Pinpointing RNA-protein crosslinks with site-specific stable isotope labeled oligonucleotides

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SUPPORTING INFORMATION

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Figure S1. Isotopic exchange of $^{18}$O label on uridine 3'(2')-monophosphate with bulk solvent under acidic conditions. The oligonucleotide preE-let-7f was isotope labeled at U11 and hydrolyzed in 50% (v/v) formic acid, observed by ESI-LC-MS. Isotope ratio of $^{16}$O:$^{18}$O vs. digest time at 80 °C (blue) and 60 °C (red) temperatures are shown. The fit lines correspond to a simple irreversible exchange model, where $^{18}$O-UMP is converted to $^{16}$O-UMP at a rate $k_{ex}$ during hydrolysis of the starting material. In this case, $k_{ex} = 1.1 \text{ h}^{-1}$ at 80 °C and 0.28 h$^{-1}$ at 60 °C.
Figure S2. Isotopic distributions of peptide cross-linked di- and trinucleotides. Tryptic peptide ions arising from U11 (red) and U12 (blue) $^{18}$O labeled RNA-protein complexes, consistent with the peptide MGFGFLSMTAR cross-linked to the compositionally-defined ribonucleotides (a) AU, (b) AUU, and (c) GAU.
Figure S3. Enzymatic digest of preE-let-7 RNA isotope labeled at U11. The isotope distribution of mononucleotides in the mass region of uridine monophosphate (UMP) were examined by negative mode ESI-MS following digestion of 90 pmol with 1U nuclease P1 for 2 h at 42 °C. The survey spectrum shows the overlapping isotopic distributions of cytidine monophosphate (CMP, measured \([M-H]^− = 322.0446 \text{ m/z}\)) and UMP (measured \([M-H]^− = 323.0296 \text{ m/z}\)), demonstrating an enrichment of the UMP + 2 Da isotope (measured \([M-H]^− = 325.0337 \text{ m/z}\)). Relative isotope ratios (normalized to 323 m/z) are shown before correction for natural abundances, and the measured stoichiometry is shown after correction. The calculated isotope distribution of CMP and UMP from digesting the U11-labeled sequence GGGGUAGUGAU\(^{11}\)UUUACCCUGGAGAU is shown (red boxes). The expected stoichiometry of \(^{16}\text{O}-\text{UMP}:^{18}\text{O}-\text{UMP}\) is 7.84:1, given that the sequence has 8 uridines, one of which is labeled with \(\sim 90%\) \(^{18}\text{O}\) enrichment. The observed \(^{16}\text{O}-\text{UMP}:^{18}\text{O}-\text{UMP}\) stoichiometry after correcting for natural abundances is 7.90:1, indicating negligible exchange of the mass label with bulk solvent. The natural abundance correction was performed by calculating integrated intensities, \(I\), for each species as: 

\[ I_{\text{CMP}} = I_{322\text{m/z}}, \quad I_{^{16}\text{O}-\text{UMP}} = I_{323\text{m/z}} - 0.1128 \times I_{\text{CMP}}, \quad \text{and} \quad I_{^{18}\text{O}-\text{UMP}} = I_{325\text{m/z}} - 0.0239 \times I_{^{16}\text{O}-\text{UMP}} - 0.002 \times I_{\text{CMP}}. \]
Supplementary Methods

Synthesis of phosphodiester isotope labeled oligonucleotides. Solid phase oligonucleotide synthesis was performed using the phosphoramidite method and typical procedures on an automated synthesizer (Expedite 8909). Positions were mass labeled using an oxidation step that draws from the AUX line, typically used for phosphorothioate synthesis. Oxidation for unlabeled positions used typical I$_2$/THF/pyridine/water oxidation mix (Glen Research), whereas labeled positions were oxidized using a fresh preparation of “heavy oxidation mix” containing 20 mM I$_2$ and 2:78:20 (v:v:v) H$_2^{18}$O:THF:pyridine, starting from 97% enriched H$_2^{18}$O water (Cambridge Isotopes). The AUX line was thoroughly washed with anhydrous acetonitrile and dried with nitrogen prior to priming with the heavy oxidation mix. This mix was used for oxidation only to generate isotopically enriched nucleotide positions carrying a 3’ phosphodiester labeled with $^{18}$O, whereas all other positions were oxidized with manufacturer-supplied oxidation mix (Glen Research) that is formulated with natural abundance water and 20 mM I$_2$. RNA phosphoramidites (Chemgenes or Glen Research) were used for synthesis and deprotected based on the manufacturer’s guidelines. Briefly, columns were treated in 1:1 (v:v) aqueous ammonium hydroxide:methylamine (AMA, mixed in equal parts from 30% aqueous ammonium hydroxide, 40% aqueous methylamine) at 65 °C for 10 min, followed by drying under a stream of nitrogen. Desilylation was performed in DMSO with 1:3 HF:TEA for 2.5 h at 65°C and quenched in Tris buffer (Glen Research). Crude oligonucleotides were purified by the DMT-on method using C18 cartridges with on-column detritylation in aqueous 2% TFA, as per the cartridge manufacturer’s protocol (Glen Research), followed by HPLC purification.

