

Development of a Facile Approach for Generating Chemically Modified CRISPR/Cas9 RNA

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The RNA-guided, modified type II prokaryotic CRISPR with CRISPR-associated proteins (CRISPR/Cas9) system represents a simple gene-editing platform with applications in biotechnology and also potentially as a therapeutic modality. The system requires a small guide RNA (sgRNA) and a catalytic Cas9 protein to induce non-homologous end joining (NHEJ) at break sites, resulting in the formation of inactivating mutations, or through homology-directed repair (HDR) can engineer in specific sequence changes. Although CRISPR/Cas9 is a powerful technology, the effects can be limited as a result of nuclease-mediated degradation of the RNA components. Significant research has focused on the solid-phase synthesis of CRISPR RNA components with chemically modified bases, but this approach is technically challenging and expensive. Development of a simple, generic approach to generate chemically modified CRISPR RNAs may broaden applications that require nuclease-resistant CRISPR components. We report here the development of a novel, functional U-replaced transactivating RNA (tracrRNA) that can be *in vitro* transcribed with chemically stabilizing 2'-fluoro (2'F)-pyrimidines. These data represent a unique and facile approach to generating chemically stabilized CRISPR RNA.

INTRODUCTION

CRISPR/Cas9, in its native function, provides adaptive immunity in bacteria by the targeted DNA cleavage of pathogenic viruses and plasmids.¹ A breakthrough in this technology was the identification of the minimal Cas9 components needed for functional gene editing in human cells.² CRISPR/Cas9 is a facile system consisting of a modular guide RNA, targeted by a 20-nt complementary sequence, and a catalytic Cas9 protein. The CRISPR/Cas9 system can be adapted to target virtually any gene in any organism with the only restrictive requirement for DNA targeting being a protospacer adjacent motif (PAM), which for a wild-type (WT) Cas9 is typically [NGG]. CRISPR/Cas9 holds significant potential for therapeutic gene editing and has been rapidly developed for applications as an anti-viral,³ inhibitor of cancer,⁴ and gene-editing platform for monogenetic diseases,⁵ and in diagnostic methodologies.⁶

The target guide RNA of CRISPR/Cas9 can be utilized as either a dual-guide RNA (dgRNA) consisting of a targeting CRISPR RNA

(crRNA) annealed to the Cas9 recognition transactivating RNA (tracrRNA), or a small guide RNA (sgRNA), which is a single fusion RNA whereby the crRNA is linked to the tracrRNA by a tetra loop.⁷ Both systems consist of RNA, which makes them highly susceptible to cellular and serum nucleases. This susceptibility may be apparent when delivering sgRNA with a Cas9 translated from mRNA, because degradation can occur prior to Cas9 expression and the downstream interaction with the guide RNA.⁸ Furthermore, chemical modification of CRISPR RNA has been used to prevent interferon (IFN) activation of sgRNAs in immune cells.⁹ However, solid-phase synthesis of long, structured RNA, like the tracrRNA, with chemically modified bases can be technically challenging and financially prohibitive. Therefore, a simplified and cost-effective method to generate chemically modified CRISPR RNA components is needed.

One approach around the pitfalls of chemical synthesis of CRISPR RNAs is *in vitro* transcription of RNA. A mutant Y639F/H784A T7 RNA polymerase (T7 RNAP), with promiscuity for modified nucleotides, is used to incorporate non-natural bases into the *in-vitro*-transcribed RNA.¹⁰ Standard commercial kits to *in-vitro*-transcribed 2'-fluoro (2'F) RNA are readily available, or the mutant polymerase can be bulk prepared in-house using established purification protocols and used with commercially available modified triphosphates, which can be used to generate large amounts of chemically modified RNA from standard DNA oligo templates. This approach has been used extensively for generating nuclease-resistant RNA for aptamer technology,¹¹ and typically *in vitro* transcription of aptamer libraries with 2'-fluoro (2'F)-pyrimidines has been used to stabilize RNA.¹²

In this work, we find that 2'F chemical modification of uridines is detrimental to Cas9 activity within *in-vitro*-transcribed sgRNAs and the tracrRNA. By systemically replacing uridines within the tracrRNA sequence, we developed a novel, chemically stabilized

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tracrRNA that is functional when *in vitro* transcribed with chemically modified bases.

RESULTS

sgRNAs and tracrRNAs Are Intolerant of 2′F-Uridines

Little was known about the tolerance of *in-vitro*-transcribed sgRNAs with 2′F-pyrimidines (Figure S1B). To test the functionality of the 2′F sgRNAs, we targeted the essential trans-activation response (TAR) loop in the long-terminal repeat (LTR) of human immunodeficiency virus (HIV). The TAR-targeted sgRNAs introduce mutations into the TAR loop, which will inactivate the promoter and prevent reporter expression^{13,14} (Figure 1B). The sgRNAs were *in vitro* transcribed with either 2′F-U, 2′F-C, or 2′F-CU bases, and the levels of activity were determined using an *in vitro* cleavage assay, which measures Cas9 activity through cutting efficiency of a target dsDNA template. We find that generally sgRNAs lose more activity with 2′F-U and 2′F-CU bases compared with unmodified gRNAs (Figure S1A). All of the sgRNAs had high levels of cleavage activity when transcribed with 2′F-C bases, suggesting 2′F-U bases were negatively affecting Cas9 function.

Next, we determined whether these sgRNAs were functional under cellular conditions. All of the sgRNAs lost knockdown activity with 2′F-U and 2′F-CU in an LTR-GFP reporter cell line (pMo-C6) that stably expressed *spCas9*, except for 2′F-U TAR7, but its knockdown was attenuated (Figure S1C). The activity was abolished with 2′F-U, but not with 2′F-C, and notably, knockdown with 2′F-C was slightly higher than that of unmodified RNA. The comparable or improved knockdown activity was also noted with sgRNAs preloaded into Cas9 ribonucleoproteins (RNPs) (Figure S1D). Collectively, these data demonstrate that sgRNAs are intolerant of 2′F-U but are functional with 2′F-Cs.

Next, we explored to what extent specific uridine nucleotides contribute to the observed loss of activity. However, the sgRNA is complicated by a random 20-nt target sequence and to simplify this investigation, we utilized a dgRNA system, which consists of two RNAs: a targeting crRNA and Cas9 recognition tracrRNA (Figure 1A). In this format, an unmodified crRNA was annealed to an *in-vitro*-transcribed, chemically modified 2′F tracrRNA, and like previous observations, 2′F-U and 2′F-CU modifications abolish knockdown of GFP in the pMo-C6-*spCas9* cell line (Figure 1B). To determine those uridines in the tracrRNA responsible for the loss of activity, we synthesized a series of tracrRNAs with 2′F-U in select positions in the tracrRNA (Figure 1C). Guided by the crystal structure of *spCas9* bound to an sgRNA,¹⁵ there are uridines that have hydrogen bonds with the Cas9 protein through the 2′OH group, which may be affected by the replacement with 2′F nucleotides. Notably, we find that the internal loop has several uridines interacting with Cas9 and was largely, although not completely, responsible for the loss of activity (2′F-UInt). There was a slight loss of activity with other internal non-2′OH-interacting uridines (2′F-Uint-Non-OH). The U12 has four interactions with Cas9 and one is a 2′OH interaction,¹⁵ and a 2′F-U12 tracrRNA also lost activity (2′F-U12). Lastly, 2′F bases in

external uridines modestly affected knockdown activity (2′F-Uext). Overall, 2′F-uridines appear to be poorly tolerated in the tracrRNA.

