Noncanonical crRNAs derived from host transcripts enable multiplexable RNA detection by Cas9

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CRISPR-Cas systems recognize foreign genetic material using CRISPR RNAs (crRNAs). In type II systems, a trans-activating crRNA (tracrRNA) hybridizes to crRNAs to drive their processing and utilization by Cas9. While analyzing Cas9-RNA complexes from Campylobacter jejuni, we discovered tracrRNA hybridizing to cellular RNAs, leading to formation of “noncanonical” crRNAs capable of guiding DNA targeting by Cas9. Our discovery inspired the engineering of reprogrammed tracrRNAs that link the presence of any RNA of interest to DNA targeting with different Cas9 orthologs. This capability became the basis for a multiplexable diagnostic platform termed LEOPARD (leveraging engineered tracrRNAs and on-target DNAs for parallel RNA detection). LEOPARD allowed simultaneous detection of RNAs from different viruses in one test and distinguished severe acute respiratory syndrome coronavirus 2 and its D614G (Asp614—Gly) variant with single-base resolution in patient samples.

Cellular RNAs bound to Cas9 from C. jejuni resemble crRNAs

Our prior work interrogating RNAs bound to Cas9 from Campylobacter jejuni NCTC1168 (CjeCas9) revealed crRNA-guided RNA targeting by CjeCas9 (9). To further explore RNA binding partners of CjeCas9, we repeated the immunoprecipitation and RNA sequencing (RIP-seq) approach of epitope-tagged Cas9 using C. jejuni strain CG8421 harboring only two spacers in its endogenous type II-C CRISPR-Cas system (Fig. 1, A and B, and fig. S1, A and B). RIP-seq identified the CRISPR-tracrRNA locus as well as 205 RNA fragments derived from cellular RNAs enriched with Cas9-3xFLAG (Fig. 1C, fig. SIC, and table S1). Analyses of the enriched fragments using MEME (10) revealed two significant sequence motifs across all three replicates (Fig. 1D). Motif #1 was complementary to 13 nucleotides (nts) within the guide portion of crRNA2, in line with RNA targeting by crRNAs in NCTC1168 (9). Motif #2 was complementary to 21 nts within the tracrRNA anti-repeat domain. As this domain normally hybridizes to the crRNA repeat as part of crRNA biogenesis (fig. S2, A and B), motif #2 raised the possibility that these cellular RNAs were hybridizing with the tracrRNA, potentially becoming RNAs that function like crRNAs.

We explored this possibility through two routes. First, for the enriched RNA fragments with motif #2, we aligned the motif within each RNA fragment with a spacer-repeat pair, and we measured the length of each fragment corresponding to the spacer or repeat (Fig. 1E). Most frequently, the spacer part was 15 nt longer than a canonical crRNA spacer whereas the repeat part was the same size as a canonical crRNA repeat, similar to slightly extended versions of crRNAs. Second, we predicted how each RNA fragment base pairs with the tracrRNA anti-repeat (11). Predicted binding affinities were significantly higher for RNA fragments with motif #2 than for fragments without the motif (p = 3 × 10^-7) (Fig. 1F). However, multiple RNA fragments were predicted to strongly pair with the tracrRNA anti-repeat despite lacking motif #2 (Fig. 1F), likely due to bulges in the RNA duplex creating discontinuities in the motif. For these RNA fragments and those containing motif #2, the predicted interactions between each RNA and the tracrRNA anti-repeat consistently contained imperfect RNA duplexes, with the most extensive pairing near the 3' end of the anti-repeat (Fig. 1G and fig. S2). We made similar observations for motif #1 (fig. SID).

