

Circulating microRNAs as stable blood-based markers for cancer detection

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Improved approaches for the detection of common epithelial malignancies are urgently needed to reduce the worldwide morbidity and mortality caused by cancer. MicroRNAs (miRNAs) are small (~22 nt) regulatory RNAs that are frequently dysregulated in cancer and have shown promise as tissue-based markers for cancer classification and prognostication. We show here that miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity. miRNAs originating from human prostate cancer xenografts enter the circulation, are readily measured in plasma, and can robustly distinguish xenografted mice from controls. This concept extends to cancer in humans, where serum levels of *miR-141* (a miRNA expressed in prostate cancer) can distinguish patients with prostate cancer from healthy controls. Our results establish the measurement of tumor-derived miRNAs in serum or plasma as an important approach for the blood-based detection of human cancer.

biomarker | miR-141 | plasma | serum | prostate cancer

The development of minimally invasive tests for the detection and monitoring of common epithelial malignancies could greatly reduce the worldwide health burden of cancer (1). Although conventional strategies for blood-based biomarker discovery (e.g., using proteomic technologies) have shown promise, the development of clinically validated cancer detection markers remains an unmet challenge for many common human cancers (2). New approaches that can complement and improve on current strategies for cancer detection are urgently needed.

MicroRNAs (miRNAs) are small (typically ~22 nt in size) regulatory RNA molecules that function to modulate the activity of specific mRNA targets and play important roles in a wide range of physiologic and pathologic processes (3, 4). We hypothesized that miRNAs could be an ideal class of blood-based biomarkers for cancer detection because: (i) miRNA expression is frequently dysregulated in cancer (5, 6), (ii) expression patterns of miRNAs in human cancer appear to be tissue-specific (7), and (iii) miRNAs have unusually high stability in formalin-fixed tissues (8–10). This third point led us to speculate that miRNAs may have exceptional stability in plasma and serum as well. We show here that miRNAs are in fact present in clinical samples of plasma and serum in a remarkably stable form. Furthermore, we establish proof-of-principle for blood-based miRNA cancer detection by using both a xenograft model system and clinical serum specimens from patients with prostate cancer. Our results lay the foundation for the development of miRNAs as a novel class of blood-based cancer biomarkers and raise provocative questions regarding the mechanism of stability and potential biological function of circulating miRNAs.

Results

Identification and Molecular Cloning of Endogenous miRNAs from Human Plasma. Prior reports have suggested that RNA from human plasma (the noncellular component of blood remaining after

removing cells by centrifugation) is largely of low molecular weight (11). We directly confirmed that human plasma contains small RNAs in the size range of miRNAs (18–24 nt) by characterizing the size of total RNA isolated from plasma by using radioactive labeling. PAGE and phosphorimaging of 5' ³²P-labeled plasma RNA demonstrated RNA species ranging from 10 to 70 nt in size, including a discernable species of size ~22 nt characteristic of most miRNAs [supporting information (SI) Fig. S1]. The detected signal was sensitive to RNase treatment but insensitive to DNase I treatment, confirming that the signal originated from RNA (Fig. S1).

To directly determine whether miRNAs are present in human plasma, we isolated the 18- to 24-nt RNA fraction from a human plasma sample from a healthy donor (see *SI Text* for details on blood collection and plasma RNA isolation) and used 5' and 3' RNA–RNA linker ligations followed by RT-PCR amplification to generate a small RNA cDNA library (Fig. 1A). Of the 125 clones sequenced from this library, 27 corresponded to spiked-in size marker oligos or linker–linker dimers. Ninety-one of the other 98 sequences (93%) corresponded to known miRNAs, providing direct confirmation that mature miRNAs are present in human plasma and indicating that the vast majority of 18- to 24-nt plasma RNA species cloned by our protocol are indeed miRNAs (Fig. 1A). To quantitate specific miRNAs, we used *TaqMan* quantitative RT-PCR (qRT-PCR) assays (12) to measure three miRNAs (*miR-15b*, *miR-16*, and *miR-24*) in plasma from three healthy individuals. These three miRNAs, chosen to represent moderate- to low-abundance plasma miRNAs (based on the sequencing results described above), were all readily detected in the plasma of each individual at concentrations ranging from 8,910 copies/μl plasma to 133,970 copies/μl plasma, depending on the miRNA examined (Fig. 1B).

