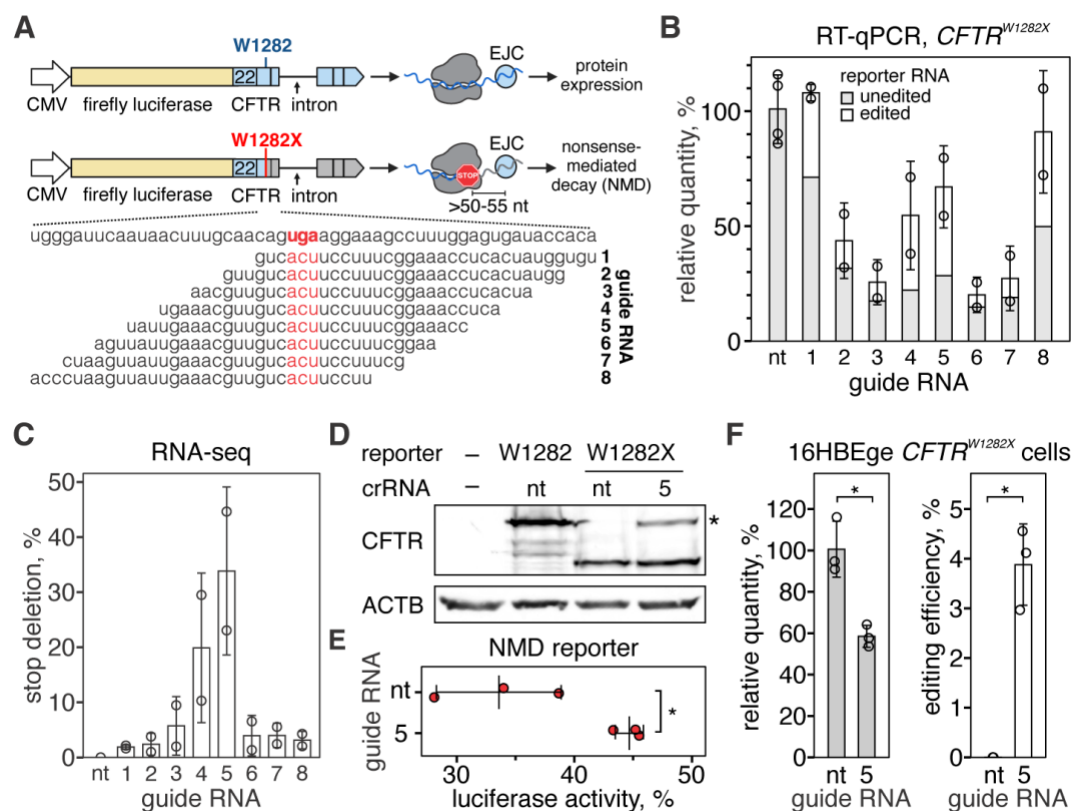


Fig. 5. Programmable excision of a non-sense mutation in the *CFTR* transcript restores translation. (A) Diagram of a luciferase-based reporter for *CFTR*^{W1282X} mutation (fLuc-CFTR, top) and guide RNA design (bottom). Top: Firefly luciferase was genetically linked to exons 22-27 of the *CFTR* cDNA, and synthetic intron sequence was inserted between exons 24 and 25. See Methods for additional details. Bottom: Eight crRNAs were tiled across the mutation (W1282X) to guide the excision of the stop codon (UGA, highlighted with red). (B) RT-qPCR was used to quantify fLuc-*CFTR* transcript targeted with Csm complexes. Amplicons were deep-sequenced to quantify edited vs. unedited reporter RNA. See fig. S6A for sequencing depth plots. (C) Quantification of deletions that remove stop codon (W1282X). (D) Western blot with antibodies against CFTR amino acid residues 1204-1211 with lysates from 293T cells expressing fLuc-CFTR or fLuc-CFTR^{W1282X} and Csm complexes with non-targeting (nt) guide RNA or CFTR-targeting guide RNA 5. See fig. S6B for uncropped images. (E) Quantification of luciferase activity in 293T cells transfected with fLuc-CFTR^{W1282X} and Csm complexes with non-targeting guide RNA or targeting guide RNA 5. Middle bar shows mean of three biological replicates (red dots). Error bars show mean $\pm$ SD. * $P < 0.05$, Welch's t-test. (F) Left: RT-qPCR was used to quantify *CFTR* transcript in HBE16ge *CFTR*^{W1282X} cells transfected with plasmids encoding for Csm complexes with non-targeting guide RNA (nt) or CFTR-targeting guide RNA 5. Right: qPCR amplicons were deep-sequenced, and deletions removing W1282X codon were quantified. See fig. S6C for depth plot. Data is shown as the mean of three biological replicates $\pm$ SD. * $P < 0.05$, Welch's t-test.



Supplementary Materials for

Repair of CRISPR-guided RNA breaks enables site-specific RNA excision in human cells

Anna Nemudraia, Artem Nemudryi, Blake Wiedenheft

Corresponding authors: Artem Nemudryi, artem.nemudryi@gmail.com; Blake Wiedenheft, bwiedenheft@gmail.com

Science **384**, 808 (2024)
DOI: 10.1126/science.adk5518

The PDF file includes:

Materials and Methods
Supplementary Text
Figs. S1 to S6
Tables S1 to S7
References

Other Supplementary Material for this manuscript includes the following:

MDAR Reproducibility Checklist
Data S1

Materials and Methods

Plasmids

pDAC627 (Addgene plasmid #195240; <http://n2t.net/addgene:195240>; RRID: Addgene_195240) and pDAC446 (Addgene plasmid # 195239; <http://n2t.net/addgene:195239>; RRID: Addgene_195239) expressing type III CRISPR-associated proteins from *Streptococcus thermophilus* (Csm2 – Csm5, Cas10, and Cas6) fused to a nuclear localization signal (NLS) and RNA guide, as well as pDAC569 (Addgene plasmid # 195241; <http://n2t.net/addgene:195241>; RRID: Addgene_195241) expressing nuclease-inactive SthCsm complex were gifts from Jennifer Doudna (19). To clone SthCsm guides targeting *PPIB*, *PARK7*, *XIST*, *CFTR*^{W1282X}, or reporter transcripts (stop-GFP, GFP-stop-Luc, fLuc-CFTR, and fLuc-CFTR^{W1282X}), pairs of complementary DNA oligos (table S1) were annealed and phosphorylated with T4 polynucleotide kinase [New England Biolabs (NEB)] as previously described (3). The resulting fragments were cloned using the *Bbs*I restriction enzyme (NEB) into pDAC627, pDAC446, or pDAC569. The unmodified vectors were used as the non-targeting control. Guide RNAs targeting *PPIB* and *PARK7* transcripts were designed using the *cas13design* algorithm (<https://gitlab.com/sanjanalab/cas13>), which was customized to select 32-nucleotide-long guide RNAs (54). Highest-ranking guide RNAs that target different regions of the transcripts were selected. The plasmids expressing crRNA alone or crRNA with Cas6-NLS protein were generated by ligation of PCR-amplified DNA from template plasmid pDAC627 (table S2).

To generate the plasmid expressing SthCsm complex without NLS tags (pAN052), the gene fragments were designed using the NEBuilder online tool, synthesized (Twist Bioscience), and assembled using NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520). Plasmid expressing crRNA within the 5'UTR of mCherry mRNA (pAN053) was generated using Q5 site-directed mutagenesis of pAN-CMV-mCherry-HA plasmid (SthCR_RFP_F and SthCR_RFP_R, table S3). The SthCsm guides were cloned as described above.

pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid #48139; <http://n2t.net/addgene:48139>; RRID: Addgene_48139). Cas9 sgRNAs targeting the *RTCB* gene were cloned as previously described (table S4) (3). Guide sequences were selected in the second and third exons of the gene using the inDelphi web interface based on predicted knockout efficiency and editing precision (<https://indelfphi.giffordlab.mit.edu/>) (60).

pDF0159 pCMV - huDisCas7-11 mammalian expression plasmid was a gift from Omar Abudayyeh and Jonathan Gootenberg (Addgene plasmid #172507; <http://n2t.net/addgene:172507>; RRID: Addgene_172507) (32). To add the NLS tag to the N-terminus of the DisCas-11 protein and to introduce mutations (D1579R, Y311K, D987K) (33), the plasmid pDF0159 was modified using Q5 site-directed mutagenesis (table S3).

pDF0114 pu6-eco31i-eco31i-dis7-11-mature-dr-guide-scaffold was a gift from Omar Abudayyeh and Jonathan Gootenberg (Addgene plasmid #186981; <http://n2t.net/addgene:186981>; RRID: Addgene_186981) (61). To clone DisCas7-11 guides, two partially complementary DNA oligos were annealed and phosphorylated (table S5). The resulting duplexes were cloned into pDF0114 using *Bsa*I restriction enzyme (NEB) to substitute a non-targeting guide sequence.

To generate reporter plasmids pStop-GFP (pAN007) and pGFP-stop-Luc (pAN013), dscGFP-2A-Luciferase gene cassette was amplified from pGreenFire1-ISRE (EF1 α -puro) vector (System Biosciences, TR016PA-P) using PCR and cloned in the pEGFP-N1 vector (Clontech) at the *Kpn*I and *Not*I restriction sites. Restriction sites and Flag-HA tag at the N-terminus of the GFP were

added into the overhangs of the oligonucleotide primers that were used for PCR. Stop codons were inserted in the 5'- or the 3'-end of the *gfp* gene using Q5 site-directed mutagenesis.

To generate the reporter plasmids fLuc-CFTR (pAN056) and fLuc-CFTR^{W1282X} (pAN057), the gene fragments were synthesized (Twist Bioscience) and assembled using NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520). The firefly luciferase (fLuc) gene was fused with exons 22-27 of the *CFTR* gene, with or without the nonsense mutation. A synthetic intron (200 nt from 5'-end and 200 nt from 3'-end of the natural intron) was introduced between exons 24 and 25, such that the exon-exon junction in the spliced transcript is more than 50-55 nucleotides downstream of the stop codon, which is required for triggering the NMD (35).

Plasmid encoding for *RTCB* cDNA (pCMV-RTCB) was ordered from Genscript (Cat. No. OHu24215D).

All plasmid sequences were confirmed with whole-plasmid sequencing at Plasmidsaurus (<https://www.plasmidsaurus.com/>) (see plasmid sequences in Data S1 file).

Nucleic acids

All DNA oligos were purchased from Eurofins (table S1-S7).

Antibodies

Antibodies used for western blot: rabbit anti-RTCB (Proteintech, Cat:19809-1-AP, 1:1000), rabbit anti-ACTB (ABclonal, Cat: AC026, 1:20,000), goat anti-rabbit IgG peroxidase-conjugated (Jackson ImmunoResearch, Cat: 111-035-003, 1:10,000), goat anti-mouse AlexaFluor488 (Invitrogen, A11001, 1:2000), goat anti-rabbit AlexaFluor 594 (Invitrogen, A11012). The CFTR antibodies mouse anti-NBD2 (596; dilution 1:2500) were purchased through the Cystic Fibrosis Foundation Therapeutics Antibody Distribution Program (CFF, Bethesda, MD, USA).

