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Supporting Information for

A Live-Cell Assay for the Detection of pre-microRNA-Protein Interactions

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A. General Materials and Methods

General cell culture methods. Flp-In™-293 cells stably expressing either SmBiT-HaloTag or SmBiT-HaloTag-NLS were grown in DMEM (Corning cat #10-017-CV) supplemented with 10% FBS (Atlanta Biologicals S11550), L-glutamine (Gibco cat #25030081), and hygromycin B (100 µg/mL) (Gibco cat #10687010) at 37 °C with 5% CO_2 in a humidified incubator, passaged at least once before use for an experiment. Cells were passaged using Trypsin-EDTA (0.25%) (Gibco cat #25300054) approximately 10 times, and no more than 15 times, before returning to low passage stocks. To count cells, cells were harvested and 10µL of the cell suspension was mixed with 10µL Trypan Blue (Gibco cat #15250061) ([final] = 0.2% trypan blue) and counted using a hemocytometer.

General assay and data analysis methods. Chemiluminescence data was collected on a BioTek Cytation3 plate reader. All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com). All normalized chemiluminescence is reported as the signal of each well divided by the average signal of quadruplicate pre-miR-21 wells. The only exception is Fig. 2C, in which signal is normalized by dividing by the average of quadruplicate wells containing no unlabeled probe and multiplying by 100.

Statistical analysis. All statistical tests were performed using Prism (v8). One- or two-way ANOVA tests were run for each set of data. Details of multiple comparisons tests are included in table legends. Graphs show mean ± standard deviation.

Materials. Chemically synthesized pre-microRNAs (deprotected, desalted and HPLC purified), containing aminoallyl uridine or aminohexylacrylamino uridine modifications and biotin attached to the 5'-end of the sequence by an 18-atom spacer, were purchased from Dharmacon and used as received for the labeling reaction. HaloTag Succinimidyl Ester (O2) and (O4) Ligands were purchased from Promega and used as received (cat #1691 and #P6751). HaloTag Succinimidyl Ester containing PEG6 linker was synthesized following previously published protocol.[1] Note that the HaloTag Succinimidyl Ester Ligands should be dissolved and immediately portioned into single use aliquots stored at -80 °C to avoid degradation. Flp-In™-293 cells and associated vectors were purchased from ThermoFisher Scientific (Invitrogen cat #75007 and #601001, respectively). The Nano-Glo Live Cell Assay System was purchased from Promega and used as received (cat #N2012). HaloTag® TMR Ligand (Promega cat #G8251) and Lipofectamine™ RNAiMAX (Invitrogen cat #13778100) were used as received.

B. Cloning

SmBiT-HT cloning. SmBiT-HT was amplified from a pFC30K construct containing a N-terminal SmBiT and C-terminal HaloTag (HT) fusion protein and inserted into pcDNA5/FRT using standard PCR cloning techniques with KpnI and NotI restriction enzymes.

Primers:

SmBiT-HT
5' GTACGGTACCGCCACCATGGTGACCGGCTACCGG
5' CAGTGCGGCCGCTCACTATTAGTGGTGATGGTGATGATG

SmBiT-HT-NLS cloning. To insert a C-terminal SV40 NLS, the multiple cloning site of the pcDNA5/FRT + SmBiT-HT construct was modified to include a Blp restriction site using standard PCR cloning techniques with KpnI and NotI restriction enzymes.

Primers:

SmBiT-HT-Blp
5’ GTACGGTACCGCCACCATGGTGACCGGCTACCGG
5’ GACTGCGGCCGCTCATTAGCTCAGCCCACCGGAAATCTCCAGAGTAG

The SV40 NLS (5’ CCAAAGAAAAAGAGAAAGTA) was then appended to the C-terminus of SmBiT-HT by annealing oligos and inserting them into pcDNA5/FRT + SmBiT-HT-Blp digested with Blp restriction enzyme.