Stability of Endogenous miRNAs in Human Plasma. We next sought to investigate the stability of miRNAs in plasma, given that this is an important prerequisite for utility as a biomarker. Incubation of plasma at room temperature for up to 24 h (Fig. 2A Upper) or subjecting it to up to eight cycles of freeze-thawing (Fig. 2A Lower) had minimal effect on levels of *miR-15b*, *miR-16*, or *miR-24* as

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The authors declare no conflict of interest.

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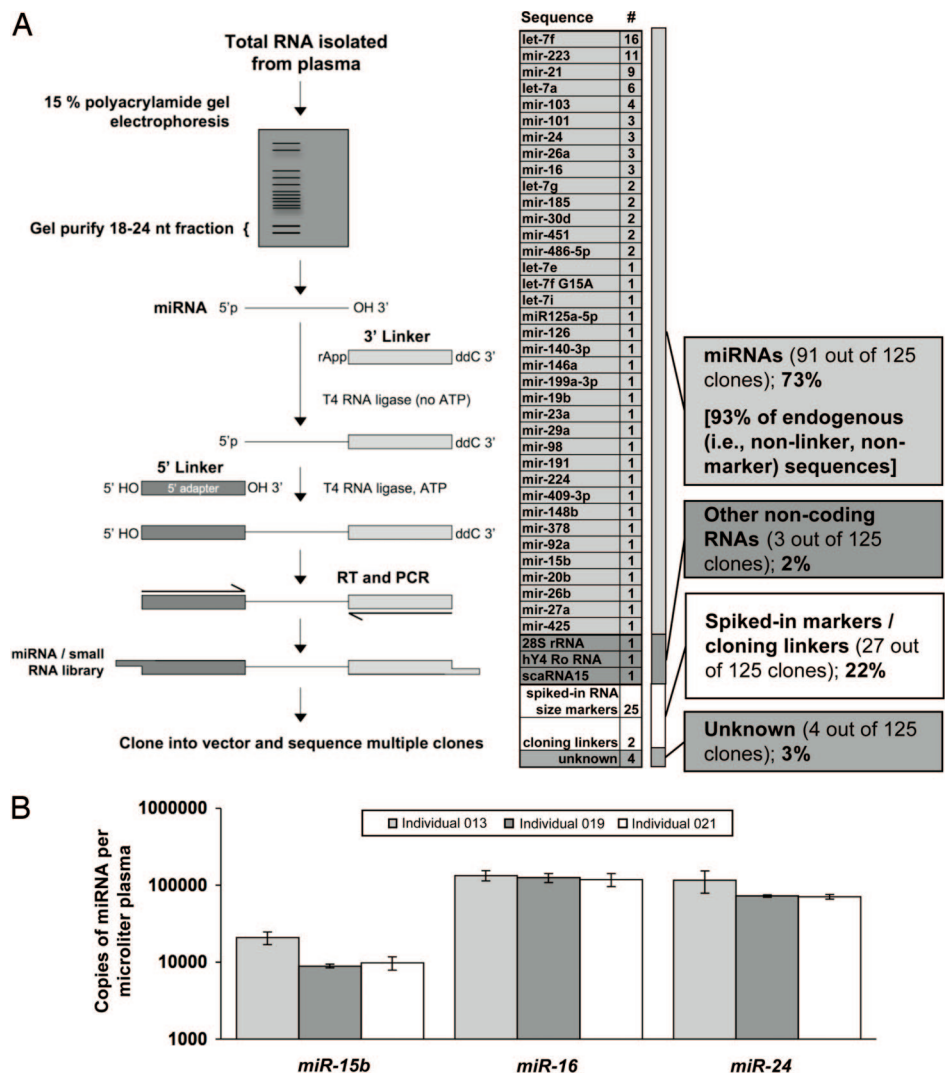
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Fig. 1. Identification of miRNAs in human plasma. (A) Cloning and sequencing of miRNAs from human plasma. The schematic diagram depicts the preparation of a small RNA library from human plasma. Briefly, the 18- to 24-nt fraction from ≈ 250 ng of plasma total RNA from a single donor (individual 006; described in Table S6) was isolated by PAGE. Purified miRNAs were then 3' and 5' ligated to single-stranded oligonucleotides that contained universal primer sequences for reverse transcription and PCR. Reverse transcription and PCR generated a library of small RNA cDNA molecules that were ligated into a plasmid vector (pCR4-TOPO) and transformed into *Escherichia coli*. Inserts from a total of 125 individual colonies yielded high-quality sequence. Sequences were compared to a reference database of known miRNA sequences (miRBase Release v.10.1) (19) and to GenBank. Seventy-three percent of sequences corresponded to known miRNAs as shown. The next most abundant species were matches to the sequence of synthetic RNAs spiked in as radiolabeled 18- and 24-nt molecular size markers during gel isolation steps. When only endogenously derived RNA sequences are considered, miRNAs represent 93% (91 of 98) of the recovered sequences. The miRNA read designated as "let-7f G15A" denotes a sequence matching the known *let-7f* miRNA except for a G-to-A substitution at nucleotide position 15. (B) Quantification of representative miRNAs in normal human plasma by *TaqMan* qRT-PCR. The graph indicates the number of copies of each of three representative miRNAs measured in plasma obtained from three healthy individuals. In each case, values represent the average of two replicate reverse transcription reactions followed by real-time PCR. For each miRNA assay, a dilution series of chemically synthesized miRNA was used to generate a standard curve that permitted absolute quantification of molecules of miRNA/ μ l plasma as shown here (see Fig. S2 for standard curve plots). Values were median-normalized by using measurements of synthetic normalization controls spiked in immediately after addition of denaturing solution during RNA isolation (see SI Text for full details). The absence of amplification in reverse transcriptase-negative controls indicated that amplification was originating from an RNA template (real-time PCR plots corresponding to plasma RNA samples and negative controls are provided in Fig. S3).