Development of a U-Replaced tracrRNA

Guided by these observations, we developed a U-replaced tracrRNA by substituting Cas9-interacting uridines with A or Gs in the internal loop (Figure 2A), with the rationale being that if the 2′OH interacting base is a purine, this should maintain this interaction in the presence of 2′F-pyrimidines. It was first determined whether the sgRNAs were functional with a U-replaced sequence by screening the sgRNAs with an altered sequence in a knockdown assay. The U-replaced sgRNAs were expressed off double-stranded DNA (dsDNA) templates driven by a U6 Pol-III promoter and transfected into the pMo-C6-*spCas9* cells, and knockdown activity was compared with a WT sgRNA (Table S1; Figure S2). We started by replacing each 2′OH interacting uridine in the internal loop, and replacements tolerated individually were combined with additional sequence changes. However, U replacements tolerated as single-nucleotide changes were not always able to maintain activity in combination. U12 was able to maintain comparable knockdown to the original sequence when replaced with an A (sgRNA-2), but lost significant activity when combined with other U replacements (sgRNA-22, 24–26, 127–134). Nevertheless, a functional sgRNA was obtained that replaced internal 2′OH interacting uridines in positions U11A, U31G, U32G (sgRNA-23). The modifications in sgRNA-23 were combined with sequence changes to the internal non-2′OH-interacting uridines U24A and U27A (sgRNA-29). External uridines were replaced in two steps: first, by combining the sgRNA-29 changes with U5A, U34A, U58G (sgRNA-97); and second, 2′F-Cs were better tolerated than 2′F-U (Figure 1B), and this promoted the design of an sgRNA with cytosines in place of the remaining external uridines, which demonstrated levels of activity comparable with a sgRNA-WT (sgRNA-183). The 3′-tail uridines (U65, U66, U67) were replaced with cytosines at these positions (sgRNA-124). Significant U-replaced sgRNA sequence intermediates that have high levels of activity are summarized in Figure 2A (right graph).

Based on these observations, U-replaced tracrRNAs were designed (Figure 2B, tracrRNA V1-4). The tracrRNAs were *in vitro* transcribed with 2′F-CUs and transfected into the pMO-C6-*spCas9* cell line to determine whether these U-replaced tracrRNAs were tolerant of chemical modification. The different versions resulted in a steady increase in GFP knockdown compared with the unmodified WT tracrRNA with 2′F-U (Figure S3) or 2′F-CU modifications (Figure 2C). Furthermore, the tracrRNA-V4 with a near-pan replaced of uridines was *in vitro* transcribed with 2′F-CU bases and able to knock down GFP at comparable levels to that of the WT tracrRNA without modification (Figure 2C). There was also an increase in Cas9 enzyme activity with 2′F-CU U-replaced tracrRNA-V4 as determined by an *in vitro* cleavage assay (Figure 2D). To further characterize the activity of the U-replaced tracrRNA-V4, a TAR6 crRNA was annealed to the tracrRNA and transfected into a pMo-C6-*spCas9* cell line in decreasing amounts, and similar levels of activity were observed at lower doses compared with an unmodified WT tracrRNA (Figure 2E). A second TAR crRNA was tested, TAR5, which had