Oligos:

SmBiT-HT-Blp
5’ TGAGTGGAGGTGGTCCAAAGAAAAAGAGAAAAGTATGGC
5’ TCAGCCATACTTTTCTCTTTTTCTTTGGACCACCTCCAC

Lin28A-LgBiT cloning. Mouse Lin28A-LgBiT was amplified from pFN29K-Lin28A-LgBiT, previously cloned in the lab[2], and inserted into pcDNA3 using standard cloning techniques with KpnI and NotI restriction enzymes. Primers insert a Kozak sequence on the N-terminus.

Primers:

Lin28A-LgBiT
5’ GTACGGTACCGCCACCATGGCCTCGGTGTCCAACC
5’ CAGTGCGGCCGCTCATTAAACACTGTTGATGGTTACTCG

LgBiT-Lin28A cloning. First, a pcDNA3 construct with LgBiT at the N-terminal position was generated by amplifying LgBiT and insertion into pcDNA3 using standard cloning techniques with BamHI and XhoI restriction enzymes. Mouse Lin28A was amplified from the pcDNA3-Lin28a-LgBiT construct and ligated into a pcDNA3 construct with LgBiT inserted at the N-terminal position using standard cloning techniques with XhoI and XbaI restriction enzymes. Primers insert a Kozak sequence on the N-terminus.

Primers:

LgBiT
5’ GTAGGATCCGCCACCATGGTCTTCACACTC
5’ CATCTCGAGACTGTTGATGGTTACTC

Lin28A
5’ ATGCCTCGAGATGGGCTCGGTGTCCAACC
5’ CATCTAGAATTCTGGGCTTCTGGGAGCA

Lin28B-LgBiT cloning. Human Lin28B was amplified from pFC30K vector containing Lin28B and ligated into previously cloned pcDNA3 vector containing LgBiT inserted at the C-terminal position (see Lin28A-LgBiT cloning) using standard cloning techniques with KpnI and AsiSI restriction enzymes. Primers insert a Kozak sequence on the N-terminus.

Primers:

5’ ATGGTACCGCCACCATGGCCGAAGGCG
5’ ATGCGATCGCTGTCTTTTTCCT

CSD-LgBiT cloning. The cold shock domain (CSD) of mouse Lin28A was amplified from pcDNA3 vector containing Lin28A-LgBiT and ligated into previously cloned pcDNA3 vector containing LgBiT

inserted at the C-terminal position (see Lin28A-LgBiT cloning) using standard cloning techniques with KpnI and AsiSI restriction enzymes. Primers insert a Kozak sequence on the N-terminus.

Primers:

5’ ATGGTACCGCCACCATGGCAGCGGAGAAGGCG
5’ TTGCGATCGCTCCTTTGGATCTTCG

ZKD-LgBiT cloning. The zinc knuckle domain (ZKD) of mouse Lin28A was amplified from pcDNA3 vector containing Lin28A-LgBiT and ligated into previously cloned pcDNA3 vector containing LgBiT inserted at the C-terminal position (see Lin28A-LgBiT cloning) using standard cloning techniques with KpnI and AsiSI restriction enzymes. Primers insert a Kozak sequence on the N-terminus.

Primers:

5’ ATGGTACCGCCACCATGAGTGAGCGGCGGCCAA
5’ AAGCGATCGCGCCCTGCTGGGCCT

C. Bioconjugation Methods

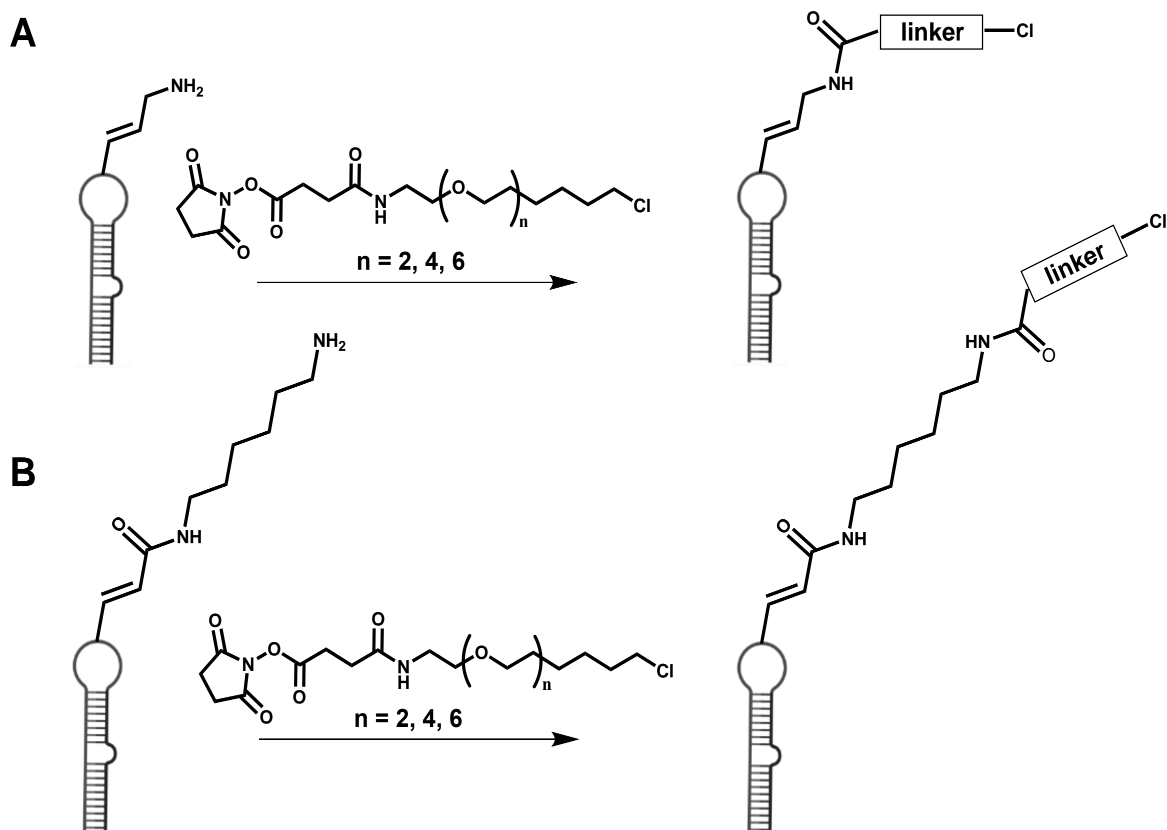

Figure S1. RNA labelling scheme with (A) 5-aminoallyl uridine and (B) 5-aminohexylacrylamino uridine modifications.

Protocol. Amino-modified pre-miRNA (1.0 mM in 100 mM phosphate buffer, pH 8.0) was mixed with an equivalent volume of HaloTag ligand (10 mM in DMSO for O2 and O4; 20 mM in DMSO for O6). The reaction was then allowed to proceed at 25 °C for 1 h. pre-miRNA-Cl was precipitated by the addition of 0.11× volume of 3.0 M sodium acetate (pH 5.2) and 4 volume equivalents of cold ethanol, and pelleted at 20,000 × g for 40 min at 4 °C. The pellet was then re-suspended in 100 mM phosphate buffer (pH 8.0) at a concentration of 1.0 mM and stored at -80 °C.

Table S1. Sequence and modifications of terminal loop-labeled pre-microRNA probes. See Fig. S6C and Fig. S6F for effect of biotinylation on signal in RiPCA.