Antibodies used for immunocytochemistry: anti-FLAG (Sigma, F1804, 1:2000), goat anti-mouse AlexaFluor488 (Invitrogen, A11001, 1:2000).

Immunocytochemistry

293T cells were transfected with plasmid DNA using FuGENE HD reagent (Promega) according to the manufacturer's instructions. 24 hours after transfection, cells were detached and seeded at 50% confluency on glass coverslips (Fisherbrand, #12-545-80) pre-coated with poly-D-lysine (Cultrex, #3439-100-01) in a 24-well plate. The next day, media was removed, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. The cells were washed three times with PBS and permeabilized with 0.5% TritonX-100 in PBS for 10 min at RT. Cells were blocked with 1% BSA in PBS with 0.05% Tween-20 for 1 hour at RT. After blocking, the cells were stained overnight at 4°C with anti-FLAG antibodies diluted in a blocking buffer. The next day, cells were washed three times in PBS and stained with secondary antibodies diluted in a blocking buffer for 1 hour at RT in the dark (goat anti-mouse AlexaFluor488; Invitrogen, A11001). After 3x PBS washes, nuclei were stained with Hoechst 33342 in PBS for 5 minutes at RT. Coverslips were then mounted with ProLong Gold antifade mounting media (Invitrogen, P36934) on Superfrost Plus microscope slides (#22-037-246). Immunostained cells were imaged using Nikon Ti-Eclipse inverted microscope (Nikon Instruments) equipped with a SpectraX LED excitation module (Lumencor) and emission filter wheels (Prior Scientific). Fluorescence imaging used excitation/emission filters and dichroic mirrors for GFP and DAPI (Chroma

Technology Corp.). Images were acquired with Plan Fluor 20Ph objective and an iXon 896 EM-CCD camera (Andor Technology Ltd.) in NIS-Elements software.

Cell cultures

293T cells (ATCC, CRL-3216) were maintained at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, cat. #12100-061) supplemented with 10% fetal bovine serum (FBS, ATLAS Biologicals, Lot. #F31E18D1), sodium bicarbonate (3.7 g/L), 50 I.U./mL penicillin and 50 mg/mL streptomycin. CFF-16HBEge CFTR W1282X cells (Cystic Fibrosis Foundation, Bethesda, MD, USA) were maintained at 37°C and 5% CO₂ in Modified Eagle Medium (MEM, GIBCO, cat. #12100-061) supplemented with 10% fetal bovine serum (FBS, ATLAS Biologicals), 50 I.U./mL penicillin and 50 mg/mL streptomycin. The cells tested PCR-negative for mycoplasma.

RTCB depletion

To deplete RTCB, 293T cells were transfected using Lipofectamine 3000 (ThermoFisher Scientific) with the plasmid encoding for Cas9, puromycin resistance protein, and a guide RNA targeting the *RTCB* gene. Twenty-four hours post-transfection, the media was changed to the media with puromycin (1 µg/mL), and the cells were selected for three days. After selection, cells were seeded on 96-well plates at 3 cells per well density to generate clonal cell lines. Cell clones were monitored for 14 days, and wells with single colonies were marked and used to determine knockout efficiencies. To determine knock-out efficiency, the genomic DNA from bulk populations or clonal cell lines was extracted using DNeasy Blood & Tissue Kit (QIAGEN), and targeted genome regions were amplified with Q5 polymerase (NEB) (table S6). Amplicons were Sanger-sequenced (Psomagen), and resulting traces (i.e., ab1 files) were used to quantify indels with the ICE software from Synthego (<https://ice.synthego.com>) (62).

Plasmid transfection

293T cells were seeded one or two days before the experiment and transfected when ~80-90% confluency was reached. For the experiment shown in Fig. 1B and Fig. 2C, 500 ng of plasmid DNA encoding for SthCsm complex and guide RNA was mixed with 1.5 µL of FuGENE HD reagent (Promega), incubated for 15 min, and added to the well of a 24-well plate. Twelve hours post-transfection, the cells were detached and seeded on 12-well plates in a media with puromycin (1 µg/mL). Three days after puromycin selection, the total RNA was extracted from cells using RNeasy Plus Mini Kit (QIAGEN, # 74134). For the experiment shown in Fig. 1E-H, transfection was performed as described above but without puromycin selection.

For the experiment shown in Fig. 4, the cells were seeded in a 96-well plate in ~80-90% confluency. For editing with SthCsm complex, 100 ng of plasmid encoding for SthCsm and 50 ng of plasmid encoding for a reporter (stop-GFP or GFP-stop-Luc) were mixed with 0.8 µL FuGENE HD reagent (Promega), incubated for 10 min, and added to the well. For editing with DisCas7-11, 100 ng of plasmid encoding for DisCas7-11, 50 ng of the plasmid encoding for guide RNA, and 50 ng of the plasmid encoding for the GFP-stop-Luc reporter were mixed with 1.1 µL FuGENE HD reagent (Promega), incubated for 10 min, and added to the wells. Forty-eight hours post-transfection, the cells were imaged with the Nikon Ti-Eclipse inverted microscope (Nikon Instruments) equipped with a SpectraX LED excitation module (Lumencor) and emission filter wheels (Prior Scientific) (for the reporter stop-GFP). Fluorescence imaging used excitation/emission filters and dichroic mirrors for GFP (Chroma Technology Corp). Images

were acquired with Plan Fluor 20Ph objective and an iXon 896 EM-CCD camera (Andor Technology Ltd.) in NIS-Elements software. For luciferase assays, media was removed, and cells were lysed in 50 μ L of the 1X Cell Culture Lysis Reagent (Promega). Lysates were used for luciferase assays (20 μ L) with Luciferase assay system (Promega) and RNA extraction (20 μ L) with RNeasy Plus Mini Kit (QIAGEN, # 74134).

For the experiment shown in Fig. 5, the CFF-16HBE cells were transfected using the reverse-transfection method. First, 2 μ g of the plasmid DNA was mixed with 6 μ L FuGENE HD reagent (Promega), incubated for 10 min, and added to the well of a 12-well plate. Then, 160,000 cells were added per well. Twelve hours post-transfection, the media was changed for the media with puromycin (0.75 μ g/mL). Two days after puromycin selection, the total RNA was extracted from cells using the RNeasy Plus Mini Kit (QIAGEN, # 74134).

RT-qPCR

Total RNA from cells was extracted with RNeasy Plus Mini Kit (QIAGEN, # 74134). The reverse-transcription (RT) reactions were performed with 2X LunaScript RT SuperMix Kit (NEB) with 5 μ L of RNA input (250 ng of RNA total) in 10 μ L total reaction volume. The RT reactions containing cDNA were diluted 100-fold, and 5 μ L was used for qPCR reactions with 2X Universal SYBR Green Fast qPCR Mix (ABClonal). Each qPCR reaction contained 5 μ L of cDNA, 4.2 μ L of Nuclease-free Water, 0.4 μ L of 10 mM Primers (table S7), and 10 μ L of 2X mastermix. Nuclease-free water was used as no template control (NTC). Three technical replicates were performed for each sample. Amplification was performed in QuantStudio 3 Real-Time PCR System instrument (Applied Biosystems) as follows: 95°C for 3 min, 40 cycles of 95°C for 5 s, and 60°C for 30 s, followed by Melt Curve analysis. Results were analyzed in Design and Analysis application at Thermo Fisher Connect Platform.

Nanopore sequencing

Amplicons from RT-qPCR reactions were purified with magnetic beads (Mag-Bind® TotalPure NGS), and ~13 ng of DNA was used to prepare sequencing libraries as described in SQK-LSK114 protocol using Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96). Edited *XIST* RNA was reverse-transcribed using the LunaScript RT SuperMix Kit (NEB), and cDNA was used for PCR with primers *XIST_seq_F* and *XIST_seq_R* (table S7) and Q5 polymerase (NEB). Amplicons were purified with magnetic beads (Mag-Bind® TotalPure NGS), and ~13 ng of DNA was used to prepare sequencing libraries as described in SQK-LSK114 protocol using Native Barcoding Kit 24 V14 (SQK-NBD114.24). ~20 fM of the library was loaded on the Nanopore MinION [R10.4.1 flow cell, accurate mode (260 bases per second)]. The flow cell was primed, and sequencing libraries were loaded according to the Oxford Nanopore protocol (SQK-LSK114). The sequencing run was performed in the fast base calling mode in the MinKNOW software. After the run, raw sequencing data (POD5 files) was re-basecalled with the super-accuracy model (dna_r10.4.1_e8.2_260bps_sup@v4.1.0) using and demultiplexed with Dorado basecaller (v0.3.3, Oxford Nanopore).

Sequencing data analysis

Sequencing reads were aligned to the reference using *minimap2* (v2.17 -r954-dirty) with Nanopore preset (-ax map-ont setting). Alignments were converted to BAM format, sorted, and indexed using *samtools* v1.13. Sequencing reads were trimmed to remove primer binding regions

using the *samtools ampliconclip*. To make sequencing depth plots, a subset of reads was randomly selected using *samtools view*. Sequencing depth was computed with the *samtools depth*. Deletions in sequencing reads were quantified using the *extract-junctions.py* script (github.com/hyeshik/sars-cov-2-transcriptome/blob/master/nanopore/_scripts/extract-junctions.py) (63). Deletion counts at the SthCsm target sites were normalized to the number of reads that span the target region, which was computed by extracting read information from BAM files using the *bamtobed* function in *bedtools* v2.30.0 package and filtering reads by start and end coordinate (Data S1). All plots were created using *ggplot2* v3.3.5 package in RStudio software v2023.03.0.

Western blot

Cells were washed two times with PBS and lysed in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.2% SDS, 50 mM Tris, pH 8.0) at 4°C for 30 min. The lysates were clarified by centrifugation (20 min at 10,000 g) and stored at -80°C. Lysates were mixed with 6X Laemmli buffer, heated at 98°C for 5 min, resolved in 12% SDS-PAGE gel, and transferred onto a nitrocellulose membrane using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, #1703930). Membranes were stained with Ponceau S, imaged, washed, blocked, and probed with indicated antibodies. The proteins were visualized using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, #32106) and exposed to X-Ray film (sc-201696, Santa Cruz Biotech).

Statistical analysis

All experiments were performed in biological triplicates. All RT-qPCR reactions were performed in three biological replicates, each with three technical replicates. Pairwise comparisons were performed in R with Welch's unequal variances *t*-test using the *t.test* function from the *stats* package. One-way ANOVA was performed using the *aov* function from the *stats* package in R. Post-hoc multiple pairwise comparisons were performed with Dunnett's test using the *glht* function from the *multcomp* R package (Fig. 4E) and Tukey's test using the *TukeyHSD* function from the *stats* R package (Fig. 2F). Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Supplementary Text

RTCB knockout

There are three copies of the *RTCB* gene in 293T cells, and after testing 41 clones, we have not identified any with a complete knockout (64) (**fig. S3D**). These results are consistent with work showing that *RTCB* is essential (24-26). However, we have identified clones with a knockout of two copies of the gene, which results in a substantial depletion of the RTCB protein (**Fig. 2B; fig. S3**).