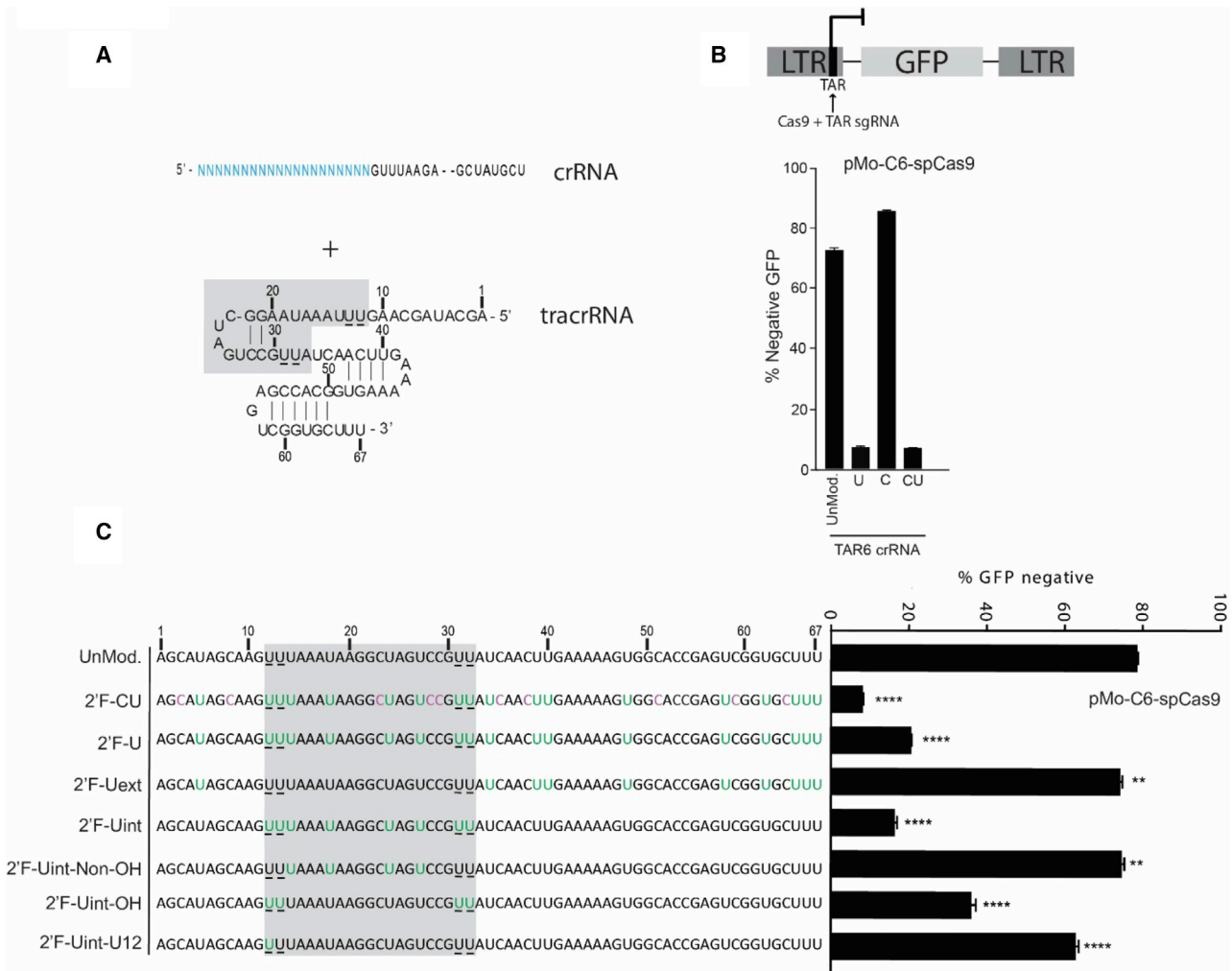


Figure 1. The Tolerance of spCas9 tracrRNA for 2'F-Uridines

(A) Schematic of the dual-guide RNA (dgRNA). The crRNA is annealed to the tracrRNA to form the dgRNA. The “internal” loop region of the tracrRNA is highlighted in a gray box. Those uridines that have 2’OH interactions with Cas9 are underlined. (B) The tracrRNAs were *in vitro* transcribed with 2’F-Urs, 2’F-Cs, or 2’F-CUs and annealed to a TAR6 crRNA before transfection into a pMo-C6-spCas9 cell line. The knockdown of GFP was assessed at 48 h post-transfection. A schematic of the LTR-GFP reporter is represented. (C) The tracrRNA was synthesized with 2’F-uridines (green) or 2’F-cytosines (purple) at the sites specified in the schematic. The tracrRNA was either unmodified (UnMod.), all uridines and cytosines modified (2’F-CU), all uridines modified (2’F-U), external uridines modified (2’F-Uext), uridines within the “internal” loop modified (2’F-Uint), modifications of the uridines with the “internal” loop that interact with Cas9 through 2’OH groups (2’F-Uint-OH) or non-interacting uridines (2’F-Uint-Non-OH), or only the U12 uridine modified (2’F-Uint-U12). The synthesized tracrRNAs were annealed to a TAR6 crRNA and transfected into the pMo-C6-spCas9 cell line, and GFP levels were measured 48 h later by FACS. (B and C) The errors bars are calculated as SEM from experiments that were performed in duplicate. **p < 0.005, ****p < 0.001 were determined using a one-way ANOVA compared with unmodified tracrRNA.

comparable levels at high concentrations but lost some activity at lower amounts (Figure S4). Overall, these data suggest that a U-replaced tracrRNA with 2’F-pyrimidines is functional with spCas9 but may have slightly lower activity compared with unmodified WT tracrRNA at certain target sites.

U-replaced tracrRNAs Are Stabilized and Reduce IFN Activity

To determine whether the 2’F-CU U-replaced tracrRNAs were stabilized, we exposed the tracrRNA to 10% serum, and sustainable

amounts of tracrRNA were present after a 1-h exposure, whereas the unmodified WT tracrRNA was rapidly degraded (Figure 3A). The serum assays were also performed on a commercial Alt-R tracrRNA, which was similarly degraded in serum (Figure S5A). The cellular stability of the U-replaced tracrRNA was then determined when the 2’F-CU U-replaced tracrRNA-V4 was electroporated into HEK293 cells, and the levels of tracrRNA were significantly higher at 30 min compared with the unmodified WT tracrRNA (Figure 3B). Furthermore, the 2- and 4-h time points were made relative