Sequence	5' modification	Sequence (CSD binding site bolded, ZKD binding site underlined, site of modified U bolded and underlined)	Length	Uridine modification
pre-miR-21	Biotin (18-atom spacer)*	UAGCUUAUCAGACUGAUGUUGACUGUUGAA **U**CUCAUGGCAACACCAGUCGAUGGGCUGUC	61	5NU
pre-miR-34a	Biotin (18-atom spacer)*	UGGCAGUGUCUUAGCUGGUUGUUGUGAGCAA **U**AGUAAGGAAGCAAUCAGCAAGUAUACUGCCCUA	65	5NU
pre-let-7a-1	Biotin (18-atom spacer)*	UGAGGUAGUAGGUUGUAUAGUUUUAGGG**U**CACACCCACCACUGGGAGAUAACUAUACAAUCUACUGUCUUUCU	73	5LCNU
pre-let-7d	Biotin (18-atom spacer)*	AGAGGUAGUAGGUUGCAUAGUUUUAGGGCAG **GGAU**UUUGCCCACAAGGAGGUAACUAUACGACCUGCUGCCUUUCU	76	5LCNU
pre-let-7g	Biotin (18-atom spacer)*	UGAGGUAGUAGUUUGUACAGUUUGAGGGUCUA **UGAU**ACCACCCGGUACAGGAGAUAACUGUACAGGCCACUGCCUUGCU	79	5LCNU

*hexaethylene glycol, Dharmacon

Table S2. Sequence and modifications of 5'-labeled pre-microRNA probes.

Sequence	5' modification	Sequence (CSD binding site bolded, ZKD binding site underlined)	Length	Uridine modification
pre-miR-21	5' amine	UAGCUUAUCAGACUGAUGUUGACUGUUGAA UCUCAUGGCAACACCAGUCGAUGGGCUGUC	61	none
pre-let-7d	5' amine	AGAGGUAGUAGGUUGCAUAGUUUUAGGGCAG **GGAU**UUUGCCCACAAGGAGGUAACUAUACGACCUGCUGCCUUUCU	76	none

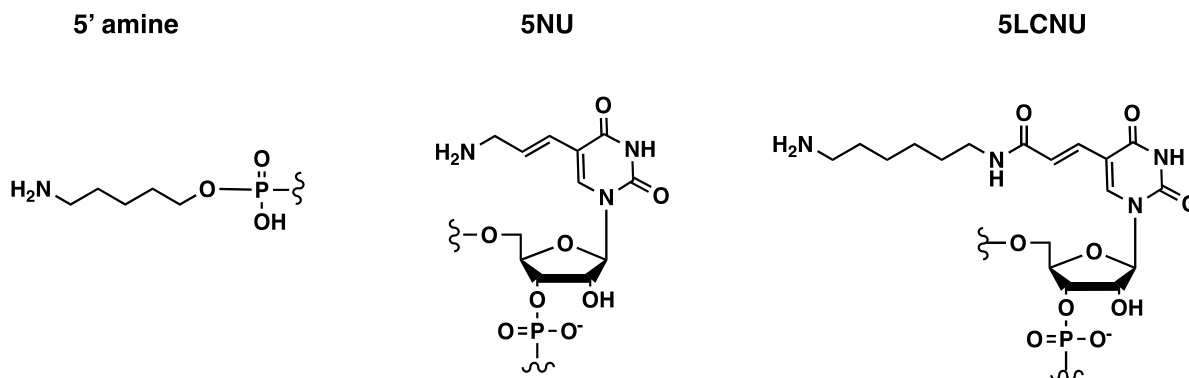

Figure S2. Structures of amino modifications.

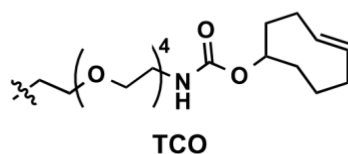

Figure S3. Structure of trans-cyclooctene conjugated to RNA probe in Fig. 2A.

D. SmBit-HT Stable Cell Lines. Flp-In-293 cells stably expressing a SmBiT-HT were generated by co-transfecting Flp-In-293 cells with 9 ng pOG44 and 1 ng pcDNA5/FRT using Lipofectamine LTX+ Plus reagent (Life Technologies) according to the manufacturer's instructions. Expression and localization of SmBiT-HaloTag or SmBiT-HaloTag-NLS were confirmed by Western blot and confocal microscopy.