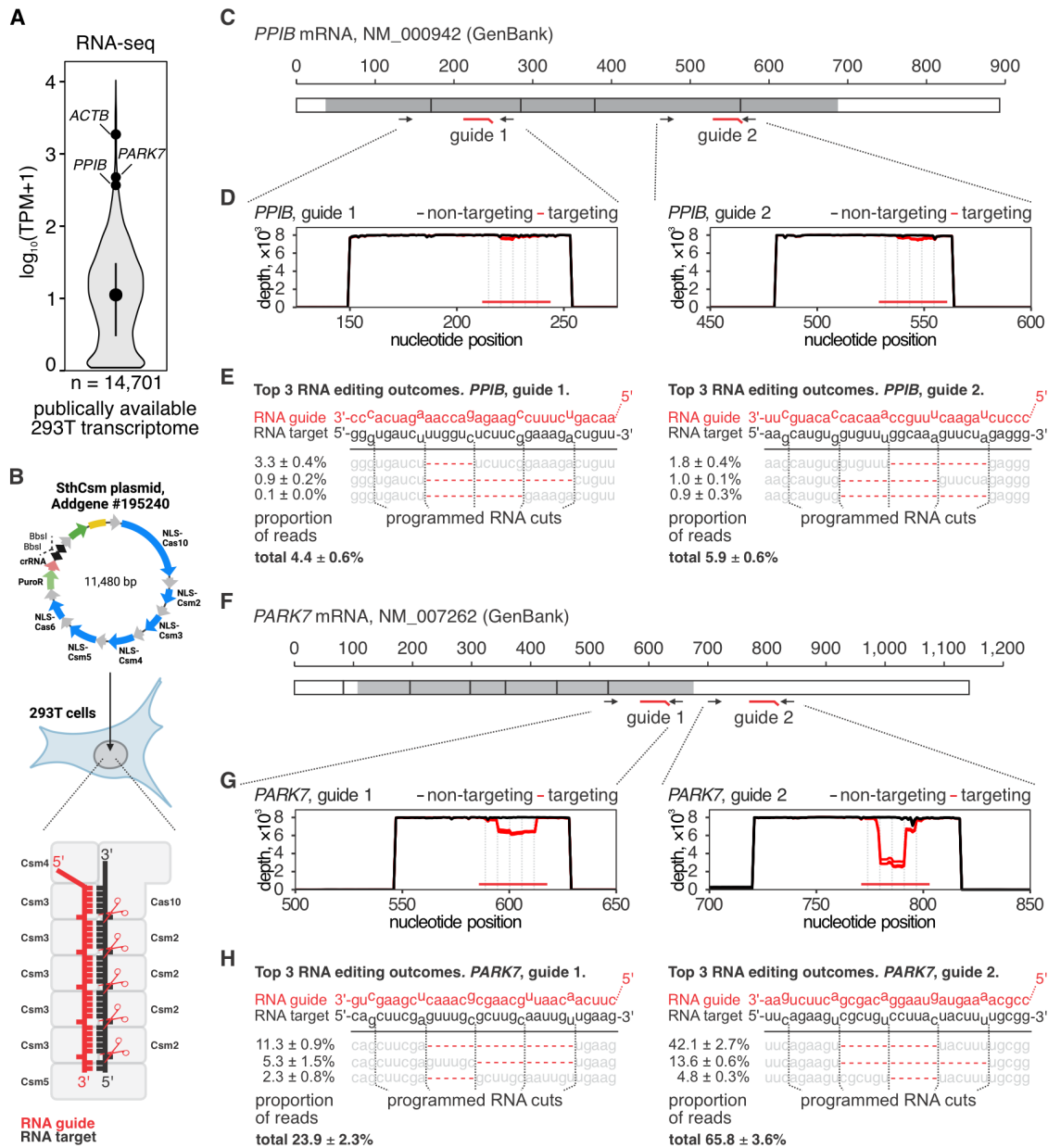
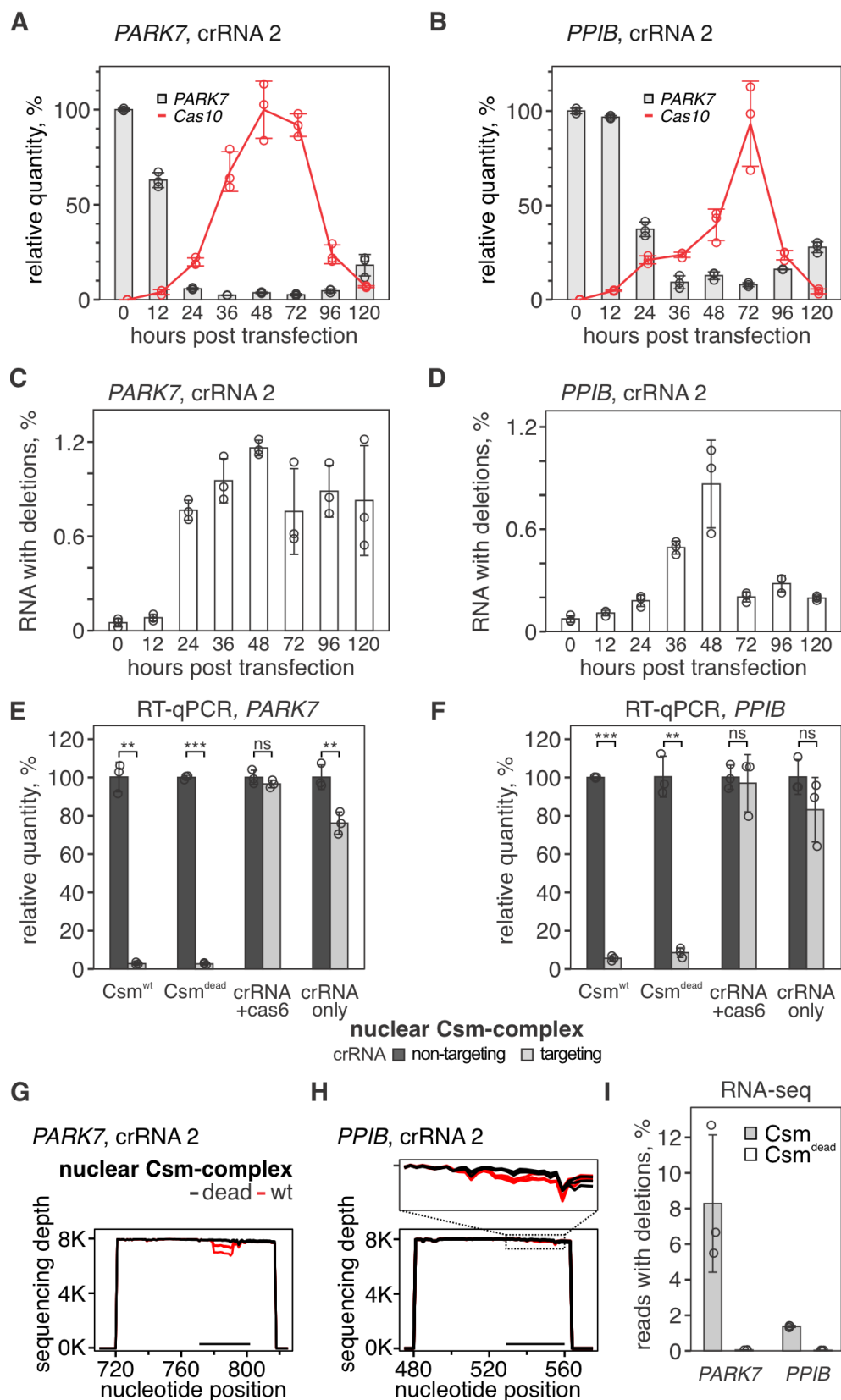


Fig. S1. The distribution and frequency of RNA editing outcomes in *PPIB* and *PARK7* mRNAs. **A)** Publicly available gene expression data from Human Protein Atlas (<https://www.proteinatlas.org/about/download>) was used to generate a violin plot. The y-axis shows expression of human genes in 293T cells (n = 14,701 genes with non-zero expression). TPM – transcripts per million. The vertical black line shows the interquartile range. The black dot shows the median expression level. **B)** *Top:* Diagram of the plasmid (Addgene #195240) encoding for human codon-optimized and NLS-tagged protein subunits of the type III-A CRISPR-Csm complex from *Streptococcus thermophilus* (SthCsm), CRISPR RNA (crRNA) - processing nuclease Cas6, and single-spacer CRISPR array. CRISPR repeats are shown as black diamonds. The sequence between the repeats has two BbsI restriction sites for cloning spacers for targeting complementary target RNAs. *Bottom:* Diagram of the assembled SthCsm ribonucleoprotein complex bound to the target RNA (black). crRNA is shown with red color.

The target RNA is cleaved in six nucleotide intervals by Csm3 nucleases in the backbone of the complex. Cleavage sites are indicated with red scissors. **C)** Diagram of the *PPIB* mRNA (NM_000942, GenBank). The scale shows nucleotide position along the transcript. Blocks show exons in the spliced mRNA, gray color indicates the position of the *PPIB* open reading frame (ORF). Red lines mark the position of the SthCsm guide RNAs. Black arrows indicate the position of the binding sites for oligonucleotide primers used for RT-qPCR in Fig. 1B. **D)** qPCR amplicons were deep-sequenced, and resulting reads were aligned to the reference sequences. A subset of 8,000 reads was randomly selected from the alignment for the representation. Plots show sequencing coverage (y-axes) along the length of the amplicons (x-axes). Each line represents a single replicate, three replicates total per guide RNA. Vertical dotted gray lines indicate predicted RNA break sites. The horizontal red line shows the position of the guide RNA with respect to the target. **E)** Top three most frequent RNA editing outcomes in *PPIB* transcript. Dotted lines indicate the positions of RNA breaks introduced with the CRISPR complex. Red dashes depict deletions identified in the sequencing data. Deletion frequency was quantified as the mean $\pm$ one standard deviation of three biological replicates. **F)** Same as in (C) but for the *PARK7* transcript (NM_007262, GenBank). **G)** Same as in (D) but for target sites in the *PARK7* transcript. **H)** Top three most frequent RNA editing outcomes at the target sites in *PARK7*. Data is shown as the mean $\pm$ one standard deviation of three biological replicates.



cas10 gene from the NLS-tagged SthCsm complex expression. The same data as in main Fig. 1E. Data was included here for comparison with *PPIB* data. **B)** Same as in **(A)** but for the *PPIB* transcript (guide 2). **C)** *PARK7* qPCR products in **(A)** were sequenced, and deletion efficiency was calculated as [relative quantity] $\times$ [fraction of reads with deletions]. The same data as in main Fig. 1F. Data was included here for comparison with *PPIB* data. **D)** Same as in **(C)** but for the *PPIB* transcript. **E)** *PARK7* knockdown efficiencies with “wildtype” NLS-tagged SthCsm complex (Csm^{wt}), catalytically inactive NLS-tagged SthCsm complex (Csm^{dead}), crRNA expressed only with Cas6 gene (no Csm genes), and crRNA alone were measured using RT-qPCR. Welch’s t-test was used to compare samples expressing targeting and non-targeting crRNA. ** - $p < 0.01$, *** - $p < 0.001$, ns – non-significant. The same data as in main Fig. 1G. Data was included here for comparison with *PPIB* data. **F)** Same as in **(E)** but for *PPIB*. **G)** RT-qPCR amplicons in **(E)** were sequenced, and reads were aligned to reference as in fig. S1G. A subset of 8,000 reads was randomly selected from the alignment for the representation. The plot shows sequencing depth (y-axis) along the length of the amplicon (x-axis). Each line represents a single replicate, with three replicates total per Csm-complex. Horizontal black bar shows position of the sequence targeted with the Csm-complex. **H)** Same as in **(G)** but for *PPIB*. The inset on top shows the zoom-in on the region targeted with Csm-complex (dotted box). **I)** Quantification of data in **(G)** and **(H)**.