measured by *TaqMan* qRT-PCR. Given that plasma has been reported to contain high levels of RNase activity (13), we sought to determine whether the stability of miRNAs is intrinsic to their small size or chemical structure, or whether it is caused by additional extrinsic factors. We introduced synthetic miRNAs corresponding to three known *Caenorhabditis elegans* miRNAs (*cel-miR-39*, *cel-miR-54*, and *cel-miR-238*), chosen because of the absence of homologous sequences in humans, into human plasma either before or after the addition of a denaturing solution that inhibits RNase activity. RNA extraction followed by measurement of synthetic miRNAs showed that the synthetic miRNAs rapidly degraded when added directly to plasma (the time between addition of synthetic miRNAs and subsequent addition of denaturing solution was < 2 min), as compared with their addition after adding denaturing solution to plasma (Fig. 2B). These results confirm the presence of RNase activity in plasma and the sensitivity of naked miRNAs to degradation. The levels of endogenous miRNAs (i.e., *miR-15b*, *miR-16*, and *miR-24*) were not significantly altered in any of the experimental samples (Fig. 2B), indicating that endogenous plasma miRNAs exist in a form that is resistant to plasma RNase activity.

Comparison of miRNA Levels Between Plasma and Serum. Given that clinical specimens of serum (the supernatant remaining after whole

blood is permitted to clot) are more plentiful than plasma samples in many retrospective clinical sample repositories, we sought to determine whether miRNA measurements are substantially different in serum compared with plasma by measuring *miR-15b*, *miR-16*, *miR-19b*, and *miR-24* in matched samples of serum or plasma collected from a given individual at the same blood draw (Fig. 2C). Measurements obtained from plasma or serum were strongly correlated, indicating that both serum and plasma samples will be suitable for investigations of miRNAs as blood-based biomarkers.