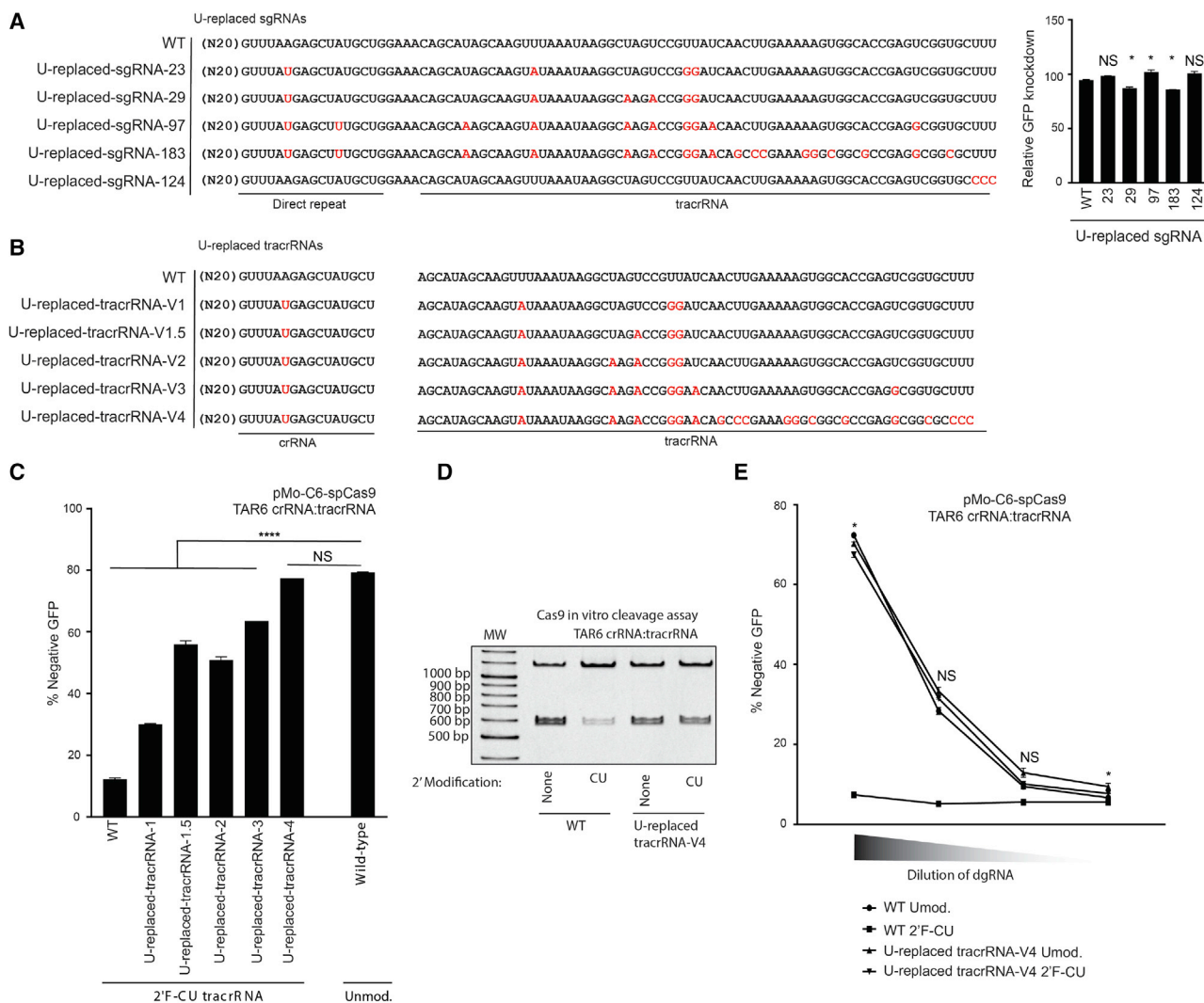


Figure 2. The U-Replaced tracrRNA Can Tolerate 2'F-Pyrimidines

(A) An alignment of the WT sgRNA sequence and the nucleotide changes in the U-replaced sgRNAs (left panel). The relative knockdown activity of the U-replaced sgRNAs compared with a WT sgRNA is shown in the right graph. (B) Sequence alignments of the WT and U-replaced tracrRNA V1-V4. U-replacements are highlighted in red. (C) The U-replaced tracrRNAs were *in vitro* transcribed with 2'F-CUs and annealed to a TAR6 crRNA before transfection into a pMo-C6-spCas9 cell line. The knockdown of GFP was assessed at 48 h post-transfection. The levels of knockdown were compared with a WT tracrRNA with 2'F-CU bases and without chemical modification. (D) The dgRNAs were incubated with a Cas9 RNP and then used to digest a linear target dsDNA, and the digestion products were resolved by PAGE. (E) The dgRNAs with a WT or U-replaced tracrRNA either unmodified or *in vitro* transcribed with 2'F-CU were diluted to 1:10, 1:50, and 1:100 and transfected into the pMo-C6-spCas9 cells. The levels of GFP were assessed 48 h post-transfection. **p* < 0.05, *****p* < 0.0001 were determined using a one-way ANOVA compared with the unmodified tracrRNA.

to the 30-min measurement, which highlighted the further ~90% reduction of the unmodified WT tracrRNA at later time points (Figure S5B), but the 2'F-CU U-replaced tracrRNA-V4 was reduced by ~50% compared with the 30-min time point. The 2'F-CU U-replaced tracrRNA-V4 had a similar cellular stability at 4 and 8 h compared with the Alt-R modified tracrRNA (Figure 3C).

The *in vitro* transcription of RNA results in a 5'-triphosphate, which is detected by pathogen-recognition receptors (PRRs) to activate

downstream IFN- β ,¹⁶ but RNA chemical modification reduces IFN activation.⁹ The 2'F-CU U-replaced tracrRNA was electroporated into HEK293 cells and resulted in an attenuated activation of IFN- β mRNA that was almost resolved by 4 h post-transfection (Figure 3D). The unmodified WT tracrRNA resulted in significant activation of IFN- β mRNA at 2 and 4 h post-electroporation. These data show that the 2'F-CU U-replaced tracrRNAs have been chemically stabilized in serum and cells, and the 2'F-CU modifications attenuate IFN activation.

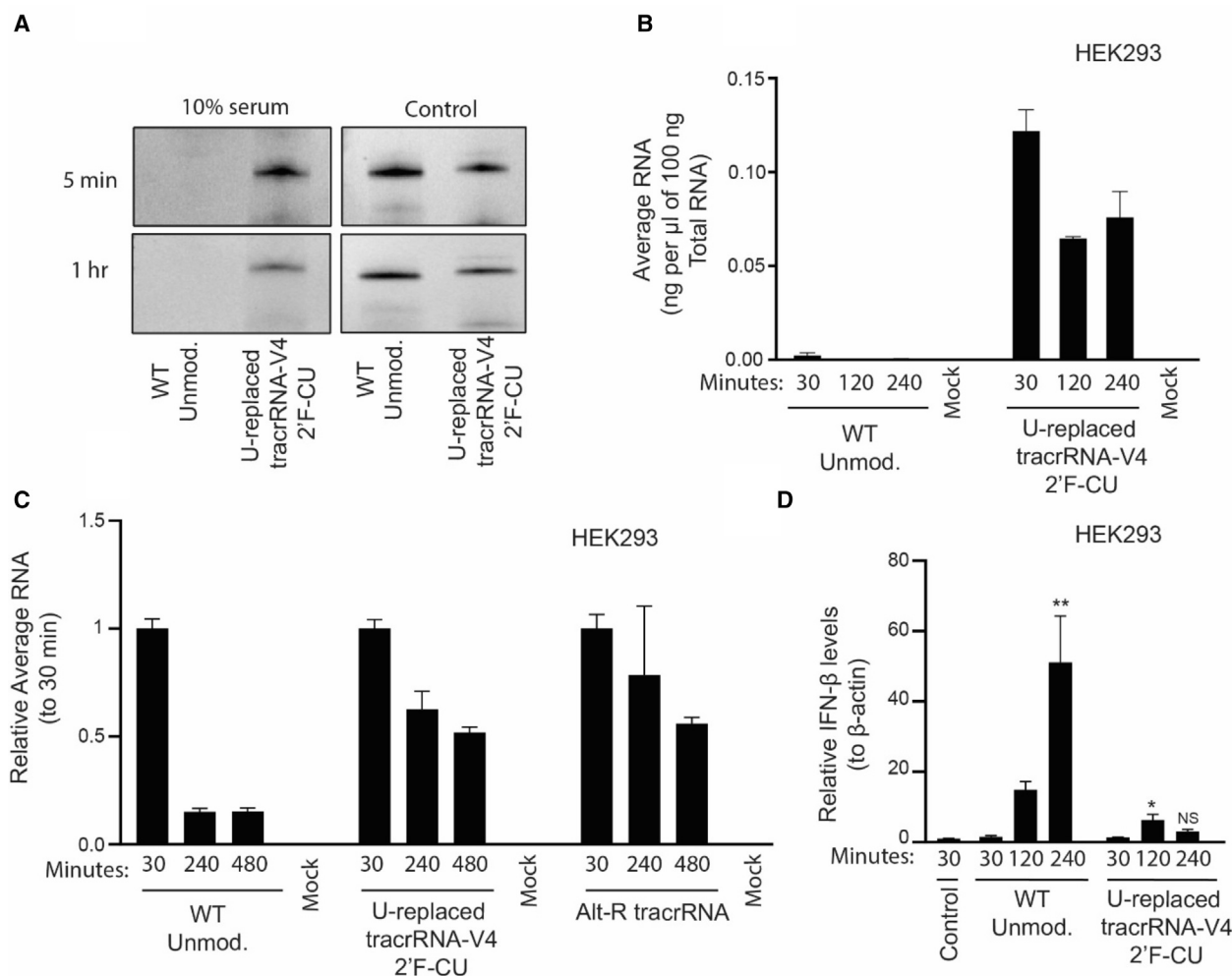


Figure 3. The 2'F-CU U-Replaced tracrRNAs Are Stabilized with Reduced Interferon Activation