These crRNA-like RNAs raised the question of whether these same RNAs were present in our prior RIP-seq analysis with strain NCTC1168 (9). We found that 7 of the 96 enriched fragments were predicted to bind the tracrRNA anti-repeat more tightly than at least one crRNA (fig. S3, A and B). Two of these RNAs (derived from flfF and dctA mRNAs) matched those found in CG8421 (Fig. 1G and figs. S2D and S3C). The RNA fragment derived from the flfF mRNA could be detected by Northern blot in both total RNA and RIP-seq samples yet disappeared following deletion of cas9 (Fig. 1H and fig. S4, A and B). The dctA RNA fragment was only weakly detected in one strain (fig. S4). Although cas9 deletion did not significantly perturb FlfF protein concentrations in vivo under standard growth conditions (fig. S5), deleting the CRISPR array in NCTC1168 increased levels of the flfF RNA fragment (fig. S4C). Finally, the flfF RNA fragment was a processing product, as confirmed with Terminator exonuclease treatment (Fig. 1G). These crRNA-like RNAs thus are also present in C. jejuni NCTC1168 and likely exist in other C. jejuni strains on account of the shared tracrRNA binding site in flfF (fig. S6).

Communoprecipitated RNAs can function as noncanonical crRNAs that direct DNA targeting by Cas9

Cas9 binding, predicted tracrRNA pairing, and the length distribution of many of these enriched RNA fragments suggested that the tracrRNA pairs with endogenous RNAs, resulting in “noncanonical” crRNAs (ncrRNAs) (Fig. 2A and fig. S2A). The ncrRNAs therefore would be expected to direct Cas9 to complementary DNA targets flanked by a protospacer-adjacent motif (PAM), similar to a canonical crRNA (12). As none of the genes giving rise to the detected ncrRNAs has a correctly placed PAM, the ncrRNAs are not expected to direct Cas9 to cleave their originating genomic site (table S1).

To evaluate ncrRNA-dependent targeting, we exploited a cell-free transcription-translation (TXTL) assay previously used to characterize CRISPR-Cas systems (13–15). As part of the assay, DNA constructs encoding CjeCas9, an
RNA guide, and a green fluorescent protein (GFP) reporter harboring a target sequence flanked by a recognized PAM are added to the TXTL reaction. GFP fluorescence is then measured over time as a readout of DNA binding and cleavage by CjeCas9 (Fig. 2B and fig. S7A).

We focused on examining the fliF ncrRNA given its presence in both C. jejuni strains and detection by Northern blotting. Applying this assay to the tracrRNA and mRNA comprising the entire fliF coding region (1683 nts) (Fig. 2C), we found that expressing the mRNA reduced GFP levels 2.5-fold compared with a nontargeting crRNA ($p = 5.4 \times 10^{-5}$). Expressing the equivalent crRNA reduced GFP levels 15.1-fold compared with the nontargeting control. The reduced GFP silencing for the fliF mRNA versus the crRNA potentially reflects not only reduced targeting efficiency but also delayed complex formation. Overall, the TXTL results offer evidence that mRNA-derived ncrRNAs can direct DNA targeting by Cas9.

The reduced performance of the fliF mRNA in TXTL could be due to how an ncrRNA deviates from a standard crRNA. These deviations include the crRNA repeat sequence, the secondary structure of the duplex formed with the tracrRNA anti-repeat, and 5′ or 3′ extensions to the repeat that do not undergo efficient processing. To evaluate these deviations, we systematically mutated or extended the standard crRNA, either as a single guide RNA (sgRNA) to ensure duplex formation or as a crRNA:tracrRNA pair, and evaluated GFP silencing in TXTL (Fig. 2D and table S1). CjeCas9 could accommodate some mutations within
Fig. 2. Noncanonical crRNAs can direct DNA cleavage by CjeCas9. (A) General process for ncrRNA generation. (B) Applying the TXTL assay to characterize putative ncrRNAs. (C) DNA targeting through the fliF ncrRNA in TXTL. Lines and shaded regions indicate the mean and standard deviation from four separately mixed replicates. NT, nontargeting. (D) Systematic evaluation of mutating the repeat:anti-repeat duplex for CjeCas9 with TXTL. Endpoint GFP levels are shown. Mutations and extensions to the fliF tracrRNA are indicated in red. See table S1 for sequences. (E) DNA targeting by selected ncrRNAs predicted in TXTL. Check marks indicate use of the construct above the line. mRNA(mut): mRNA encoding the ncrRNA with point mutations in the predicted “seed” region of the guide. tracrRNA(scr): tracrRNA with the anti-repeat sequence scrambled. Values in (D) and (E) represent the mean and standard deviation from four separately mixed replicates. **p < 0.001. n.s., not significant.
the region of the repeat:anti-repeat duplex in the sgRNA implicated in nuclease binding (16). The more disruptive mutations spanned more nts, were closer to the 5' end of the repeat, or resulted in a bulge in the tracrRNA (e.g., s3, s6, s7, s9, s12, s18 in Fig. 2D, left). Observed differences in GFP silencing do not appear to arise from variable sgRNA levels (fig. S7B). Extending the sgRNA-crRNA ends or mutating the region cleaved by RNase III within the crRNA had minimal impact on GFP silencing (Fig. 2D, right). Overall, the tracrRNA can tolerate deviations from a standard crRNA as long as pairing through the 3' end of the tracrRNA anti-repeat is maintained.