Tumor-Derived miRNAs Are Present in Plasma. Having demonstrated that circulating miRNAs are detectable and stable in blood collected from healthy individuals, we next sought to determine whether tumor-derived miRNAs enter the circulation at levels sufficient to be measurable as biomarkers for cancer detection. We chose to study a mouse prostate cancer xenograft model system that involves growth of the 22Rv1 human prostate cancer cell line in NOD/SCID immunocompromised mice (14–17). We established a cohort of 12 mice xenografted with 22Rv1 cells injected with Matrigel and 12 control mice inoculated with Matrigel alone (Fig. 3A). Plasma was collected 28 days later (once tumors were well established), and RNA was isolated for miRNA quantitation. Because of the lack of an established endogenous miRNA control

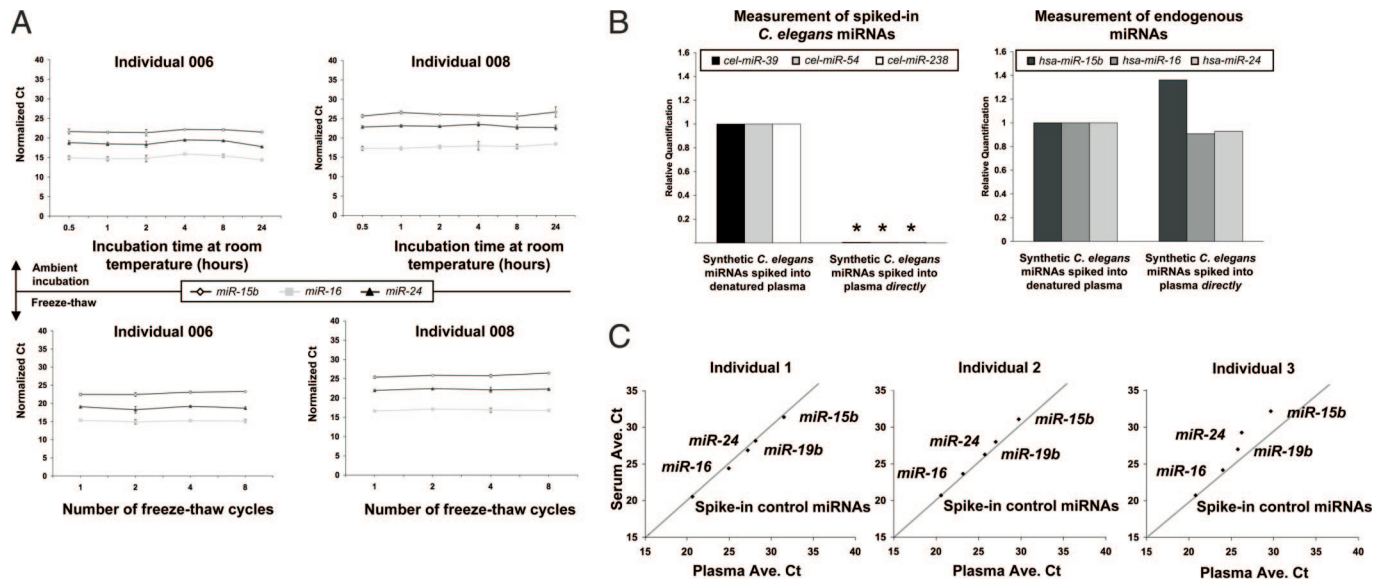


Fig. 2. Characterization of miRNA stability in human plasma. (A) miRNA levels remain stable when plasma is subjected to prolonged room temperature incubation or freeze-thawed multiple times. (Upper) The graphs show normalized Ct values for the indicated miRNAs measured in parallel aliquots of human plasma samples incubated at room temperature for the indicated times. The experiment was carried out by using plasma from the two different individuals noted. Normalization of raw Ct values across samples is based on the measurement of three nonhuman synthetic miRNAs spiked into each sample at known molar amounts after initial plasma denaturation for RNA isolation (described in detail in *SI Text*). (Lower) The graphs show normalized Ct values for the indicated miRNAs measured in parallel aliquots of human plasma samples subjected to the indicated number of cycles of freeze-thawing. Raw Ct values were normalized across samples by using the same approach as described above. (B) Exogenously added miRNAs are rapidly degraded in plasma, whereas endogenous miRNAs are stable. Three *C. elegans* miRNAs (chosen for the absence of sequence similarity to human miRNAs) were chemically synthesized and added either directly to human plasma (from individual 003; described in *Table S6*) or added after the addition of denaturing solution (containing RNase inhibitors) to the plasma (referred to as “denatured plasma”). RNA was isolated from both plasma samples, and the abundance of each of the three *C. elegans* miRNAs was measured by TaqMan qRT-PCR (Left), as was that of three endogenous plasma miRNAs (Right). Asterisks indicate that the abundance ratios of *cel-miR-39*, *cel-miR-54*, and *cel-miR-238* added to human plasma directly, relative to addition to denatured plasma, were 1.7×10^{-5} , 9.1×10^{-6} , and 1.1×10^{-5} , respectively and therefore too low to accurately display on the plot. (C) Abundance of miRNAs in serum and plasma collected from the same individual is highly correlated. Each plot depicts the average Ct values (average of two technical replicates) of the indicated miRNAs measured in serum and plasma samples collected from a given individual at the same blood draw. Results from three different individuals are shown. miRNA measurements were highly correlated in both sample types. Results shown for synthetic *C. elegans* miRNAs spiked into each plasma or serum sample (after addition of denaturing solution) demonstrate that experimental recovery of miRNAs and robustness of subsequent qRT-PCR is not affected by whether it is plasma or serum that is collected.