(A) The U-replaced tracrRNA-V4 was *in vitro* transcribed with 2'F-CUs and mixed with 10% FBS for 5 min or 1 h, and the RNA was resolved using PAGE. An unmodified WT tracrRNA was included as a comparison. (B) The 2'F-CU U-replaced tracrRNA-V4 or unmodified WT tracrRNA was electroporated in HEK293, and RNA was extracted at 30, 120, and 240 min. The amount of tracrRNA was determined using qRT-PCR. (C) The 2'F-CU U-replaced tracrRNA-V4, unmodified WT tracrRNA, or commercially synthesized Alt-R tracrRNA was electroporated into HEK293 cells, and RNA was extracted at 30, 240, and 480 min. The levels of RNA were determined using qRT-PCR and made relative to the 30-min treatment, which was set at 100%. Errors are calculated as SEM from experiments performed in triplicate. (D) The 2'F-CU U-replaced tracrRNA-V4 or unmodified WT tracrRNA was electroporated in HEK293; RNA was extracted at 30, 120, and 240 min; and the levels of IFN- β mRNA expression were determined by qRT-PCR. Errors are calculated as SEM from experiments performed in duplicate. * $p < 0.05$, ** $p < 0.005$ were determined using a one-way ANOVA compared with the 30-min treated sample.

U-replaced tracrRNAs Are Functional When Used with Cas9 mRNA

To determine whether the U-replaced tracrRNA (Figure 4A) could function with systems other than stable Cas9-expressing cell lines, the 2'F-CU tracrRNA-V4 was annealed to a TAR-5 crRNA with Alt-R modifications (Alt-R TAR5) and transfected with a Cas9 mRNA into the pMo-C6 cell line. At 72 h post-transfection, knockdown of the LTR (Figure 4B) and insertions/deletions (indel) formation (Figure 4C) were comparable with a WT tracrRNA. To further characterize the U-replaced tracrRNA activity to target other sites, the 2'F-CU U-replaced tracrRNA-V4 was annealed to a crRNA

targeting C-C Chemokine Receptor 5 (Alt-R CCR5 crRNA) and transfected with Cas9 mRNA into a pMo-C6 cell line engineered to stably express CCR5. At 72 h post-transfection, there was significant knockdown of CCR5 (Figure 4D) and indels at the target site (Figure 4E), but the levels were lower in the 2'F-CU tracrRNA-V4-treated cells. To verify these results in a different cell line, we transfected the 2'F-CU U-replaced tracrRNA-V4 into a TZM-bl cell line,¹⁷ which has an LTR driving luciferase and stably expresses CCR5. The TAR-targeted crRNAs with the 2'F-CU U-replaced tracrRNA-V4 had comparable levels of knockdown to the unmodified WT tracrRNA (Figure S6A), but CCR5 knockdown was similarly reduced