We applied insights from our mutational analyses to prioritize putative ncrRNAs from C. jejuni CG8421 for functional tests in TXTL. In total, we identified eight RNA fragments predicted to base pair extensively with the 3' end of the tracrRNA anti-repeat (Fig. 2E and fig. S8). We then assessed GFP silencing by expressing up to 350 nts upstream and downstream of each associated ncrRNA-encoding gene with tracrRNA, CjeCas9, and the GFP reporter harboring each cognate DNA target. Of the eight tested RNAs, three (from rseP, nuoL, and dctA) yielded a >twofold reduction in GFP reporter levels compared with a nontargeting crRNA control (p < 0.001). Furthermore, targeting was directed specifically through the predicted ncrRNA, as mutating the "seed" region of the putative ncrRNA (17), scrambling the tracrRNA anti-repeat, or replacing CjeCas9 with the orthogonal FnCas12a nuclease fully relieved GFP repression (Fig. 2E). Multiple factors, such as mRNA folding or accessibility during translation, may explain why the other five ncrRNAs did not exhibit targeting activity in TXTL, as a linear-regression model built around the sgRNA mutants had limited ability to predict the targeting activity of these ncrRNAs (supplementary text S1). The current lack of predictability parallels guide design for RNA-targeting Cas13a nucleases, which only became predictable with extensive datasets and machine learning (18).

Beyond TXTL, we assessed ncrRNA function as part of DNA targeting in C. jejuni CG8421 and in Escherichia coli. For CG8421, transformation interference assays did not yield any significant DNA targeting directed by ncrRNAs derived from the rseP, dctA, and nuoL mRNAs (fig. S9A), likely due to low ncrRNA abundance compared with the strain's crRNAs under the examined growth conditions. For E. coli, overexpressing the dctA mRNA, CjeCas9, and the tracrRNA led to moderate (15.5-fold) clearance of a transformed plasmid with the putative dctA ncrRNA target (p = 0.0036), but not when the tracrRNA anti-repeat was scrambled (1.6-fold) (p = 0.068) (fig. S9B). We therefore conclude that ncrRNAs derived from mRNAs can elicit DNA targeting in both in vivo and cell-free systems.

The tracrRNA can be reprogrammed to direct Cas9 activity by an RNA of interest

The conversion of a cellular RNA into an ncrRNA was based on sequences bearing complementarity to the tracrRNA anti-repeat, analogous to natural crRNA biogenesis (fig. S2A). What if the tracrRNA anti-repeat sequence could be changed to hybridize to other RNAs while maintaining the appropriate structure for Cas9 recognition? If so, then the resulting reprogrammed tracrRNA (Rptr, pronounced "raptor") could specifically derive an ncrRNA from a cellular RNA. The resulting ncrRNAs...
can then guide Cas9 to matching DNA targets (Fig. 3A). Although tracrRNA engineering has rarely been explored outside of sgRNAs or crRNA:tracrRNA duplexes (19), multiple studies have shown that the repeat-anti-repeat duplex of the sgRNA for the *Streptococcus pyogenes* Cas9 (SpyCas9) can be extensively modified as long as the secondary structure is maintained (20, 21).