Oligonucleotide UV cross-linking to LIN28A. Recombinant protein preparation of the loop-minimized Lin28ΔΔ variant of Lin28A and purification from E. coli has been previously described$^1$. Complexes of Lin28ΔΔ and preE-let-7f were crosslinked in 20 mM Bis-Tris, pH 7, 100 mM NaCl, 50 μM ZnCl$_2$, and 1 mM DTT by 254 nm irradiation in three pulses of 300 mJ/cm$^2$ in a fluorescent bulb crosslinker (Stratagene 1800). Crosslinked complexes were enriched by denaturing PAGE and electroelution, followed by denaturation in 8 M urea, 50 mM Bis-Tris, pH 7. Protein was digested enzymatically with Trypsin/Lys-C (Promega) added to a final ratio of 1:25 (w/w) at 37 °C for 3 h, followed by dilution to <1 M urea and overnight incubation at 37 °C. Modified RNAs were enriched by anion exchange chromatography on diethylaminoethyl resin in
20 mM Bis-Tris, pH 6, 10% glycerol and 5 mM DTT using a linear gradient from 0 – 2 M NaCl. Eluted fractions containing RNA were pooled, flash frozen, and lyophilized. Crosslinked tryptic peptide-RNA conjugates were prepared for MS analysis by hydrolysis in 50% (v/v) formic acid (FA) at 60 °C for 2 h, flash frozen, and lyophilized to dryness. Shorter digests tend to enrich for dimer and trimer species, and it may often be preferable to use a 30 min digest at 60 °C to observe these species in greater abundance. Dried samples were resuspended in LC-MS grade water for analysis.

**LC-MS analysis.** Samples were separated and analyzed on an Agilent 1200 HPLC coupled to an Agilent 6520 accurate-mass Q-TOF equipped with a dual ESI source, solvent degasser, auto sampler, diode array detector and column oven. RNA oligonucleotide characterization was performed in negative mode with ion pairing reverse phase chromatography. RNA sequencing was performed by digesting 45 pmol of purified oligonucleotides for 5 min at 40 °C in 50% (v/v) FA/water, followed by flash freezing, lyophilization, resuspension in water, and LC-MS analysis. On-line separations were performed with aqueous mobile phase (A) as 200 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) with 1.25 mM triethylamine (TEA) at pH 7.0 and organic mobile phase (B) as methanol across a 100 mm × 1 mm i.d. Xbridge C18 column with a particle size of 3.5 μm (Waters). Cross-linked nucleotide-peptide conjugates were mass analyzed as recently described. Briefly, separations were performed using an Agilent ZORBAX SB-C18 column 1.0 mm i.d. × 150 mm length with 3.5 μm particle size with a solvent elution gradient from (A) water with 0.1 % formic acid to (B) acetonitrile with 0.1 % formic acid. The flow rate was 0.1 mL/min, and all separations were performed with the column maintained at 40 °C. Mass analysis was performed in positive mode, and tandem mass analysis was performed by collision induced dissociation (CID) using nitrogen as the collision gas and a 4 m/z isolation width.

**Characterization of phosphodiester mass label exchange during hydrolysis**

Total RNA digests for isotope exchange studies were generated either by enzymatic or acid hydrolysis. To examine 

\[ ^{18}\text{O} \] isotope exchange when the hydrolytic product carries a 3’ or 2’ monophosphate, U11-labeled RNA was hydrolyzed in 50% (v/v) formic acid at either 60 °C or 80 °C in a thermocycler with a heated lid, and samples were taken at the indicated time points, frozen on dry ice, and lyophilized. In both cases, the isotope distribution of product nucleotides were analyzed by ESI-LC-MS using the ion pairing separation method described above. The exchange rate, \( k_X \), was estimated by fitting the observed ratio of \(^{16}\text{O}-\text{UMP} \) to \(^{18}\text{O}-\text{UMP}, l/h, over\)
time, where the observable \( l \) and \( h \) species (light and heavy, respectively) were estimated by considering hydrolysis and exchange of the UMP content of the starting material, \( L \) and \( H \) respectively, to be first-order irreversible processes under these conditions. We considered an approximated reaction scheme,

\[
(1) \quad H \xrightarrow{k} h \xleftarrow{k_x} l \xleftarrow{k} L ,
\]

where \( k \) is the hydrolysis rate of both \( L \) and \( H \) starting material (neglecting any kinetic isotope effect) and \( h_0 \) and \( l_0 \) are the maximum observable counts of heavy and light monomer, respectively, within the starting material, which were constrained based on their known initial stoichiometry. We solved the system of first-order differential equations to obtain the time-dependent observable counts of \( l \) and \( h \), where we assumed no significant difference in ionization efficiency between them:

\[
(2) \quad h = \frac{k}{k_x - k} h_0 \left( e^{-kt} - e^{-k_x t} \right)
\]

\[
(3) \quad l = \frac{k}{k_x - k} h_0 e^{-k_x t} - \left( \frac{k_x}{k_x - k} h_0 + l_0 \right) e^{-kt} + h_0 + l_0 .
\]

Alternatively, to examine \(^{18}\text{O}\) isotope exchange with bulk solvent when the product nucleotide carries the labeled monophosphate in the ribose 5’ position, the preE-let-7 RNA isotope labeled at U11 (90 pmol) was digested enzymatically with 1 U nuclease P1 from \( P. \) citrinum (USBio) for 2 h at 42 \(^\circ\)C in the enzyme’s diluted storage buffer alone (3 mM NaOAc, pH 5.3, 0.5 mM ZnCl\(_2\), and 5 mM NaCl).

References

(1) Nam, Y.; Chen, C.; Gregory, R. I.; Chou, J. J.; Sliz, P. Cell 2011, 147, 1080.