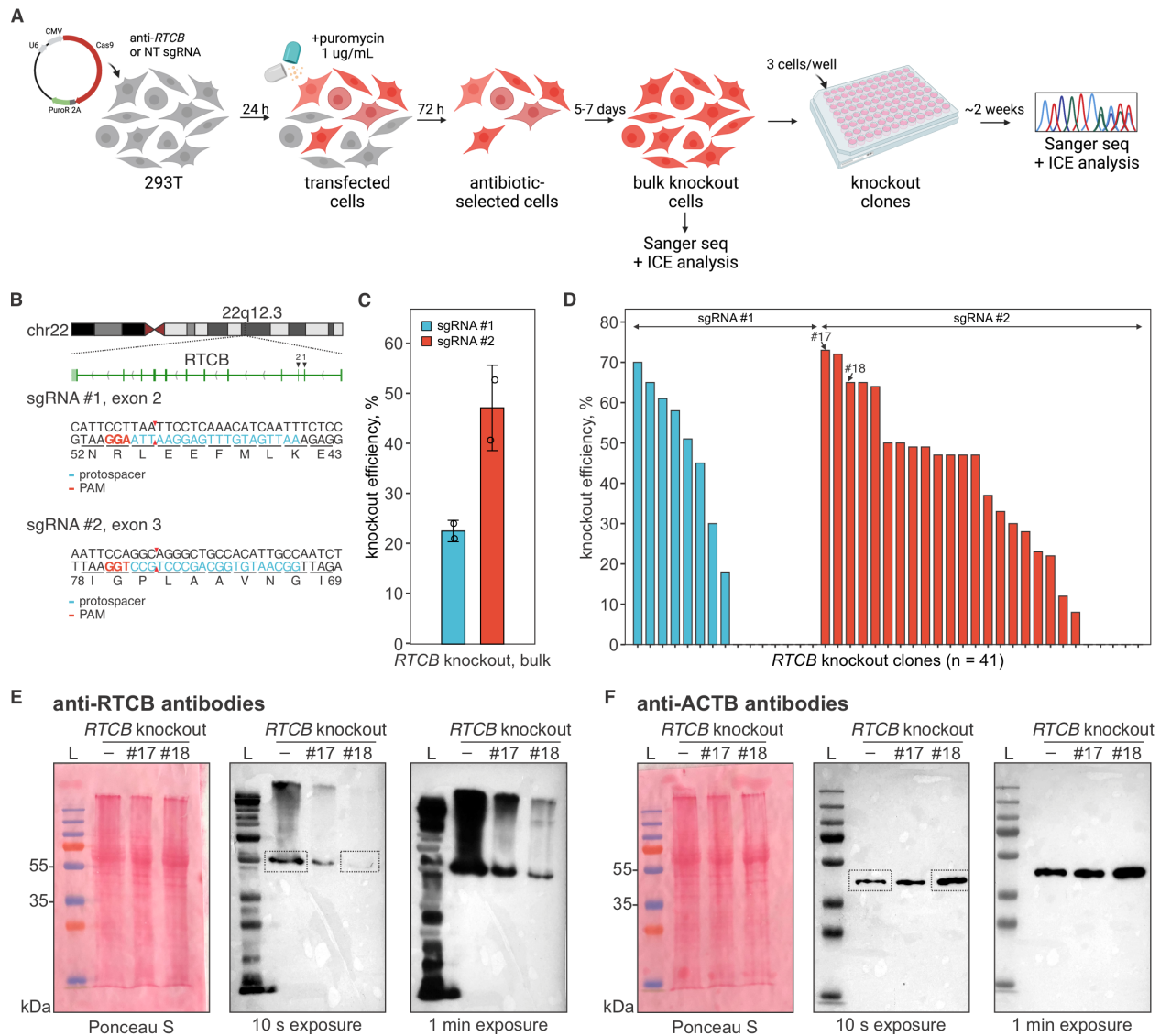


Fig. S3. Knockout of RTCB gene with CRISPR-Cas9. **A)** Schematics of the knockout strategy. **B)** Diagram showing the exon-intron structure of the *RTCB* gene (top), and sequences targeted with Cas9 (middle and bottom). **C)** *RTCB* knockout efficiency in bulk cells was quantified by analyzing Sanger traces from target site amplicons using the ICE web tool (Synthego). **D)** Quantification of *RTCB* knockout efficiency in 293T cell clones. **E and F)** Clones #17 and #18, indicated with black arrows in **(D)**, were lysed, and lysates were probed with anti-RTCB or anti-ACTB antibodies. Dotted boxes show parts of the images that were cropped out for main Fig. 2B.

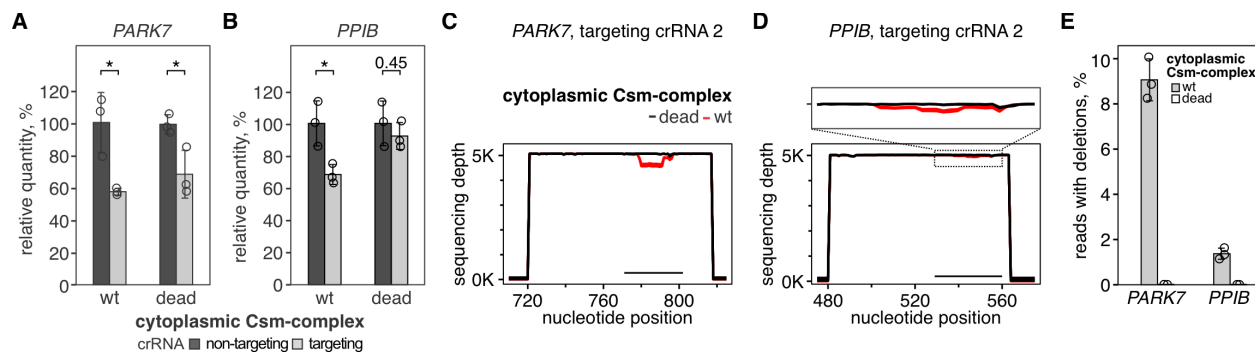


Fig. S4. Targeting nuclear-exported transcripts *PARK7* and *PPIB* with cytoplasmic SthCsm-complex. **A, B** Knockdown of *PARK7* (A) and *PPIB* (B) transcripts with cytoplasmic SthCsm (no NLS). Data in (A) is the same as in main Fig. 2H and was included for comparison with *PPIB* targeting. Transcript levels were quantified with RT-qPCR and normalized to *ACTB* and non-targeting control. “wt” – nuclease-active SthCsm, “dead” – catalytically inactivated SthCsm (Csm3^{D33A} mutation). *p-value < 0.05, ns – non-significant; Welch’s t-test. Data is shown as mean (n = 3) ± one standard deviation. **C, D** RT-qPCR amplicons in (A) and (B) were deep-sequenced, and reads were aligned to reference sequences. Analysis was performed as in fig. S1 and fig. S2. **E** Quantification of data in (C) and (D).

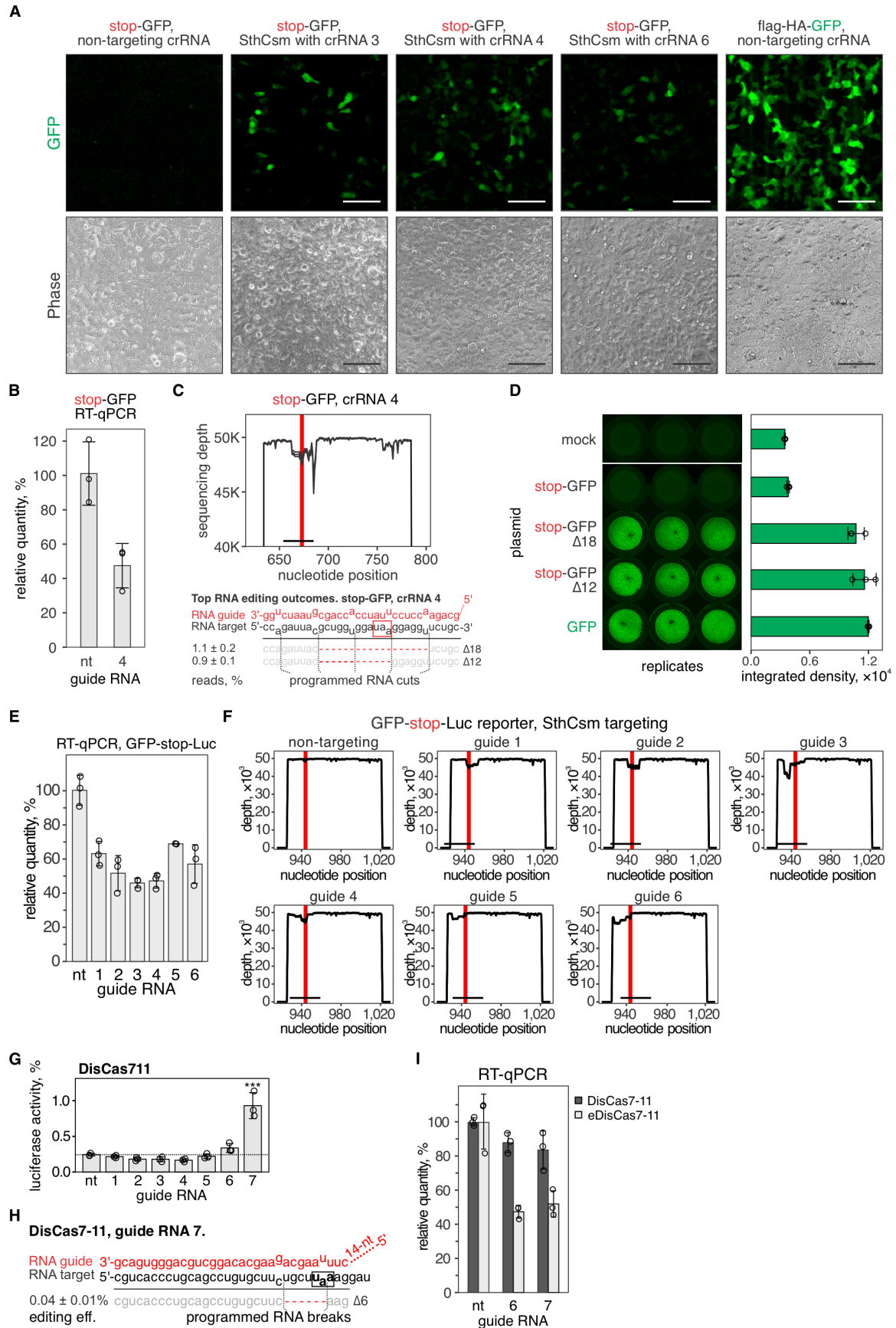


Fig. S5. Programmable deletion of stop codons in RNA restores protein expression.