CjeCas9 recognizes a perfect RNA duplex formed between the crRNA repeat and tracrRNA anti-repeat (fig. S2B) (16). Based on our mutational analysis of the crRNA repeat, we already observed that Cas9 can accommodate several mutations within the crRNA repeat: tracrRNA anti-repeat duplex (Fig. 2D). Therefore, we evaluated GFP silencing in TXTL after mutating both sides of the duplex in the *flIF* sgRNA while preserving the secondary structure (s26-s31, Fig. 3B). GFP silencing was maintained even when exchanging the sequence of the entire duplex (s31, Fig. 3B). Next, we reprogrammed the tracrRNA anti-repeat to form perfect 25-base pair duplexes with three putative ncrRNAs (derived from *flIF*, CJ8421_04245, CJ8421_04975) exhibiting at most modest GFP silencing in TXTL (Fig. 2, C and E, and fig. S8). In all three cases, GFP repression was significantly enhanced with Rptrs compared with wild-type (WT) tracrRNAs (\( p = 1 \times 10^{-8} \) to 0.001), even if repression was not as strong as with the canonical crRNA:tracrRNA pair (Fig. 3C). Finally, we reprogrammed the tracrRNA anti-repeat to base pair with entirely new regions of an mRNA (Fig. 3D). Starting with the CJ8421_04975 mRNA, we designed five different CjeCas9 Rptrs hybridizing to different locations (n1 to n5) in the mRNA (Fig. 3D and fig. S10A). Of these Rptrs, four yielded significantly reduced GFP levels compared with nontargeting crRNA controls (\( p = 6 \times 10^{-7} \) to 0.002). Notably, mutating the predicted seed region, scrambling the tracrRNA anti-repeat, or replacing CjeCas9 with FnCas12a restored GFP expression (Fig. 3D). Northern blot analysis from TXTL-extracted RNAs further revealed no detectable processed RNAs with a size resembling that of mature ncrRNA. Complete ncrRNA processing to a size similar to that of canonical crRNAs therefore may not be necessary for DNA targeting by CjeCas9 (fig. S11), in line with the dispensability of RNase III for crRNA-mediated DNA targeting through the II-C CRISPR-Cas system in *Neisseria meningitidis* (22).

Given the functionality of CjeCas9 Rptrs, we asked whether tracrRNAs for other Cas9 homologs can be similarly reprogrammed. We selected the well-characterized *Streptococcus pyogenes* Cas9 (SpyCas9) and the *Streptococcus thermophilus* CRISPR Cas9 (StlCas9) as examples. In both cases, we devised design rules for Rptrs based on the known secondary structure of the crRNA:tracrRNA duplex and the preference of RNase III to cleave double-stranded RNA with AT-rich sequences (fig. S10A) (20, 23). All 10 designed Rptrs significantly reduced GFP levels compared with the nontargeting crRNA control (\( p = 1 \times 10^{-7} \) to 1 \( \times 10^{-4} \)) (Fig. 3D). As before, GFP expression was restored by disrupting the seed sequence in the ncrRNA guide, scrambling the tracrRNA anti-repeat, or swapping either of the Cas9’s for FnCas12a. In many cases, the extent of GFP silencing approached that of the targeting crRNA control. We also evaluated plasmid clearance with Rptrs in *E. coli* for all
three Cas9 orthologs, finding that each could elicit efficient plasmid clearance for at least one tested Rptr (fig. S10B). The targeted plasmid was efficiently cleared even when expressing the sensed mRNA at low levels (fig. S12A) or when deleting RNase III (fig. S12B). Overall, the tracrRNA for different Cas9 orthologs can be converted into Rptrs to elicit DNA targeting based on the presence of a selected cellular RNA.