E. RiPCA Protocol

General protocol. Flp-In-293 cells stably expressing a SmBiT-HT protein were reverse transfected using Lipofectamine™ RNAiMAX Transfection Reagent. Cells were passaged approximately 10 times, and no more than 15 times, before returning to low passage stocks. To test "n" number of conditions, Solution A was prepared by combining 50 × (n+1) µL of room temperature Opti-MEM and 2.4 × (n+1) µL plasmid encoding selected RBP-LgBiT fusion. Solution B was prepared by adding pre-miRNA-Cl and plasmid (final concentrations 0.3 µM and 0.195 ng/µL, respectively) to 50 µL Opti-MEM™ for each separate condition to be tested. Solution B was mixed with 50 µL of Solution A to yield Solution A+B, which was incubated for at least 15 min at room temperature while cells were harvested. Cells were harvested as and counted as described above. Harvested cells were used to prepare Solution C, which was composed of 400 µL of 100,000 cells/mL. Solution C was mixed with 50 µL of Solution A+B and plated 100 µL per well, four wells per condition, in a white-bottom, tissue culture-treated 96-well plate (Corning cat #3917). The plate was incubated in a humidified incubator (37 °C and 5% CO_2) for 24 h. After incubation, the media was removed and replaced with 100 µL room temperature Opti-MEM™ and treated with 25 µL NanoGlo Live Cell Reagent diluted 1:20 according to the manufacturer's recommendation. All chemiluminescence data was collected immediately on a BioTek Cytation3 plate reader.

Representative calculations based on an assay for n = 5 conditions:

<u>Solution A: Prepared for n+1= 6</u>
6 x 50 µL → 300 µL OptiMEM™
6 x 2.4 µL → 14.4 µL Lipofectamine™ RNAiMAX

<u>Solution B:</u>
50 µL OptiMEM™
2.5 µL 3.9 ng/µL RBP-LgBiT plasmid
0.3 µL 50 µM pre-miRNA-Cl

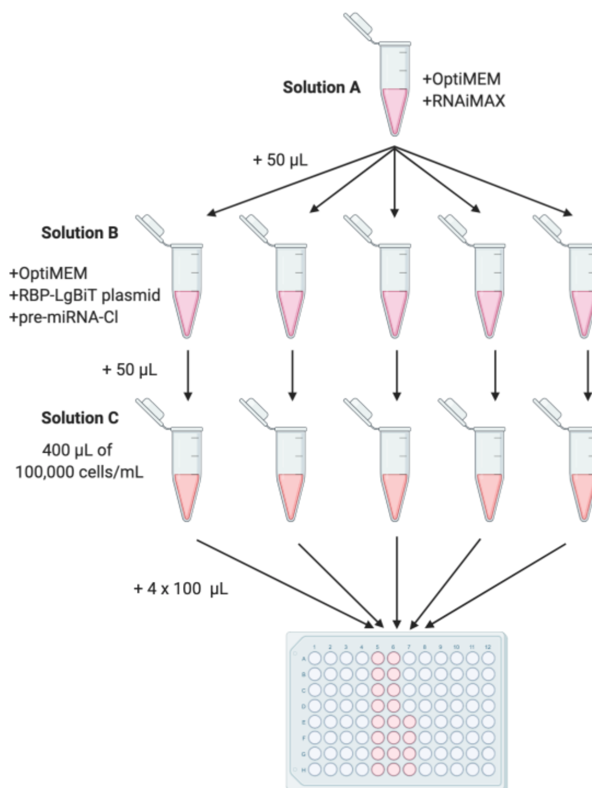

Figure S4. RiPCA transfection workflow.

DNA and RNA titration. To alter the amount of DNA transfected per well, higher concentration stocks of plasmid were used to allow addition of the same volume to each condition (final concentrations increased from 0.195 ng/μL to 0.39 ng/μL and 19.5 ng/μL). To alter the amount of RNA transfected per well, increasing volumes of 50 μM stock solution of pre-miRNA-Cl were used. The amount of Lipofectamine™ RNAiMAX was adjusted accordingly (from 2.4 × (n+1) μL for 16.7 nM/well to 1.2× (n+1) μL for 8.3 nM/well and 3.6 × (n+1) μL for 25 nM/well).