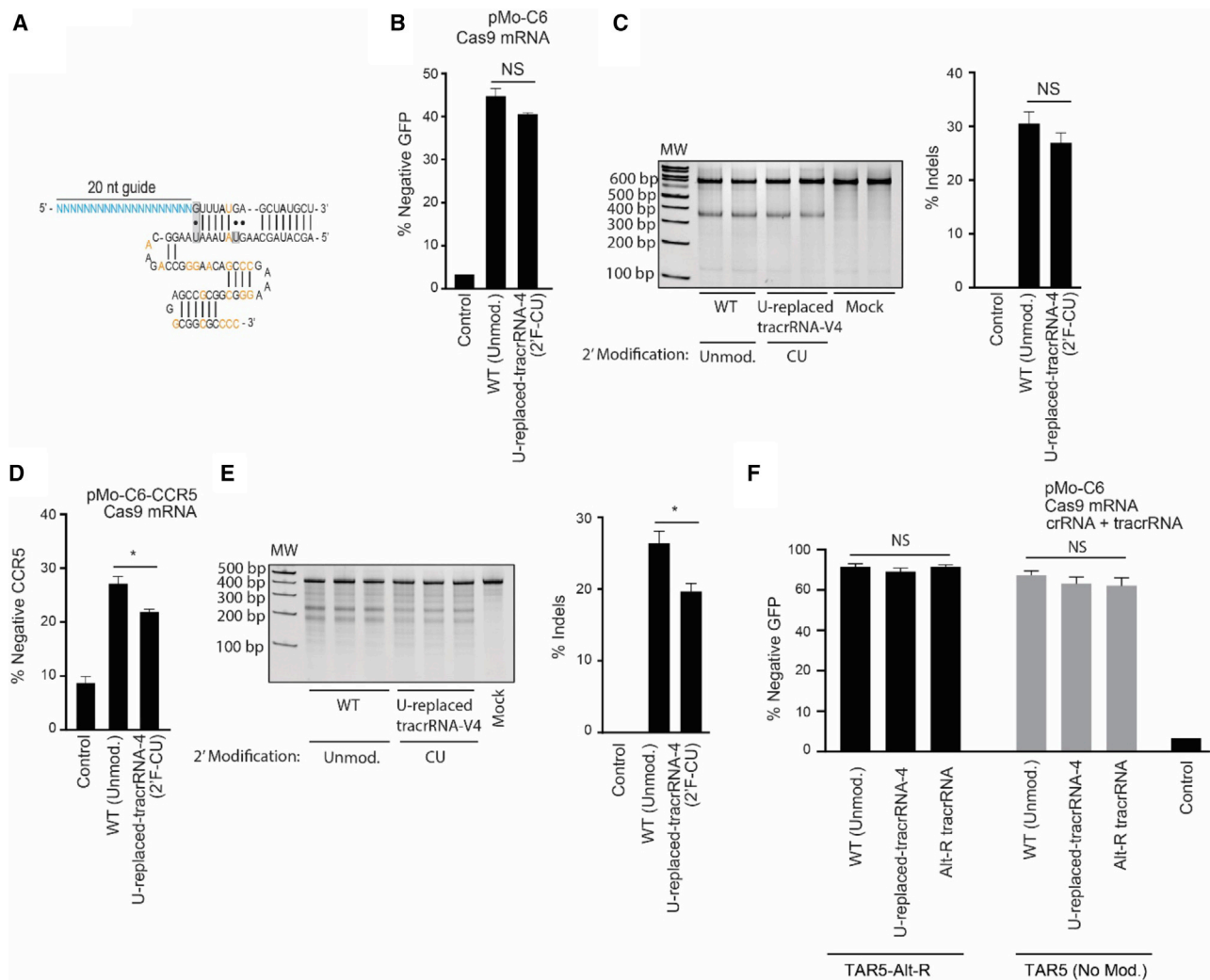


Figure 4. The U-Replaced tracrRNAs Are Functional with Other Cas9 Modalities

(A) Schematic of the U-replaced tracrRNA-4 with sequence changes highlighted in orange. Uridines that were refractory to replacement are in a gray box. (B–E) The U-replaced tracrRNAs were *in vitro* transcribed with 2'F-CUs and annealed to an Alt-R TAR5 crRNA (B and C) or an Alt-R crRNA targeting CCR5 (D and E) before transfection with Cas9 mRNA into a pMo-C6 or pMo-C6-CCR5 cell line, respectively. The knockdown of (B) GFP or (D) CCR5 was assessed at 72 h post-transfection. The levels of knockdown were compared with a WT tracrRNA without chemical modifications. A T7E1 assay was performed on the cells treated with a TAR5 crRNA (C) or CCR5 crRNA (E). The quantification of the indels (TAR5: $n = 2$, CCR5: $n = 3$) is represented as a bar graph. (F) The 2'F-CU U-replaced tracrRNA-V4 electroporated without annealing with an Alt-R or unmodified TAR5 crRNA with Cas9 mRNA into a pMo-C6 cell line. The unmodified WT tracrRNA or commercially synthesized Alt-R tracrRNA was included for comparison. The knockdown of GFP was assessed at 72 h post-transfection. Errors are calculated as SEM. * $p < 0.05$ as determined using an unpaired Student's *t* test.

(Figure S6B). To further assess activity, we tested the 2'F-CU U-replaced tracrRNA with an unmodified or Alt-R TAR5 crRNA. Unmodified crRNAs can be degraded prior to Cas9 expression, which may alter knockdown effects.⁸ The U-replaced tracrRNA was not annealed to the crRNAs and electroporated into pMo-C6 cells with Cas9 mRNA and compared with an unmodified WT tracrRNA or an Alt-R tracrRNA. In our hands, there was substantial GFP knockdown with the 2'F-CU U-replaced tracrRNA with both the Alt-R and unmodified TAR5 crRNA, and this knockdown was comparable with the unmodified WT tracrRNA and Alt-R tracrRNA (Figure 4F). Overall, these data suggest the chemically modified U-replaced tracrRNA-

V4 are functional when used with a Cas9 mRNA, although as previously observed, activity can be reduced at specific target sites.

We next sought to determine the activity of the 2'F-CU U-replaced tracrRNA-V4 when preloaded into a Cas9 RNP complex. The tracrRNA-V4 annealed to the Alt-R TAR5 crRNA was electroporated into the pMo-C6 cells and showed comparable knockdown to the WT control (Figure S7A). However, when the Cas9 RNP was transfected by lipofection, there was a 50% reduction in knockdown activity (Figure S7B). These data suggest that although Cas9 RNP has improved activity with the 2'F-CU U-replaced tracrRNA compared with

2′F-CU WT tracrRNA, Cas9 RNPs may be more negatively affected by the modifications.

DISCUSSION

The work described here shows the development of a novel U-replaced tracrRNA that is functional when *in vitro* transcribed with 2′F-pyrimidines, which represents a technically simple and cost-effective approach to generating chemically modified CRISPR components. Cas9 activity could be rescued with *in-vitro*-transcribed 2′F RNA when uridines detrimental to activity were replaced. The enhanced stability of the 2′F-CU U-replaced tracrRNA in serum compared with the Alt-R modified tracrRNA was likely as a result of RNase A degradation of unmodified pyrimidines and highlights a potential unique property of this technology. Future work could focus on characterizing U-replaced tracrRNA properties to various commercial chemically modified dgRNAs and sgRNAs, as well as novel modified CRISPR RNAs developed by various groups.^{18–21} Additionally, the mutant T7 can incorporate 2′-O-methyl bases²² that are nuclease resistant, more “natural” bases, and less expensive, which opens up the use of alternative modified bases in the U-replaced tracrRNA with unique properties.

Even though the Cas9 activity at certain target sites with the 2′F-CU U-replaced tracrRNA was comparable with an unmodified WT tracrRNA, reduced activity was noted in several assays. Also, the U-replaced tracrRNA activity never exceeded WT tracrRNA, even though it was chemically stabilized, which suggests this system could be improved. Improvement in activity may be achieved by further sequence optimization, and supporting this notion were the observations that there was a marginal increase in activity with 2′F-CU U-replaced tracrRNA-V2 and -V3 (Figure 2C), and U58A was changed in U-replaced tracrRNA-V3, which makes a direct base interaction with Cas9.¹⁵ Reverting this base back to a uridine or replacing it with a C may recreate an essential pyrimidine interaction that could improve activity. Alternatively, the activity of the U-replaced tracrRNA could be explained by the remaining detrimental uridines that are refractory to replacement (Figure 4A). U12 has four interactions with Cas9, whereas U18 has two interactions,¹⁵ and in this case combining the chemically modified U-replaced tracrRNA with directed evolution of Cas9 could yield a variant with improved activity with 2′F bases at these sites.^{23,24}

The activity at the TAR5 target site with 2′F-CU U-replaced tracrRNA-V4 with expressed Cas9 was comparable with the unmodified WT tracrRNA at high doses, but activity was noticeably lost with RNPs (Figure 4B; Figures S4 and S7B). Studies have shown that truncated tracrRNAs have reduced Cas9 RNP effector complex formation that is susceptible to interference by endogenous RNA,²⁵ and 2′F-CU U-replaced tracrRNA having lost essential interactions with the Cas9 protein may make it more susceptible to interference by competing RNA, which may explain the dissimilarity between stable Cas9, mRNA, and RNPs. Nevertheless, the binding of Cas9 RNPs to CRISPR RNA itself protects RNA from degradation (data not shown), and chemically modification only minimally improves activity.⁸ Even

though the data suggest that U-replaced tracrRNAs do have some functionality with Cas9 RNP, U-replaced tracrRNAs would likely be used with expressed Cas9 systems rather than with RNPs.

IFN activation was noted with *in-vitro*-transcribed CRISPR RNA²⁶ resulting from recognition of the 5′-triphosphate through retinoic acid-inducible gene I (RIG-1) signaling,²⁷ but removal of the triphosphate only partially reduced activation in peripheral blood mononuclear cells (PBMCs).⁹ Furthermore, even synthetic RNA activated IFN in PBMCs, which could be reduced with 2′-O-methyl chemical modification.⁹ Our data show that 2′F modification of the U-replaced tracrRNA attenuates IFN activation of *in-vitro*-transcribed RNA even with the 5′-triphosphate and could potentially be used in combination with 5′-triphosphate removal to ablate immune recognition of the U-replaced CRISPR RNA in a wide range of cell types.