Reprogrammed tracrRNAs enable sequence-specific detection by Cas9

By linking DNA targeting to an RNA of interest, Rptrs offer a valuable opportunity for RNA detection and a different paradigm for CRISPR diagnostics. Current CRISPR diagnostics principally rely on Cas12a or Cas3 searching for double-stranded DNA or RNA targets in a sample, where target recognition elicits nonspecific single-stranded DNA or RNA cleavage of a fluorescent reporter (24–26). The nonspecific readout practically limits one test to one target sequence. By contrast, Rptrs convert sensed RNAs into ncrRNAs, which would direct Cas9 to matching DNA. Cas9 binding or cleavage of a DNA sequence would then indicate the presence of the sensed RNA in the sample. Because the sequence of each DNA target is distinctive, large numbers of target sequences could be monitored in parallel in one test. We call the resulting diagnostic platform LEOPARD, for leveraging engineered tracrRNAs and on-target DNAs for parallel RNA detection (Fig. 4A).

To begin assessing LEOPARD, we performed a simplified in vitro reaction using T7-transcribed RNAs, commercially available SpyCas9 protein, and linear DNA targets (fig. S13A). We began with RNA corresponding to one of the synthetic ncrRNA loci within CJ8421_04975 (n4 under SpyCas9, Fig. 3D). Introducing an annealing step to hybridize the Rptr to the T7-transcribed ncrRNA yielded DNA target cleavage without adding RNase III or RNase A for ncrRNA:Rptr processing (fig. S13D, B and C). The cleavage efficiency was also similar to that of the equivalent crRNA:tracrRNA pair, even when the ncrRNA sequence was extended on either end (fig. S13D). The time scale of the annealing step could also be minimized by rapid cooling of the samples (fig. S13E). With the annealing step, efficient cleavage occurred with a 100-fold excess of yeast total RNA but only when the ncrRNA was present (Fig. 4B). LEOPARD therefore can report the presence of a specific RNA of interest based on cleavage of a DNA target and can be streamlined through further optimization.

LEOPARD allows for multiplexed RNA detection by Cas9 with single-base resolution

Realizing the full multiplexing potential of LEOPARD requires monitoring many DNA targets at once. To initially demonstrate this multiplex capability, we devised a readout scheme based on resolving distinct cleavage products from pooled DNA targets by gel electrophoresis (Fig. 4C). Each target is labeled with a fluorophore on one end, producing only two visualizable products—cleaved and uncleaved. We then applied this scheme to specifically detect nine ~150-nt RNA fragments associated with respiratory viruses, including two from SARS-CoV-2 coronavirus (the causative agent of COVID-19), six from other coronaviruses, and one from influenza H1N1 (Fig. 4D and fig. S14). Each DNA target was cleaved by Cas9 only in the presence of the corresponding RNA, even when detecting three or five specific RNA fragments in the same reaction (Fig. 4D and fig. S14).

As the viral RNA sequences were selected to minimize homology, we asked if LEOPARD could detect even a single-nucleotide difference. The Asp614→Gly (D614G) mutation in the spike protein of SARS-CoV-2 served as an example, as it comprises a single base change (A23403G) that increased infectivity and drove global spread (27). By placing this nt change within the seed region of the target, we could detect the WT or D614G RNA using one Rptr combined with either the WT or D614G target (Fig. 4E). The matching DNA target was preferentially cleaved when testing each target individually, although some cleavage of the nonmatching target was observed. However, combining the two targets in a single reaction yielded discernable cleavage only for the matching target, presumably through preferential binding and cleavage of the perfect target by Cas9 (28). LEOPARD therefore can confer multiplexed RNA detection in a single reaction with single-base resolution.

To extend LEOPARD beyond this proof-of-principle demonstration, we made two additions. First, we added target-specific reverse transcription–polymerase chain reaction (RT-qPCR) and in vitro transcription similar to Cas3-based diagnostics (29) to improve assay sensitivity beyond the threshold set by detection of a cleaved DNA product. Second, we resolved DNA targets using a Bioanalyzer as a more practical readout (Fig. 5A). Applying this modified workflow to sense the in vitro–transcribed WT SARS-CoV-2 RNA fragment, we could detect as little as approximately one copy, or 1.7 aM in the original dilution, of this RNA (Fig. 5B and fig. S15B). LEOPARD therefore can confer multiplexed RNA detection in a single reaction with single-base resolution.