Competition with unlabeled RNA. In competition experiments, the general RiPCA protocol was followed with the following change. In addition to the DNA and RNA added to Solution B, varying amounts of unlabeled pre-miRNA were added to Solution B (0, 0.15, 0.225, 0.3, or 0.6 uL unlabeled probe).

F. Confocal Microscopy

Protocol. Flp-In cells stably expressing SmBiT-HT or SmBiT-HT-NLS were harvested and counted using methods described above. Cells were diluted to a density of 100,000 cells/mL and 200 μL was plated in an 8-well chambered coverglass (Nunc™ Lab-Tek™ II). The chambered coverglass was incubated in a tissue culture incubator (37 °C and 5% CO_2) for 24 h to allow the cells to adhere to the glass. To stain live cells, the media was supplemented with a single stain or a combination of stains at final concentrations of 50 nM HaloTag® TMR Ligand (Promega cat #G8251), 0.44 μM Hoescht 33342 (Fisher), and 0.2 μM MitoTracker Green FM (Cell Signaling Technology). The chambered coverglass was returned to the incubator for 30 min. The media was then removed and replaced with 200 μL Opti-MEM ™. Fluorescence was visualized using Nikon A1SI Confocal microscope. Images were processed with NIS-Elements.

G. Supplemental Figures

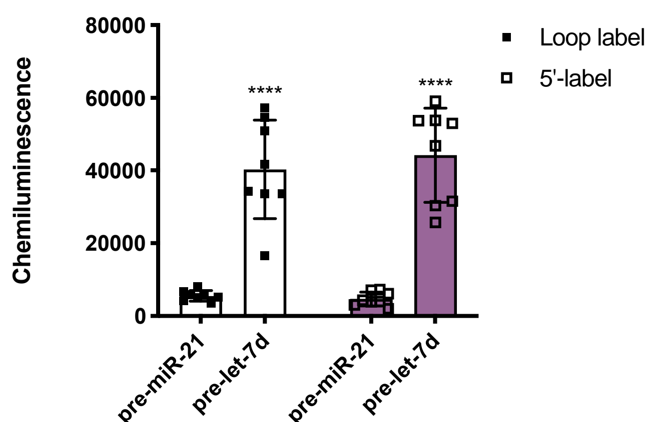

Figure S5. Chemiluminescence signal detection of interaction between Lin28A-LgBiT and 5'- or loop-labeled pre-miR-21 and pre-let-7d. See Table S2 for p values.