for plasma or serum, we introduced three synthetic *C. elegans* miRNAs (described earlier) after the addition of denaturing solution to the plasma samples and used these as normalization controls to correct for technical variations in RNA recovery (detailed in *SI Text*).

We first sought to establish that endogenous (murine) miRNAs exist in mouse plasma and determine whether the presence of cancer may lead to a general increase in plasma miRNAs, whether they be tumor- or host-derived. *miR-15b*, *miR-16*, and *miR-24* (which are perfectly conserved in mature sequence between human and mouse) were all readily detectable in healthy control mice (Fig. 3B) and were not expressed at substantially different levels in xenograft-bearing mice, indicating that the presence of tumor does not lead to a generalized increase in plasma miRNAs.

We next sought to identify candidate tumor-derived miRNAs for examination in plasma by first profiling the expression of 365 known miRNAs in 22Rv1 cells by using a microfluidic TaqMan low-density miRNA qRT-PCR array (Applied Biosystems). We identified two miRNAs, *miR-629** and *miR-660*, that (i) were expressed in these cells as indicated by low cycle threshold (Ct) values and (ii) did not have known mouse homologs and therefore would be expected to be tumor-specific markers in this setting (*Table S1*). We next analyzed plasma samples from control and xenograft mice for the levels of *miR-629** and *miR-660* by TaqMan qRT-PCR. Levels of *miR-629** and *miR-660* were generally undetectable in the control mice, whereas they were readily detected (ranging from 10 to 1,780 copies/ μ l plasma for *miR-629** and 5,189–90,783 copies/ μ l for *miR-660*) in all of the xenograft mice (Fig. 3C). Levels of both

*miR-629** and *miR-660* were able to independently differentiate xenografted mice from controls with 100% sensitivity and 100% specificity. These data establish proof of the principle that tumor-derived miRNAs reach the circulation where their measurement in plasma can serve as a means for cancer detection.

To understand the basis for the wide variation in miRNA abundance observed among the different xenografted mice, we compared plasma levels of *miR-629** and *miR-660* with tumor mass in each mouse. Levels of these miRNAs were moderately correlated with tumor mass (Fig. S4 and see *Table S7*), indicating that variation in miRNA abundance across animals reflects, at least in part, the differences in tumor burden.

Tumor-Derived miRNAs in Plasma Are Not Cell-Associated. We sought to further explore the mechanism of protection of tumor-derived miRNAs from plasma RNase activity by testing the hypothesis that they are present inside circulating tumor cells that might have escaped pelleting during primary centrifugation for plasma isolation. We took two approaches to address this hypothesis. In the first experiment, we filtered pooled plasma generated from the xenograft or control groups through a 0.22- μ m filter, followed by RNA extraction from the filtrate and the material retained on the filter (referred to as the retentate). Measurement of *miR-629** and *miR-660* by qRT-PCR in each of the samples demonstrated that virtually all of the tumor-derived miRNAs passed through the 0.22- μ m filter (Fig. S5A). As expected, tumor-derived miRNAs were essentially undetectable in all of the samples from the control group. In a second independent experiment, we subjected plasma

The available data indicate that *miR-141* is expressed in an epithelial cell type-specific manner in a range of common human cancers. Given this, we speculate that it could have value in the setting of detecting cancer recurrence for cancer types for which clinically validated blood biomarkers are lacking (e.g., lung cancer, breast cancer, etc.). We also anticipate that advances in miRNA qRT-PCR assay design and assay optimization, and the application of alternative miRNA quantitation strategies, will substantially improve the approach and will likely be needed to detect cancer at lower tumor burdens (i.e., early-stage disease). It is likely that other blood-based miRNA markers that are specific for particular cancer types will be discovered. The results presented here establish the foundation and rationale to motivate future global investigations of miRNAs as circulating cancer biomarkers for a variety of common human cancers.