Fig. 5. LEOPARD with RNA preamplification and Bioanalyzer readout allows for multiplexed detection of SARS-CoV-2 in patient samples. (A) General workflow for LEOPARD with target-specific preamplification and DNA target resolution on a Bioanalyzer. (B) Sensitivity of the workflow for detecting dilutions of an in vitro–transcribed WT SARS-CoV-2 RNA fragment. One microliter was added for each test. Bars represent the average of independent duplicates. (C) Multiplexed detection of five RNAs in patient samples confirmed positive or negative for SARS-CoV-2 by RT-qPCR. (D) Sanger sequencing results for the detected region in SARS-CoV-2 cDNA from the positive patient samples. Blue bar: Position of the ncrRNA, with the thick part indicating the resulting ncrRNA portion.
mRNA (n4 under SpyCas9, Fig. 3D) as a non-human negative control, and the mRNA encoding human RNase P to confirm correct administration of the nasal swab using four Rptrs [one for both WT and ΔD14G SARS-CoV-2 (Fig. 4E)] and five DNA targets. Of the four SARS-CoV-2–positive and five SARS-CoV-2–negative samples tested, RNase P mRNA but not the C08421_04975 mRNA or H1N1 RNA was detected in all nine samples (Fig. 5C and Fig. S16). Notably, we detected the ΔD14G variant of SARS-CoV-2 in all four positive samples, which was confirmed by sequencing preamplified cDNA (Fig. 5D). Although the sample size is small, detection of this variant suggests that it was spreading in Germany when the samples were collected. WT or ΔD14G SARS-CoV-2 RNA was not detected in any of the negative samples, paralleling the RT-qPCR results (Fig. 5C). Each reaction allowed for parallel testing for five different RNAs, including controls, that would require separate reactions for other diagnostic platforms. These findings demonstrate the practical utility of LEOPARD for multiplexed RNA detection.

**Discussion**

Starting from the characterization of a native CRISPR-Cas9 system in the bacterial pathogen *C. jejuni*, we discovered that cellular transcripts can be the source of noncanonical crRNAs through hybridization with the tracrRNA. This discovery adds ncRNAs to the list of RNA guides that account for potential off-targeting as well that similarly pair with the tracrRNA anti-repeat, and thus function like standard crRNA:tracrRNA duplex, we found that anti-repeat hybridization and guide-dependent targeting may limit off-targeting activity. Although we did not observe any detectable off-targeting in vitro or in vivo, future work could devise design rules for Rptrs that account for potential off-targeting as well as on-target activity, similar to existing sgRNA design algorithms (36). In turn, these rules would help advance the utilization of any RNA into a sequence-specific guide for CRISPR technologies.

**REFERENCES AND NOTES**


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**SUPPLEMENTARY MATERIALS**

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Materials and Methods
Secondary Text Figs. S1 to S51 Table S1 References (39–52) MDAR Re producibility Checklist

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Cellular RNAs guide CRISPR-Cas9

The Cas9 nuclease widely used for genome editing is derived from natural bacterial defense systems that protect against invading viruses. Cas9 is directed by RNA guides to cut matching viral DNA. Jiao et al. discovered that RNA guides can also originate from cellular RNAs unassociated with viral defense (see the Perspective by Abudayyeh and Gootenberg). They rendered this process programmable, linking the presence of virtually any RNA to cutting of matching DNA by Cas9. This capability is the basis of a new CRISPR diagnostic method developed by the authors that can detect many biomarkers at once. Named LEOPARD, this method can detect, for example, RNAs from severe acute respiratory syndrome coronavirus 2 and other viruses, thereby translating a new CRISPR discovery into a powerful diagnostic tool.

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