A) Fluorescent imaging of cells transfected with plasmids for the stop-GFP reporter and SthCsm with a non-targeting guide (left), stop-GFP reporter and SthCsm with targeting guides, or GFP reporter and SthCsm with the non-targeting guide (right). Scale bars – 50 μm . **B)** RT-qPCR was used to quantify stop-GFP reporter RNA in cells transfected with plasmids encoding for SthCsm complex and non-targeting crRNA (NT) or targeting crRNA 4. **C) Top:** RT-qPCR products were sequenced, and sequencing depth (*y-axis*) was calculated and plotted against the position in the reporter RNA (*x-axis*) (*top*). The horizontal black bar marks the target sequence in the reporter. The vertical red line highlights the stop codon. *Bottom:* two most frequent deletions in the target RNA. The red box marks the stop codon (UAA). **D)** Site-directed mutagenesis was used to make deletions identified in **(C)** into the stop-GFP reporter plasmid. Mutagenized plasmids were used to transfect 293T cells, and cell culture plates were imaged 48 hours post-transfection using the Typhoon scanner (*left*). The fluorescent signal (integrated density) was quantified in ImageJ and plotted as a bar graph (*right*). **E)** RNA was extracted from the same lysates that were used in luciferase assays shown in Fig. 4E. RT-qPCR was performed with oligonucleotide primers that flank the region targeted with SthCsm in the GFP-stop-Luc reporter transcript. Transcript quantities are normalized to *ACTB* and non-targeting guide RNA control using the $\Delta\Delta\text{Ct}$ method. The mean $\pm$ one standard deviation of three biological replicates is shown. nt – non-targeting guide RNA. **F)** Deep sequencing of qPCR amplicons in **(E)**. Reads were aligned to the reference sequence of the GFP-stop-Luc reporter. Graphs show sequencing depth (*y-axes*) at the amplified region of the transcript (*x-axes*). Every line shows a biological replicate ($n = 3$). The horizontal black bar indicates a region complementary to the guide RNA of the SthCsm complex. Vertical red lines mark the position of the stop codon targeted with SthCsm. **G)** Luciferase activity was measured in cell lysates 48 hours after transfection with GFP-stop-Luc and DisCas7-11 plasmids. Luciferase activity is normalized to a control transfected with a reporter plasmid without the stop codon. Data are shown as mean $\pm$ one standard deviation of three replicates. Means were compared using one-way ANOVA, and samples with targeting guide RNAs were compared to the non-targeting control using one-tailed Dunnett's test. *** $p < 0.001$. **H)** Frequency of RNA editing with DisCas7-11 and guide 7. Editing efficiency was quantified as mean $\pm$ one standard deviation of three biological replicates. The black box shows the stop codon that was targeted by DisCas7-11. **I)** RNA was extracted from the same lysates that were used in luciferase assays shown in **(G)**(DisCas7-11) and main Fig. 4H (eDisCas7-11). RT-qPCR was performed with oligonucleotide primers that flank the target region in the GFP-stop-Luc reporter transcript. Transcript quantities are normalized to *ACTB* and non-targeting guide RNA control using the $\Delta\Delta\text{Ct}$ method. The mean $\pm$ one standard deviation of three biological replicates is shown. nt – non-targeting guide RNA.

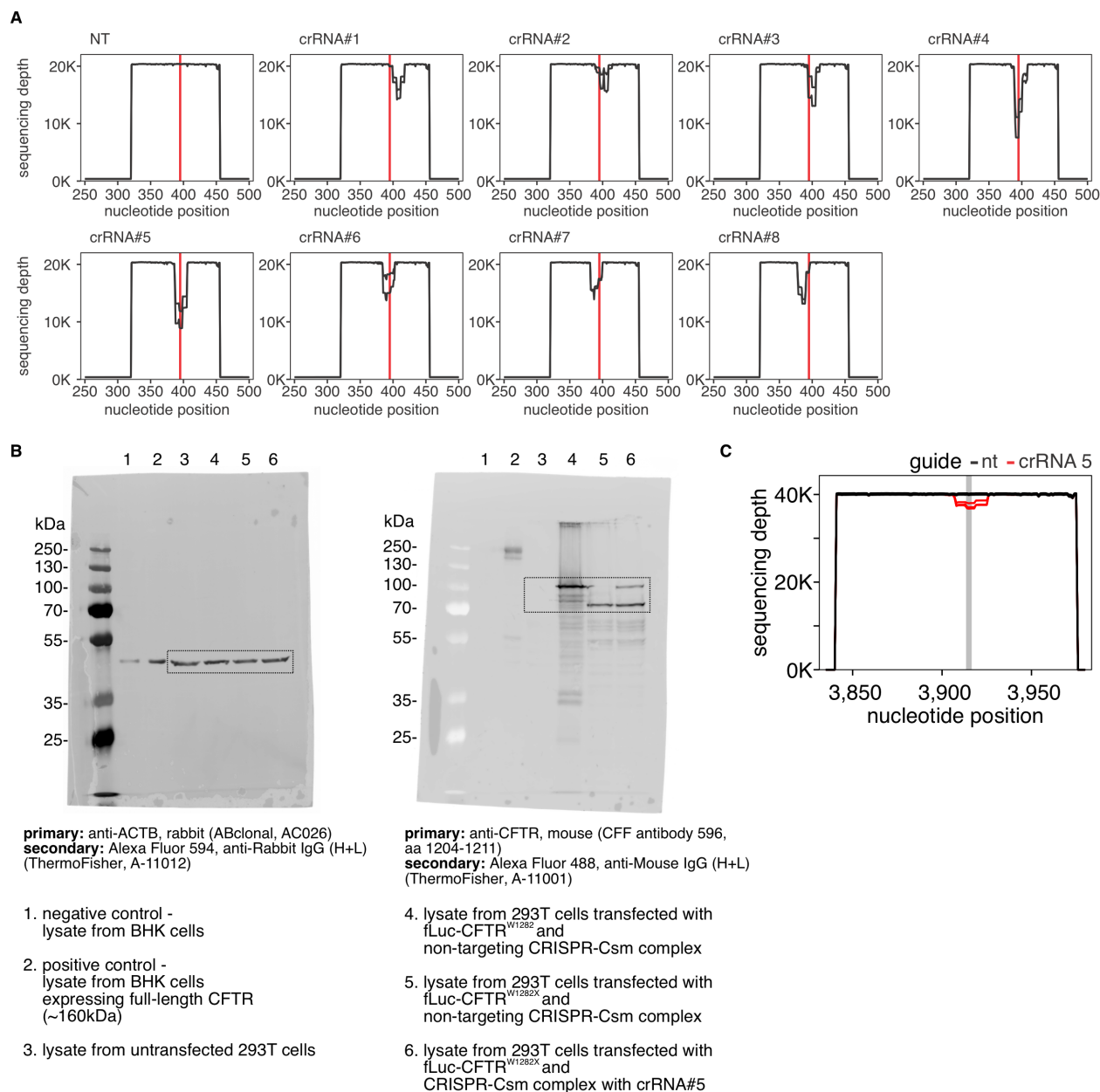


Fig. S6. Programmable excision of W1282X premature stop codon in *CFTR* transcript. **A)** Deep-sequencing of qPCR amplicons in Fig. 5B. Reads were aligned to the reference sequence of the fLuc-CFTR reporter. Graphs show sequencing depth (y-axes) at the amplified region of the transcript (x-axes). Every line shows a biological replicate (n = 2). Vertical red lines mark the position of the stop codon targeted with SthCsm. **B)** Uncropped western blot images for Fig. 5D. Dotted boxes show parts of the images that were cropped out. **C)** Deep-sequencing of qPCR amplicons in Fig. 5F. Reads were aligned to the reference sequence of the *CFTR* (NM_000492.4, GenBank). Graph show sequencing depth (y-axis) at the amplified region of the transcript (x-axis). Every line shows a biological replicate (n = 3).

Table S1.

Oligonucleotides used for cloning SthCsm guide RNAs

Name	Sequence 5'-3'
<i>Guides targeting endogenous transcripts</i>	
PPIB_g1F	AAACAACAGTCTTTCCGAAGAGACCAAAGATCACCC
PPIB_g1R	TATCGGGTGATCTTTGGTCTCTTCGGAAAGACTGTT
PPIB_g2F	AAACCCCTCTAGAACTTTGCCAAACACCACATGCTT
PPIB_g2R	TATCAAGCATGTGGTGTGTTGGCAAAGTTCTAGAGGG
PARK7_g1F	AAACCTTCAACAATTGCAAGCGCAAACCTCGAAGCTG
PARK7_g1R	TATCCAGCTTCGAGTTTGCCTTGCAATTGTTGAAG
PARK7_g2F	AAACCCGCAAAAAGTAGTAAGGACAGCGACTTCTGAA
PARK7_g2R	TATCTTCAGAAGTCGCTGTCTTACTACTTTTTCGG
XIST_cr1F	AAACTAAAACCAGGTATCCGCAGCCCCGATGGGCAA
XIST_cr1R	TATCTTGCCCATCGGGGCTGCGGATACCTGGTTTTA
XIST_cr2F	AAACAAAAGCAGATATCCGTCACCCCGATGGGCCAG
XIST_cr2R	TATCCTGGCCCATCGGGGTGACGGATATCTGCTTTT
XIST_cr3F	AAACAAAAAGCAGGTATCCGAAGCCCCGATGGGCCA
XIST_cr3R	TATCTGGCCCATCGGGGCTTCGGATACCTGCTTTTT
XIST_cr4F	AAACAAAATCAAAGCAGGTATCCGCGGCCCGATGG
XIST_cr4R	TATCCCATCGGGGCCGCGGATACCTGCTTTGATTTT
<i>Guides targeting stop-GFP reporter</i>	
sthcsm_stop_gfp_g1F	AAACGCGGCGGGGGCAGAACCTCCTTATCCACCAGC
sthcsm_stop_gfp_g1R	TATCGCTGGTGGATAAGGAGGTTCTGCCCCCGCCGC
sthcsm_stop_gfp_g2F	AAACGCGGGGGCAGAACCTCCTTATCCACCAGCGTA
sthcsm_stop_gfp_g2R	TATCTACGCTGGTGGATAAGGAGGTTCTGCCCCGC
sthcsm_stop_gfp_g3F	AAACGGGGCAGAACCTCCTTATCCACCAGCGTAATC
sthcsm_stop_gfp_g3R	TATCGATTACGCTGGTGGATAAGGAGGTTCTGCCCC
sthcsm_stop_gfp_g4F	AAACGCAGAACCTCCTTATCCACCAGCGTAATCTGG
sthcsm_stop_gfp_g4R	TATCCCAGATTACGCTGGTGGATAAGGAGGTTCTGC
sthcsm_stop_gfp_g5F	AAACGAACCTCCTTATCCACCAGCGTAATCTGGAAC
sthcsm_stop_gfp_g5R	TATCGTTCCAGATTACGCTGGTGGATAAGGAGGTTCT
sthcsm_stop_gfp_g6F	AAACCCTCCTTATCCACCAGCGTAATCTGGAACATC
sthcsm_stop_gfp_g6R	TATCGATGTTCCAGATTACGCTGGTGGATAAGGAGG
<i>Guides targeting GFP-stop-Luc reporter</i>	
csm_gfp_stop_luc_g1F	AAACTCACCGGTCACATTGATCCTTTAAGCAGAAGC
csm_gfp_stop_luc_g1R	TATCGCTTCTGCTTAAAGGATCAATGTGACCGGTGA
csm_gfp_stop_luc_g2F	AAACCCGGTCACATTGATCCTTTAAGCAGAAGCACA
csm_gfp_stop_luc_g2R	TATCTGTGCTTCTGCTTAAAGGATCAATGTGACCGG
csm_gfp_stop_luc_g3F	AAACGTACATTGATCCTTTAAGCAGAAGCACAGGC