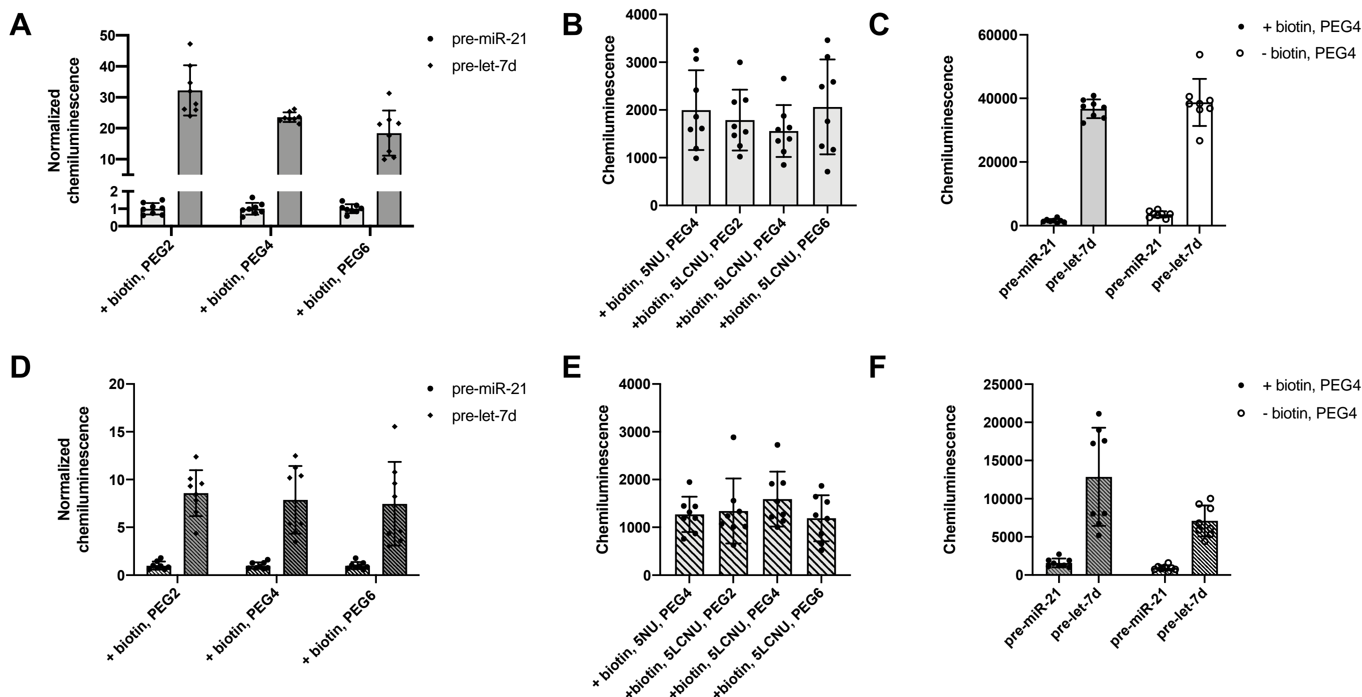

Figure S6. (A and D) Chemiluminescence signal detection of interaction between Lin28A-LgBiT and biotinylated pre-miR-21 and pre-let-7d probes conjugated to HaloTag succinimidyl ester ligand containing PEG2, PEG4, or PEG6 linkers in cytoplasmic and nuc-RiPCA, respectively. Signal was normalized to pre-miR-21 in each case. (B and E) Differences in pre-miR-21 linker length and modification do not significantly change signal produced in RiPCA with Lin28A-LgBiT linkers in cytoplasmic and nuc-RiPCA, respectively. (C and F) Effect of 5' biotinylation of the RNA probes on signal/background in cytoplasmic and nuclear RiPCA, respectively.

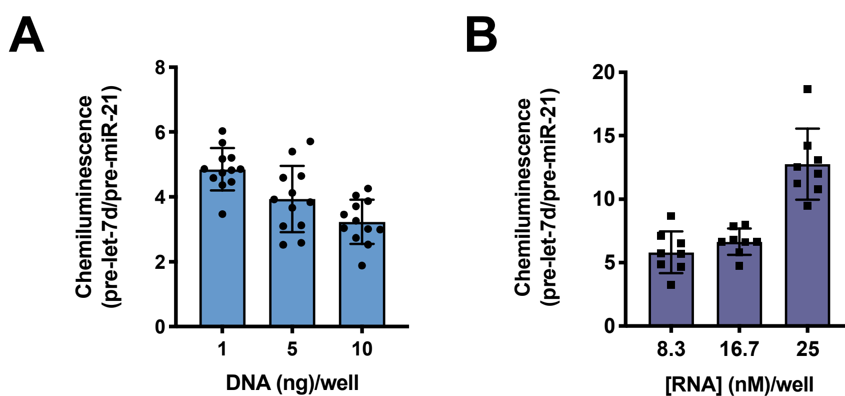

Figure S7. (A) Dependence of S/B on the amount of Lin28A-LgBiT plasmid and (B) pre-miRNA-Cl transfected in SmHT-expressing cells. Normalized chemiluminescence is reported as pre-let-7d/pre-miR-21.