The U-replaced tracrRNA-V4 sequence deviates ~26% from the original sequence, suggesting a high level of tolerance for sequence modification, which has been observed in studies of the tracrRNA structures.²⁸ Several U-replaced tracrRNA variants were generated, but only certain intermediates resulted in significant gain-of-function with 2′F-pyrimidines (Figure 2C). The U-replaced tracrRNA-V1 had replacements at 2′OH interactions (U11A, U31G, and U32G), resulting in increased Cas9 activity, and these data suggest that recreating these 2′OH with Cas9 was important for functionality but did not fully recapitulate activity, possibly signifying other disruptive effects by 2′F-uridines. Interestingly, the single U27A change doubled knockdown activity (Figure 2C) and suggests that this uridine is highly influenced by 2′F modification, but the U27 base does not have interactions with Cas9. A 2′F modification increases Watson-Crick bonding strength and base stacking,²⁹ and a 2′F-U27 may reduce activity by distorting the tracrRNA structure, or may result in steric clashes with Cas9. Intriguingly, 2′F-CU U-replaced tracrRNA activity was improved by replacing several uridines with cytosines (Figure 2C), and represents differences in the chemical properties and tolerance of the fluorinated bases. Furthermore, 2′F-C improves activity of sgRNAs with Cas9 RNPs (Figure S1D), and the reason for this improvement is unknown but may reflect increased guide RNA binding to its target DNA site through improved base pairing²⁹ or enhanced sgRNA stability. A better understanding of how 2′-fluorination alters the properties of bases in RNA may be able to better predict the functional effects on CRISPR and other RNA modalities.

There still needs to be further optimization of this system, as well as validation at additional target sites, in more stringent models, such as primary cells and *in vivo*, and with alternative chemical modifications in order to fully elucidate the range functionally of the U-replaced tracrRNA system. Nevertheless, this work has expanded upon current efforts to generate chemically modified CRISPR RNA components through a novel approach by sequence modification. The U-replaced tracrRNA may represent a generic, simplified approach to produce chemically modified tracrRNAs with significantly reduced cost for use in various research and biotechnological applications *in vitro* and *in vivo*.

MATERIALS AND METHODS

Vectors

The CCR5-T2A-Puro sequence was ordered as a gBLOCK sequence and cloned into a NheI and PmeI digested pcDNA3.1 (+) mammalian expression vector using the NEBuilder HiFi DNA Assembly Master Mix according to the manufacturer's instructions (NEB, MA, USA). Positive clones were confirmed by automated sequencing. The px459 vector was obtained from Addgene (#62988).

Cell Culture

The pMo-C6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA) and incubated at 37°C and 5% CO₂. The pMo-C6-*spCas9* and CCR5 cell lines were cultured in DMEM + 10% FBS + 1.5 µg/mL puromycin.

Cell Lines

The MoHIV cell line was generated as described by Shrivastava et al.,³⁰ but briefly lentiviral vectors were generated and packaged with a vector driving GFP off the LTR promoter (pMoHIV). HEK293 cells were transduced with the pMoHIV vector and had variable GFP expression as a result of the random integration of the vector into the genome. The cells were clonally expanded by limiting dilution to get a single pMoHIV clone 6 (pMo-C6) that had high levels of ubiquitous GFP expression (data not shown) and could be used to assess LTR promoter inactivation by target CRISPR systems. To generate the *spCas9* overexpression cell line, the px459, which contains a cytomegalovirus (CMV)-expressed *spCas9* in-frame with puromycin separated by a T2A ribosomal skip peptide, was linearized with BbsI (to prevent expression of the U6-expressed sgRNA within the vector) and purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). A total of 1 µg of linear DNA was transfected into the pMo-C6 cell line using Lipofectamine 3000 as per manufacturer's instructions, and at 48 h post-transfection the media were replaced with DMEM+10% FBS containing 0.5 µg/mL GIBCO puromycin (Thermo Fisher Scientific, MA, USA). Gradually, the cells were split into higher concentrations of puromycin until 1.5 µg/mL to select for resistant cells expressing the *spCas9* (pMo-C6-*spCas9*). Similarly, in order to create the pMo-C6 cells that stably expressed CCR5, we transfected the pMo-C6 cell with a linearized CCR5-T2A-Puro and selected it for puromycin resistance. Surface CCR5 expression was confirmed by fluorescence-activated cell sorting (FACS; data not shown).

Screening of U-replaced tracrRNA

The sgRNAs were ordered as gBlocks (IDT, CA, USA) with a U6 Pol-III promoter driving expression of the sgRNA. The U6-sgRNA gBlocks were reverse transfected into the pMo-C6 cell line by diluting the gBlocks to 3 µM and adding 1.5 µL to 0.75 µL of Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, MA, USA) incubated at room temperature for 20 min in 50 µL of Opti-MEM (Thermo Fisher Scientific, MA, USA). The 50 µL sgRNA mixture was added to a 96-well plate, and 200 µL of pMo-C6-*spCas9*

cells diluted to 6.4×10^5 cells/mL was added to the well. The levels of GFP were measured at 48 h post-transfection by FACS on Beckman Coulter FC500 Cytometer with 10,000 events collected and analyzed using FlowJo v.X3.05470 software.

Generation of Chemically Modified tracrRNAs

The templates for the tracrRNA transcription were ordered as complementary oligomers (250-µmol scale, PAGE purified; IDT, CA, USA) and contained a T7 promoter at the 5' end of the tracrRNA sequence (Table S3). The oligomers were mixed at an equal concentration at 500 ng/µL and heated to 95°C for 5 min and slowly cooled to room temperature. The tracrRNAs were *in vitro* transcribed from the templates using the DuraScribe kit according to the manufacturer's instructions (Lucigen, WI, USA). To generate 2'F-C or 2'F-U or unmodified RNA, we supplemented the 2'F bases with unmodified ribose nucleotides in the reaction (NEB, MA, USA). An RNA Clean and Concentrator-25 kit (Zymo Research Corporation, CA, USA) was used to purify the RNA according to the manufacturer's instructions, eluted in 50 µL of RNase-free water, and precipitated overnight at -20°C with 5 µL of 3 M sodium acetate and 125 µL of 100% ethanol. The reaction was centrifuged at 4°C for 30 min; the pellet was washed with 70% ethanol, resuspended in 20 µL of duplex buffer (IDT, CA, USA), and stored at -80°C until ready to use. The crRNAs were ordered as high-performance liquid chromatography (HPLC)-purified RNA oligomers with or without Alt-R modifications (IDT, CA, USA), resuspended in duplex buffer at 100 µM concentration, and stored at -80°C until needed (Table S2). The tracrRNAs with specific 2'F-U within the tracrRNA were synthesized in-house by the DNA/RNA Core at City of Hope (Figure 1C).

Cas9 In Vitro Cleavage Assays

The LTR target substrate was amplified from a pNL4-3 vector with the primers in Table S3 (TAR F2 and TAR R2) using KAPA2G Robust HotStart Ready mix (Sigma, St. Louis, MO, USA) and purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Recombinant Cas9 (NEBMA, USA) was mixed with a sgRNA or annealed dgRNA at a final concentration of 30 nM and incubated at room temperature for 10 min in Cas9 buffer (NEBMA, USA). The target substrate was added at a final concentration of 3 nM and incubated for 1 h at 37°C, and then the Cas9 was inactivated at 65°C for 10 min. The digestion products were resolved on a Novex 10% Tris-borate-EDTA (TBE) gel, stained with ethidium bromide, and visualized on a Gel Doc Ez imager (Bio-Rad, CA, USA).