csm_gfp_stop_luc_g3R	TATCGCCTGTGCTTCTGCTTAAAGGATCAATGTGAC
csm_gfp_stop_luc_g4F	AAACACATTGATCCTTTAAGCAGAAGCACAGGCTGC
csm_gfp_stop_luc_g4R	TATCGCAGCCTGTGCTTCTGCTTAAAGGATCAATGT
csm_gfp_stop_luc_g5F	AAACTTGATCCTTTAAGCAGAAGCACAGGCTGCAGG
csm_gfp_stop_luc_g5R	TATCCCTGCAGCCTGTGCTTCTGCTTAAAGGATCAA
csm_gfp_stop_luc_g6F	AAACATCCTTTAAGCAGAAGCACAGGCTGCAGGGTG
csm_gfp_stop_luc_g6R	TATCCACCCTGCAGCCTGTGCTTCTGCTTAAAGGAT

Guides targeting fLuc-CFTR(W1282X) reporter and CFTR

CFTR_1282_cr1F	AAACTGTGGTATCACTCCAAAGGCTTTCCTTCACTG
CFTR_1282_cr1R	TATCCAGTGAAGGAAAGCCTTTGGAGTGATACCACA
CFTR_1282_cr2F	AAACGGTATCACTCCAAAGGCTTTCCTTCACTGTTG
CFTR_1282_cr2R	TATCCAACAGTGAAGGAAAGCCTTTGGAGTGATACC
CFTR_1282_cr3F	AAACATCACTCCAAAGGCTTTCCTTCACTGTTGCAA
CFTR_1282_cr3R	TATCTTGCAACAGTGAAGGAAAGCCTTTGGAGTGAT
CFTR_1282_cr4F	AAACACTCCAAAGGCTTTCCTTCACTGTTGCAAAGT
CFTR_1282_cr4R	TATCACTTTGCAACAGTGAAGGAAAGCCTTTGGAGT
CFTR_1282_cr5F	AAACCCAAAGGCTTTCCTTCACTGTTGCAAAGTTAT
CFTR_1282_cr5R	TATCATAACTTTGCAACAGTGAAGGAAAGCCTTTGG
CFTR_1282_cr6F	AAACAAGGCTTTCCTTCACTGTTGCAAAGTTATTGA
CFTR_1282_cr6R	TATCTCAATAACTTTGCAACAGTGAAGGAAAGCCTT
CFTR_1282_cr7F	AAACGCTTTCCTTCACTGTTGCAAAGTTATTGAATC
CFTR_1282_cr7R	TATCGATTCAATAACTTTGCAACAGTGAAGGAAAGC
CFTR_1282_cr8F	AAACTTCCTTCACTGTTGCAAAGTTATTGAATCCCA
CFTR_1282_cr8R	TATCTGGGATTCAATAACTTTGCAACAGTGAAGGAA

Table S2.

Oligonucleotides used for generating plasmids encoding for crRNA or crRNA with Cas6-NLS gene

Name	Sequence 5`-3`
AN_Cas6F	GCCGGCCGGAGAATAAAAAG
AN_PuroF	GCGATAGAGGGATCCCGC
AN_Afl II R	CTTAAGAAAAAGGCCGCGTTGC

Table S3.

Oligonucleotide primers used for Q5 SDM

Name	Sequence 5'-3'
SthCR_RFP_F	CGCGATGAAGCGATTTCAGCGTCTCTGATATAAACCTAATTACCTCGAGAGGGGAC GCGGCCGCCACCATG
SthCR_RFP_R	TCTCGGTTTCCGTCCCCTCTCGAGGTAATTAGGTTTATATCGGATCTCTAGCGGAT CTGACGGTTCATAAACCAGCTC
NLS- DisCas711_F	AAGCGGAAGGTCGGCGGTAGCACTACTATGAAGATTTCATTGAATTC
NLS- DisCas711_R	CTTCTTTGGGGCCATGGTGGCGGCTCTCCCTATAGTG
Cas711_F1_F	AGAACTCAAACGCGGGGAATTCAAGAAAG
Cas711_F1_R	GATATCCCAGAGTTTATGGTCATCC
Cas711_F2_F	GGATGACCATAAACTCTGGGATATC
Cas711_F2_R	CTGGGAGAAATTTTGCAGCACA
Cas711_F3_F	TGTGCTGCAAAAATTTCTCCCAG
Cas711_F3_R	CTTTCTTGAATTCCCCGCGTTTGAGTTCT

Table S4.

Oligonucleotides used for cloning Cas9 guides targeting RTCB gene

Name	Sequence 5`-3`
RTCB_sg1F	CACCGAATTGATGTTTGAGGAATTA
RTCB_sg1R	CAAATAATTCCTCAAACATCAATTC
RTCB_sg2F	CACCGGCAATGTGGCAGCCCTGCC
RTCB_sg2R	CAAAGGCAGGGCTGCCACATTGCC

Table S5.

Oligonucleotides used for cloning DisCas7-11 guide RNAs

Name	Sequence 5'-3'
discas711_gfp_stop_luc_g1F	GAACTTGATCCTTTAAGCAGAAGCACAGGCTGCAGG
discas711_gfp_stop_luc_g1R	AAAACCTGCAGCCTGTGCTTCTGCTTAAAGGATCAA
discas711_gfp_stop_luc_g2F	GAACTGATCCTTTAAGCAGAAGCACAGGCTGCAGGG
discas711_gfp_stop_luc_g2R	AAAACCCTGCAGCCTGTGCTTCTGCTTAAAGGATCA
discas711_gfp_stop_luc_g3F	GAACGATCCTTTAAGCAGAAGCACAGGCTGCAGGGT
discas711_gfp_stop_luc_g3R	AAAAACCCCTGCAGCCTGTGCTTCTGCTTAAAGGATC
discas711_gfp_stop_luc_g4F	GAACATCCTTTAAGCAGAAGCACAGGCTGCAGGGTG
discas711_gfp_stop_luc_g4R	AAAACACCCTGCAGCCTGTGCTTCTGCTTAAAGGAT
discas711_gfp_stop_luc_g5F	GAACTCCTTTAAGCAGAAGCACAGGCTGCAGGGTGA
discas711_gfp_stop_luc_g5R	AAAATCACCCCTGCAGCCTGTGCTTCTGCTTAAAGGA
discas711_gfp_stop_luc_g6F	GAACCCTTTAAGCAGAAGCACAGGCTGCAGGGTGAC
discas711_gfp_stop_luc_g6R	AAAAGTCACCCTGCAGCCTGTGCTTCTGCTTAAAGG
discas711_gfp_stop_luc_g7F	GAACCTTTAAGCAGAAGCACAGGCTGCAGGGTGACG
discas711_gfp_stop_luc_g7R	AAAACGTCACCCTGCAGCCTGTGCTTCTGCTTAAAG

Table S6.

Oligonucleotides used to amplify Cas9-targeted regions in the *RTCB* gene

Name	Sequence 5'-3'
RTCB_seq_F1	AGATGGAGCTTCAAATCCGTT
RTCB_seq_R1	TGTGGTTTTGAGGGTATGAGAAT
RTCB_seq_F2	GGTTATGTCTGGCTGTCCAAAG
RTCB_seq_R2	ATCTGGAATTTGAAGCTGGGTAG

Table S7.

Primers used for RT-qPCR and PCR

Name	Sequence 5'-3'
ACTB_F	AGAGCCTCGCCTTTGCC
ACTB_R	ATGCCGGAGCCGTTGTC
PPIB_cr1_qF1	GCGGCCGATGAGAAGAAGAA
PPIB_cr1_qR1	AGCTAAGGCCACAAAATTATCCAC
PPIB_cr2_qF1	CGCAGGCAAAGACACCAAC
PPIB_cr2_qR1	TTCCGCACCACCTCCA
PARK7_cr1_qF1	CCTACTCTGAGAATCGTGTGGA
PARK7_cr1_qR1	GAGCCGCCACCTCCTTG
PARK7_cr2_qF1	AGAGAAACAGGCCGTTAGGA
PARK7_cr2_qR1	GGCTGAGAAATCTCTGTGTAGTTG
gfp_stop_luc_qF1	GGATGGACCGTCACCCCTG
gfp_stop_luc_qR1	TGTTTTTGGCGTCTTCCATACC
XIST_qF. Ref. 19	GTTGTATCGGGAGGCAGTAAGAATCATCTTT
XIST_qR Ref. 19	GAAAAGCACACAGCAAAGACAAAGAGGC
XIST_seq_F	GTCTTCTTGACACGTCCTCCA
XIST_seq_R	TCTGAACACGCCCTTAGCTT
CFTR_qF1	GGAAGAGTACTTTGTTATCAGCTTT
CFTR_qF2	CAGGGAAGAGTACTTTGTTATCAGC

Data S1. (separate file)

Analysis of the sequencing data in Figs. 1-4, fig. S1 and fig. S3-6; Sequences of plasmids created in this work.