Our Study Raises Intriguing Questions Regarding the Mechanism of miRNA Stability in Plasma and Potential Biological Roles of Circulating miRNAs. The remarkable stability of miRNAs in clinical plasma samples raises important and intriguing questions regarding the mechanism by which miRNAs are protected from endogenous RNase activity. One tantalizing hypothesis is that they are packaged inside exosomes that are secreted from cells. Exosomes are 50- to 90-nm (24), membrane-bound particles that have been reported to be abundant in plasma (25) and that have recently been shown (in cell culture studies) to contain miRNAs (26). Our results on filtration (through a 0.22- μ m filter) and differential centrifugation of plasma containing tumor-derived miRNAs are certainly consistent with this hypothesis (Fig. S5). Alternative explanations include protection via association with other molecules (e.g., in a RNA-protein complex) or modifications of the miRNAs that make them resistant to RNase activity.

The high abundance of many circulating miRNAs also raises provocative questions regarding their potential biological role as extracellular messengers mediating short- and long-range cell-cell communication, reminiscent of the similar role played by small RNAs that spread within the organism in plants and *C. elegans* (27, 28). Additional studies will be needed to explore these exciting hypotheses.

Advantages and Potential of miRNAs as Blood-Based Cancer Biomarkers. The availability of powerful approaches for global miRNA characterization and simple, universally applicable assays for quantitation (e.g., qRT-PCR) suggests that the discovery-validation pipeline for miRNA biomarkers will be more efficient than traditional proteomic biomarker discovery-validation pipelines, which typically encounter bottlenecks at the point of antibody generation

and quantitative assay development for validation of biomarker candidates (2). In addition, the inherent regulatory function of miRNAs makes it likely that many miRNAs expressed in tumor tissue influence the biological behavior and clinical phenotype of the tumor. As the functional roles of miRNAs in tumor biology are unraveled, we envision that blood-based miRNA biomarkers that predict clinical behavior and/or therapeutic response will be identified.

Materials and Methods

Clinical Samples. Human plasma and serum samples from healthy donors or patients with cancer were obtained with informed consent under institutional review board-approved protocols. Samples were derived from the Pacific Ovarian Cancer Research Consortium, local healthy donors from the Seattle area, or the Department of Urology, University of Washington. Details of sample collection and processing and relevant corresponding clinical data are provided in *SI Text*.

RNA Isolation. Isolation of RNA from plasma or serum was carried out using a modification of the mirVana PARIS kit (Ambion) that is described in detail in *SI Text*.

Mouse Xenograft Experiment. Xenografts were established in NOD/SCID mice by s.c. injection of 7.5×10^5 22Rv1 cells per mouse. Mouse blood was collected by cardiac puncture at 28 days after injection. Additional details of cell culture, establishment of xenografts, plasma collection, and RNA isolation are provided in *SI Text*.

qRT-PCR. MiRNAs were quantified by using TaqMan miRNA qRT-PCR assays with modifications as described in detail in *SI Text*.

Normalization of Experimental qRT-PCR Data from Plasma or Serum Using Spiked-In *C. elegans* Controls.

Three *C. elegans* miRNAs were chosen because of a lack of sequence homology to human miRNAs and absence of empiric hybridization to human miRNA probes on miRNA microarrays (data not shown). Molar concentrations to be spiked-in were derived empirically to produce Ct values comparable to those of moderately abundant miRNAs (e.g., *miR-16*) measured in blood from human plasma from normal healthy donors (Table S9). Synthetic versions of the *C. elegans* miRNAs were spiked into plasma or serum after the addition of an equal volume of 2 \times denaturing solution (to inhibit RNases) to the samples. Full details of the use of these controls to normalize qRT-PCR results across independent RNA isolations are provided in *SI Text*.

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