Cas9 mRNA and sgRNA Transfection

To test the activity of the chemically modified dgRNAs into overexpression cell lines, we diluted the dgRNA or sgRNAs to 3 µM, and 0.5 µL of the sgRNA was mixed with 1.5 µL RNAiMAX Reagent (Thermo Fisher Scientific, MA, USA) in 50 µL of Opti-MEM. The mixture was incubated at room temperature for 20 min and then added to a 48-well plate. The pMo-C6-*spCas9* cells were diluted to 7.5×10^5 cells/mL, 200 µL was added to the well, and the levels of GFP were assessed at 48 h post-transfection.

For transfections of the U-replaced tracrRNAs with Cas9 mRNA, 400 ng of CleanCap Cas9 mRNA (Trilink, CA, USA) was mixed with 1.6 μ L of 3 μ M dgRNA and 1.6 μ L of RNAiMAX Reagent (Thermo Fisher Scientific, MA, USA) in 50 μ L of Opti-MEM. The mixture was incubated at room temperature for 20 min and then added to a 48-well plate, and 200 μ L of 6.4×10^5 cells/mL of pMo-C6 or pMo-C6-CCR5 cells was added. The levels of GFP or CCR5 were measured at 72 h post-transfection by FACS.

For electroporation of the U-replaced tracrRNAs, 800 ng of CleanCap Cas9 mRNA (Trilink, CA, USA) was mixed with 0.9 μ L of 35 μ M tracrRNA and crRNA in a total of 14 μ L of Buffer R (Thermo Fisher Scientific, MA, USA). The tracrRNA and crRNA were not pre-annealed. The Alt-R tracrRNA was obtained from IDT. The reaction was mixed with 5 μ L of 2×10^5 total pMo-C6 cells and electroporated using the 10 μ L Neon transfection system (1,700 V, 20 ms, 1 pulse). The levels of GFP were measured at 72 h post-electroporation by FACS.

In Vitro Serum Stability Assay

To determine the stability of the tracrRNA *in vitro*, we diluted the tracrRNA to 3 μ M, and 1 μ L was mixed with 1 μ L of 100% FBS in a 10- μ L reaction and incubated for the described times at room temperature. The reaction was stopped by adding 2 μ L of 20% sodium dodecyl sulfate (SDS) and by loading the RNA onto a 15% Novex TBE-UREA gel (Thermo Fisher Scientific, MA, USA), and was resolved by PAGE. The gel was stained with ethidium bromide and imaged on a Bio-Rad Gel Doc EZ system (Bio-Rad, CA, USA).

Detection of tracrRNA and IFN mRNA Levels

To determine the levels of tracrRNA in cultured cells, the tracrRNA was diluted to 40 μ M, and 1 μ L was made up to 7 μ L in Buffer R. The reaction was added to 1×10^6 total HEK293 cells and electroporated using the 10 μ L Neon transfection system (1,700 V, 20 ms, 1 pulse). The RNA was extracted at the described time points using the Maxwell RSC system with the Maxwell RSC SimplyRNA cells kit according to the manufacturer's instructions (Promega, WI, USA). The RNA was normalized to 100 ng and reverse transcribed using the QuantiTect Reverse Transcription Kit, except the RT primer mix was replaced with 14 μ M tracrRNA RT specific primer (QIAGEN, Hilden, Germany). The U-replaced tracrRNA-V4 was reverse transcribed with the U-replaced tracrRNA RT primer, and the Alt-R tracrRNA and WT tracrRNA used the WT tracrRNA RT primer (Table S3). Two microliters of the RT reaction was used with the Kapa SYBR Fast qPCR Master Mix according to the manufacturer's instructions (Roche, Basal, Switzerland). The U-replaced tracrRNA-V4 and WT tracrRNA were amplified using the tracrRNA F and R primers, whereas the Alt-R tracrRNA was amplified with the Alt-R tracrRNA F and tracrRNA R primer set (Table S3). To quantify the tracrRNAs, we generated a standard curve by the serial dilution of the corresponding tracrRNA from 1×10^1 to 1×10^{-4} ng.

The tracrRNAs were electroporated and RNA extracted as described above to determine the levels of IFN activation. The RNA was reverse

transcribed using the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions, and the levels of IFN- β and β -actin were amplified using the Kapa SYBR Fast qPCR Master Mix with primers described in Table S3. Both the tracrRNA and IFN- β qPCRs were run on the Roche LightCycler 96 and analyzed using the LightCycler 96 software (Roche, Basal, Switzerland). The PCR conditions were as follows: initial denaturation, 95°C for 3 min; denaturation, 95°C for 30 s; annealing, 60°C for 20 s for 40 \times cycles.

FACS Analysis of CCR5 Expression

For the detection of CCR5, pMo-C6-CCR5 were centrifuged at 1,600 rpm for 5 min and resuspended in 100 μ L of PBS + 1% bovine serum albumin (BSA) with 5 μ L of a Mouse APC anti-CCR5 (Cat. no 556903; BD Biosciences, CA, USA) for 30 min at room temperature in the dark. One milliliter of PBS + 1% BSA was added to the tube and centrifuged at 1,600 rpm for 5 min, and the pellet was resuspended in PBS + 1% BSA. Counts were gated on single cells, and a total of 10,000 events were collected using the BD Accuri C6 and analyzed by FlowJo vX3.05470 software.

T7E1 Assays

Genomic DNA was extracted from samples using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The TAR or CCR5 target site was amplified using KAPA2G Robust HotStart Ready mix (Sigma, MO, USA) using the primers indicated in Table S3 (TAR F and R, CCR5 F and R). The products were purified using QIAquick PCR purification kit (-QIAGEN, Hilden, Germany), quantified on a NanoDrop One, and 100 ng of PCR product was mixed with NEB 2 buffer in 10 μ L of H₂O. The mixture was heat denatured at 95°C for 5 min and slowly cooled (0.1°C/s) to 25°C. After re-annealing, 0.5 μ L of T7 endonuclease 1 (NEB, MA, USA) was added and incubated for 30 min at 37°C. The digestion products were resolved on a Novex 10% TBE gel and visualized as described above. The band intensities were quantified using ImageJ 1.50i and quantified as described elsewhere.³¹

Statistical Analysis

Graphing and statistical analyses were performed using GraphPad Prism version 8 (v.8.1.2).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.01.004>.

AUTHOR CONTRIBUTIONS

T.S. and K.V.M. conceived and designed the experiments. T.S. and C.S. conducted the experimentation. T.S. and K.V.M. wrote the main manuscript and prepared the tables and figures. All authors have reviewed and edited the manuscript.

CONFLICTS OF INTEREST

T.S. and K.V.M. have submitted a provisional patent 048440-659001WO on this technology.

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