References and Notes

1. M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012). [doi:10.1126/science.1225829](https://doi.org/10.1126/science.1225829) [Medline](#)
2. G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences* **109**, (2012).
3. F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott, F. Zhang, Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013). [doi:10.1038/nprot.2013.143](https://doi.org/10.1038/nprot.2013.143) [Medline](#)
4. M. van Overbeek, D. Capurso, M. M. Carter, M. S. Thompson, E. Frias, C. Russ, J. S. Reece-Hoyes, C. Nye, S. Gradia, B. Vidal, J. Zheng, G. R. Hoffman, C. K. Fuller, A. P. May, DNA Repair Profiling Reveals Nonrandom Outcomes at Cas9-Mediated Breaks. *Mol. Cell* **63**, 633–646 (2016). [doi:10.1016/j.molcel.2016.06.037](https://doi.org/10.1016/j.molcel.2016.06.037) [Medline](#)
5. M. Kosicki, K. Tomberg, A. Bradley, Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* **36**, 765–771 (2018). [doi:10.1038/nbt.4192](https://doi.org/10.1038/nbt.4192) [Medline](#)
6. Y. Fu, J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung, J. D. Sander, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 (2013). [doi:10.1038/nbt.2623](https://doi.org/10.1038/nbt.2623) [Medline](#)
7. P. D. Hsu, D. A. Scott, J. A. Weinstein, F. A. Ran, S. Konermann, V. Agarwala, Y. Li, E. J. Fine, X. Wu, O. Shalem, T. J. Cradick, L. A. Marraffini, G. Bao, F. Zhang, DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013). [doi:10.1038/nbt.2647](https://doi.org/10.1038/nbt.2647) [Medline](#)
8. V. Pattanayak, S. Lin, J. P. Guilinger, E. Ma, J. A. Doudna, D. R. Liu, High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843 (2013). [doi:10.1038/nbt.2673](https://doi.org/10.1038/nbt.2673) [Medline](#)
9. E. Haapaniemi, S. Botla, J. Persson, B. Schmierer, J. Taipale, CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat. Med.* **24**, 927–930 (2018). [doi:10.1038/s41591-018-0049-z](https://doi.org/10.1038/s41591-018-0049-z) [Medline](#)
10. R. J. Ihry, K. A. Worringer, M. R. Salick, E. Frias, D. Ho, K. Theriault, S. Kommineni, J. Chen, M. Sondey, C. Ye, R. Randhawa, T. Kulkarni, Z. Yang, G. McAllister, C. Russ, J. Reece-Hoyes, W. Forrester, G. R. Hoffman, R. Dolmetsch, A. Kaykas, p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat. Med.* **24**, 939–946 (2018). [doi:10.1038/s41591-018-0050-6](https://doi.org/10.1038/s41591-018-0050-6) [Medline](#)
11. O. O. Abudayyeh, J. S. Gootenberg, S. Konermann, J. Joung, I. M. Slaymaker, D. B. T. Cox, S. Shmakov, K. S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E. S. Lander, E. V. Koonin, F. Zhang, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* **353**, aaf5573 (2016). [doi:10.1126/science.aaf5573](https://doi.org/10.1126/science.aaf5573) [Medline](#)

12. O. O. Abudayyeh, J. S. Gootenberg, P. Essletzbichler, S. Han, J. Joung, J. J. Belanto, V. Verdine, D. B. T. Cox, M. J. Kellner, A. Regev, E. S. Lander, D. F. Voytas, A. Y. Ting, F. Zhang, RNA targeting with CRISPR-Cas13. *Nature* **550**, 280–284 (2017). [doi:10.1038/nature24049](https://doi.org/10.1038/nature24049) [Medline](#)
13. S. Konermann, P. Lotfy, N. J. Brideau, J. Oki, M. N. Shokhirev, P. D. Hsu, Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell* **173**, 665–676.e14 (2018). [doi:10.1016/j.cell.2018.02.033](https://doi.org/10.1016/j.cell.2018.02.033) [Medline](#)
14. J. Borrajo, K. Javanmardi, J. Griffin, S. J. St Martin, D. Yao, K. Hill, P. C. Blainey, B. Al-Shayeb, Programmable multi-kilobase RNA editing using CRISPR-mediated trans-splicing. *bioRxiv* 2023.08.18.553620. [Preprint] (2023); [doi:10.1101/2023.08.18.553620](https://doi.org/10.1101/2023.08.18.553620).
15. D. B. T. Cox, J. S. Gootenberg, O. O. Abudayyeh, B. Franklin, M. J. Kellner, J. Joung, F. Zhang, RNA editing with CRISPR-Cas13. *Science* **358**, 1019–1027 (2017). [doi:10.1126/science.aag0180](https://doi.org/10.1126/science.aag0180) [Medline](#)
16. O. O. Abudayyeh, J. S. Gootenberg, B. Franklin, J. Koob, M. J. Kellner, A. Ladha, J. Joung, P. Kirchgatterer, D. B. T. Cox, F. Zhang, A cytosine deaminase for programmable single-base RNA editing. *Science* **365**, 382–386 (2019). [doi:10.1126/science.aax7063](https://doi.org/10.1126/science.aax7063) [Medline](#)
17. C. R. Hale, P. Zhao, S. Olson, M. O. Duff, B. R. Graveley, L. Wells, R. M. Terns, M. P. Terns, RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139**, 945–956 (2009). [doi:10.1016/j.cell.2009.07.040](https://doi.org/10.1016/j.cell.2009.07.040) [Medline](#)
18. G. Tamulaitis, M. Kazlauskienė, E. Manakova, Č. Venclovas, A. O. Nwokeoji, M. J. Dickman, P. Horvath, V. Siksnys, Programmable RNA shredding by the type III-A CRISPR-Cas system of *Streptococcus thermophilus*. *Mol. Cell* **56**, 506–517 (2014). [doi:10.1016/j.molcel.2014.09.027](https://doi.org/10.1016/j.molcel.2014.09.027) [Medline](#)
19. D. Colognori, M. Trinidad, J. A. Doudna, Precise transcript targeting by CRISPR-Csm complexes. *Nat. Biotechnol.* **41**, 1256–1264 (2023). [doi:10.1038/s41587-022-01649-9](https://doi.org/10.1038/s41587-022-01649-9) [Medline](#)
20. W. T. Woodside, N. Vantsev, R. J. Catchpole, S. C. Garrett, S. Olson, B. R. Graveley, M. P. Terns, Type III-A CRISPR systems as a versatile gene knockdown technology. *RNA* **28**, 1074–1088 (2022). [doi:10.1261/rna.079206.122](https://doi.org/10.1261/rna.079206.122) [Medline](#)
21. T. Fricke, D. Smalakyte, M. Lapinski, A. Pateria, C. Weige, M. Pastor, A. Kolano, C. Winata, V. Siksnys, G. Tamulaitis, M. Bochtler, Targeted RNA Knockdown by a Type III CRISPR-Cas Complex in Zebrafish. *CRISPR J.* **3**, 299–313 (2020). [doi:10.1089/crispr.2020.0032](https://doi.org/10.1089/crispr.2020.0032) [Medline](#)
22. NIH, ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar>
23. L. V. Sharova, A. A. Sharov, T. Nedorezov, Y. Piao, N. Shaik, M. S. H. Ko, Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. *DNA Res.* **16**, 45–58 (2009). [doi:10.1093/dnares/dsn030](https://doi.org/10.1093/dnares/dsn030) [Medline](#)
24. Y. Lu, F.-X. Liang, X. Wang, A synthetic biology approach identifies the mammalian UPR RNA ligase RtcB. *Mol. Cell* **55**, 758–770 (2014). [doi:10.1016/j.molcel.2014.06.032](https://doi.org/10.1016/j.molcel.2014.06.032) [Medline](#)

25. J. Jurkin, T. Henkel, A. F. Nielsen, M. Minnich, J. Popow, T. Kaufmann, K. Heindl, T. Hoffmann, M. Busslinger, J. Martinez, The mammalian tRNA ligase complex mediates splicing of XBP1 mRNA and controls antibody secretion in plasma cells. *EMBO J.* **33**, 2922–2936 (2014). [doi:10.15252/embj.201490332](https://doi.org/10.15252/embj.201490332) [Medline](#)
26. J. Popow, M. Englert, S. Weitzer, A. Schleiffer, B. Mierzwa, K. Mechtler, S. Trowitzsch, C. L. Will, R. Lührmann, D. Söll, J. Martinez, HSPC117 is the essential subunit of a human tRNA splicing ligase complex. *Science* **331**, 760–764 (2011). [doi:10.1126/science.1197847](https://doi.org/10.1126/science.1197847) [Medline](#)
27. A. Kroupova, F. Ackle, I. Asanović, S. Weitzer, F. M. Boneberg, M. Faini, A. Leitner, A. Chui, R. Aebersold, J. Martinez, M. Jinek, Molecular architecture of the human tRNA ligase complex. *eLife* **10**, e71656 (2021). [doi:10.7554/eLife.71656](https://doi.org/10.7554/eLife.71656) [Medline](#)
28. L. E. Maquat, G. G. Carmichael, Quality control of mRNA function. *Cell* **104**, 173–176 (2001). [doi:10.1016/S0092-8674\(01\)00202-1](https://doi.org/10.1016/S0092-8674(01)00202-1) [Medline](#)
29. A. Wutz, T. P. Rasmussen, R. Jaenisch, Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nat. Genet.* **30**, 167–174 (2002). [doi:10.1038/ng820](https://doi.org/10.1038/ng820) [Medline](#)
30. J. Minks, S. E. L. Baldry, C. Yang, A. M. Cotton, C. J. Brown, XIST-induced silencing of flanking genes is achieved by additive action of repeat a monomers in human somatic cells. *Epigenetics Chromatin* **6**, 23 (2013). [doi:10.1186/1756-8935-6-23](https://doi.org/10.1186/1756-8935-6-23) [Medline](#)
31. S. P. B. van Beljouw, A. C. Haagsma, A. Rodríguez-Molina, D. F. van den Berg, J. N. A. Vink, S. J. J. Brouns, The gRAMP CRISPR-Cas effector is an RNA endonuclease complexed with a caspase-like peptidase. *Science* **373**, 1349–1353 (2021). [doi:10.1126/science.abk2718](https://doi.org/10.1126/science.abk2718) [Medline](#)
32. A. Özcan, R. Krajewski, E. Ioannidi, B. Lee, A. Gardner, K. S. Makarova, E. V. Koonin, O. O. Abudayyeh, J. S. Gootenberg, Programmable RNA targeting with the single-protein CRISPR effector Cas7-11. *Nature* **597**, 720–725 (2021). [doi:10.1038/s41586-021-03886-5](https://doi.org/10.1038/s41586-021-03886-5) [Medline](#)
33. C. Schmitt-Ulms, A. Kayabolen, M. Manero-Carranza, N. Zhou, K. Donnelly, S. Pia Nuccio, K. Kato, H. Nishimasu, J. S. Gootenberg, O. O. Abudayyeh, Programmable RNA writing with trans-splicing. *bioRxiv* 2024.01.31.578223 [Preprint] (2024); [doi:10.1101/2024.01.31.578223v1](https://doi.org/10.1101/2024.01.31.578223v1).
34. S. M. Rowe, K. Varga, A. Rab, Z. Bebok, K. Byram, Y. Li, E. J. Sorscher, J. P. Clancy, Restoration of W1282X CFTR activity by enhanced expression. *Am. J. Respir. Cell Mol. Biol.* **37**, 347–356 (2007). [doi:10.1165/rcmb.2006-0176OC](https://doi.org/10.1165/rcmb.2006-0176OC) [Medline](#)
35. E. Nagy, L. E. Maquat, A rule for termination-codon position within intron-containing genes: When nonsense affects RNA abundance. *Trends Biochem. Sci.* **23**, 198–199 (1998). [doi:10.1016/S0968-0004\(98\)01208-0](https://doi.org/10.1016/S0968-0004(98)01208-0) [Medline](#)
36. Y. J. Kim, T. Nomakuchi, F. Papaleonidopoulou, L. Yang, Q. Zhang, A. R. Krainer, Gene-specific nonsense-mediated mRNA decay targeting for cystic fibrosis therapy. *Nat. Commun.* **13**, 2978 (2022). [doi:10.1038/s41467-022-30668-y](https://doi.org/10.1038/s41467-022-30668-y) [Medline](#)

37. A. Nemudryi, A. Nemudraia, J. E. Nichols, A. M. Scherffius, T. Zahl, B. Wiedenheft, CRISPR-based engineering of RNA viruses. *Sci. Adv.* **9**, eadj8277 (2023). [doi:10.1126/sciadv.adj8277](https://doi.org/10.1126/sciadv.adj8277) [Medline](#)
38. K. Nemeth, R. Bayraktar, M. Ferracin, G. A. Calin, Non-coding RNAs in disease: From mechanisms to therapeutics. *Nat. Rev. Genet.* **25**, 211–232 (2024). [doi:10.1038/s41576-023-00662-1](https://doi.org/10.1038/s41576-023-00662-1) [Medline](#)
39. R. Batra, D. A. Nelles, E. Pirie, S. M. Blue, R. J. Marina, H. Wang, I. A. Chaim, J. D. Thomas, N. Zhang, V. Nguyen, S. Aigner, S. Markmiller, G. Xia, K. D. Corbett, M. S. Swanson, G. W. Yeo, Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9. *Cell* **170**, 899–912.e10 (2017). [doi:10.1016/j.cell.2017.07.010](https://doi.org/10.1016/j.cell.2017.07.010) [Medline](#)
40. R. Vaz-Drago, N. Custódio, M. Carmo-Fonseca, Deep intronic mutations and human disease. *Hum. Genet.* **136**, 1093–1111 (2017). [doi:10.1007/s00439-017-1809-4](https://doi.org/10.1007/s00439-017-1809-4) [Medline](#)
41. P. Irmisch, I. Mogila, B. Samatanga, G. Tamulaitis, R. Seidel, Retention of the RNA ends provides the molecular memory for maintaining the activation of the Csm complex. *Nucleic Acids Res.* gkae080 (2024). [doi:10.1093/nar/gkae080](https://doi.org/10.1093/nar/gkae080) [Medline](#)
42. Y. Ben-Ari, Y. Brody, N. Kinor, A. Mor, T. Tsukamoto, D. L. Spector, R. H. Singer, Y. Shav-Tal, The life of an mRNA in space and time. *J. Cell Sci.* **123**, 1761–1774 (2010). [doi:10.1242/jcs.062638](https://doi.org/10.1242/jcs.062638) [Medline](#)
43. K. N. D’Orazio, R. Green, Ribosome states signal RNA quality control. *Mol. Cell* **81**, 1372–1383 (2021). [doi:10.1016/j.molcel.2021.02.022](https://doi.org/10.1016/j.molcel.2021.02.022) [Medline](#)
44. G. Dugar, R. T. Leenay, S. K. Eisenbart, T. Bischler, B. U. Aul, C. L. Beisel, C. M. Sharma, CRISPR RNA-Dependent Binding and Cleavage of Endogenous RNAs by the *Campylobacter jejuni* Cas9. *Mol. Cell* **69**, 893–905.e7 (2018). [doi:10.1016/j.molcel.2018.01.032](https://doi.org/10.1016/j.molcel.2018.01.032) [Medline](#)
45. M. R. O’Connell, B. L. Oakes, S. H. Sternberg, A. East-Seletsky, M. Kaplan, J. A. Doudna, Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* **516**, 263–266 (2014). [doi:10.1038/nature13769](https://doi.org/10.1038/nature13769) [Medline](#)
46. B. A. Rousseau, Z. Hou, M. J. Gramelspacher, Y. Zhang, Programmable RNA Cleavage and Recognition by a Natural CRISPR-Cas9 System from *Neisseria meningitidis*. *Mol. Cell* **69**, 906–914.e4 (2018). [doi:10.1016/j.molcel.2018.01.025](https://doi.org/10.1016/j.molcel.2018.01.025) [Medline](#)
47. S. C. Strutt, R. M. Torrez, E. Kaya, O. A. Negrete, J. A. Doudna, RNA-dependent RNA targeting by CRISPR-Cas9. *eLife* **7**, e32724 (2018). [doi:10.7554/eLife.32724](https://doi.org/10.7554/eLife.32724) [Medline](#)
48. J. P. K. Bravo, T. Hallmark, B. Naegle, C. L. Beisel, R. N. Jackson, D. W. Taylor, RNA targeting unleashes indiscriminate nuclease activity of CRISPR-Cas12a2. *Nature* **613**, 582–587 (2023). [doi:10.1038/s41586-022-05560-w](https://doi.org/10.1038/s41586-022-05560-w) [Medline](#)
49. O. Dmytrenko, G. C. Neumann, T. Hallmark, D. J. Keiser, V. M. Crowley, E. Vialto, I. Mougiakos, K. G. Wandera, H. Domgaard, J. Weber, T. Gaudin, J. Metcalf, B. N. Gray, M. B. Begemann, R. N. Jackson, C. L. Beisel, Cas12a2 elicits abortive infection through RNA-triggered destruction of dsDNA. *Nature* **613**, 588–594 (2023). [doi:10.1038/s41586-022-05559-3](https://doi.org/10.1038/s41586-022-05559-3) [Medline](#)

50. W. X. Yan, P. Hunnewell, L. E. Alfonse, J. M. Carte, E. Keston-Smith, S. Sothiselvam, A. J. Garrity, S. Chong, K. S. Makarova, E. V. Koonin, D. R. Cheng, D. A. Scott, Functionally diverse type V CRISPR-Cas systems. *Science* **363**, 88–91 (2019). [doi:10.1126/science.aav7271](https://doi.org/10.1126/science.aav7271) [Medline](#)
51. M. Jinek, F. Jiang, D. W. Taylor, S. H. Sternberg, E. Kaya, E. Ma, C. Anders, M. Hauer, K. Zhou, S. Lin, M. Kaplan, A. T. Iavarone, E. Charpentier, E. Nogales, J. A. Doudna, Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* **343**, 1247997 (2014). [doi:10.1126/science.1247997](https://doi.org/10.1126/science.1247997) [Medline](#)
52. T. Yamano, H. Nishimasu, B. Zetsche, H. Hirano, I. M. Slaymaker, Y. Li, I. Fedorova, T. Nakane, K. S. Makarova, E. V. Koonin, R. Ishitani, F. Zhang, O. Nureki, Crystal Structure of Cpf1 in Complex with Guide RNA and Target DNA. *Cell* **165**, 949–962 (2016). [doi:10.1016/j.cell.2016.04.003](https://doi.org/10.1016/j.cell.2016.04.003) [Medline](#)
53. Y. Yuan, F. M. Stumpf, L. A. Schlör, O. P. Schmidt, P. Saumer, L. B. Huber, M. Frese, E. Höllmüller, M. Scheffner, F. Stengel, K. Diederichs, A. Marx, Chemoproteomic discovery of a human RNA ligase. *Nat. Commun.* **14**, 842 (2023). [doi:10.1038/s41467-023-36451-x](https://doi.org/10.1038/s41467-023-36451-x) [Medline](#)
54. H.-H. Wessels, A. Méndez-Mancilla, X. Guo, M. Legut, Z. Daniloski, N. E. Sanjana, Massively parallel Cas13 screens reveal principles for guide RNA design. *Nat. Biotechnol.* **38**, 722–727 (2020). [doi:10.1038/s41587-020-0456-9](https://doi.org/10.1038/s41587-020-0456-9) [Medline](#)
55. H.-H. Wessels, A. Stirn, A. Méndez-Mancilla, E. J. Kim, S. K. Hart, D. A. Knowles, N. E. Sanjana, Prediction of on-target and off-target activity of CRISPR–Cas13d guide RNAs using deep learning. *Nat. Biotechnol.* **42**, 628–637 (2023) [doi:10.1038/s41587-023-01830-8](https://doi.org/10.1038/s41587-023-01830-8) [Medline](#)
56. L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013). [doi:10.1126/science.1231143](https://doi.org/10.1126/science.1231143) [Medline](#)
57. L. Villiger, J. Joung, L. Koblan, J. Weissman, O. O. Abudayyeh, J. S. Gootenberg, CRISPR technologies for genome, epigenome and transcriptome editing. *Nat. Rev. Mol. Cell Biol.* (2024). [doi:10.1038/s41580-023-00697-6](https://doi.org/10.1038/s41580-023-00697-6) [Medline](#)
58. A. Nemudraia, A. Nemudryi, B. Wiedenheft, Amplicon sequencing of human mRNA and reporter mRNA targeted with type III-A CRISPR complex of *Streptococcus thermophilus*, NCBI (2024); <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1099688/>
59. A. Nemudraia, A. Nemudryi, B. Wiedenheft, Code for generating figures and analyzing amplicon sequencing of human mRNA and reporter mRNA targeted with type III-A CRISPR complex from *Streptococcus thermophilus*, Zenodo (2024); doi: [10.5281/zenodo.10966960](https://doi.org/10.5281/zenodo.10966960)
60. M. W. Shen, M. Arbab, J. Y. Hsu, D. Worstell, S. J. Culbertson, O. Krabbe, C. A. Cassa, D. R. Liu, D. K. Gifford, R. I. Sherwood, Predictable and precise template-free CRISPR editing of pathogenic variants. *Nature* **563**, 646–651 (2018). [doi:10.1038/s41586-018-0686-x](https://doi.org/10.1038/s41586-018-0686-x) [Medline](#)
61. K. Kato, W. Zhou, S. Okazaki, Y. Isayama, T. Nishizawa, J. S. Gootenberg, O. O. Abudayyeh, H. Nishimasu, Structure and engineering of the type III-E CRISPR-Cas7-11

- effector complex. *Cell* **185**, 2324–2337.e16 (2022). [doi:10.1016/j.cell.2022.05.003](https://doi.org/10.1016/j.cell.2022.05.003) [Medline](#)
62. D. Conant, T. Hsiao, N. Rossi, J. Oki, T. Maures, K. Waite, J. Yang, S. Joshi, R. Kelso, K. Holden, B. L. Enzmann, R. Stoner, Inference of CRISPR Edits from Sanger Trace Data. *CRISPR J.* **5**, 123–130 (2022). [doi:10.1089/crispr.2021.0113](https://doi.org/10.1089/crispr.2021.0113) [Medline](#)
63. D. Kim, J.-Y. Lee, J.-S. Yang, J. W. Kim, V. N. Kim, H. Chang, The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914–921.e10 (2020). [doi:10.1016/j.cell.2020.04.011](https://doi.org/10.1016/j.cell.2020.04.011) [Medline](#)
64. Y.-C. Lin, M. Boone, L. Meuris, I. Lemmens, N. Van Roy, A. Soete, J. Reumers, M. Moisse, S. Plaisance, R. Drmanac, J. Chen, F. Speleman, D. Lambrechts, Y. Van de Peer, J. Tavernier, N. Callewaert, Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat. Commun.* **5**, 4767 (2014). [doi:10.1038/ncomms5767](https://doi.org/10.1038/ncomms5767) [Medline](#)