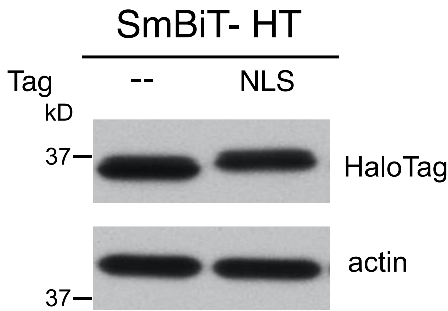

Figure S8. Expression of SmBiT-HT and SmBiT-HT-NLS constructs in stably expressing Flp-In 293 cell lines.

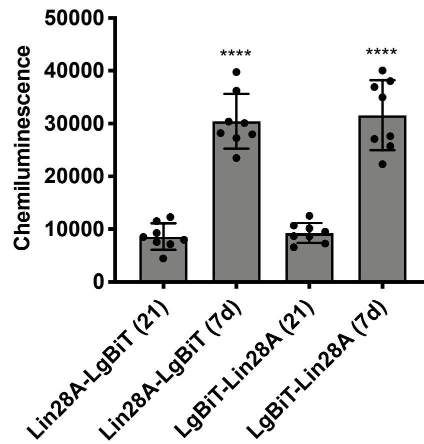

Figure S9. (A) Signal produced by SmHT-NLS-expressing cells co-transfected with pre-miR-21 or pre-let-7d and Lin28A tagged with LgBiT at the C- or N-terminus. Statistical significance determined by one-way ANOVA Sidak's multiple comparison test (n = 8); p <0.0001.

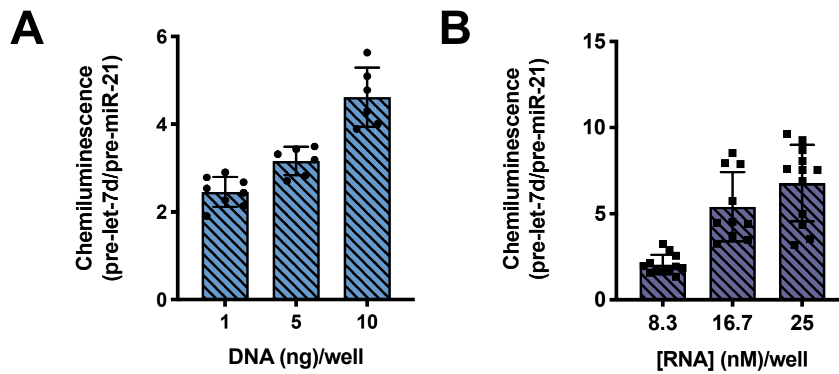

Figure S10. (A) Dependence of S/B on the amount of Lin28A-LgBiT plasmid and (B) pre-miRNA-Cl transfected in SmHT-cNLS-expressing cells. Normalized chemiluminescence is reported as pre-let-7d/pre-miR-21.

H. Supplemental Tables

Table S3. Statistical significance associated with **Fig. 2A** between chemiluminescence produced by cells co-transfected with chloroalkane-labeled pre-let-7d and Lin28A-LgBiT or LgBiT-Lin28A and cells co-transfected with either a LgBiT or TCO-labeled pre-let-7d control (ns = not significant). Statistical significance determined by one-way ANOVA Sidak's multiple comparison test (n = 8).

Construct	LgBiT control	TCO control
Lin28A-LgBiT	<0.0001 (****)	<0.0001 (****)
LgBiT-Lin28A	<0.0001 (****)	<0.0001 (****)

Table S4. Statistical significance associated with **Fig. 2B and Fig. S5** between chemiluminescence produced by cells transfected with pre-miR-21 and pre-let-7d with chloroalkane labels in either the loop or at the 5'-end of the probe. Statistical significance determined by two-way ANOVA Sidak's multiple comparison test (n = 8).

Sequence		Statistical significance
#1	#2	
pre-miR-21 (5' label)	**pre-let-7d (5' label)**	<0.0001 (****)
pre-miR-21 (loop label)	**pre-let-7d (loop label)**	<0.0001 (****)
pre-miR-21 (5' label)	**pre-miR-21 (loop label)**	>0.9999 (ns)
pre-let-7d (5' label)	**pre-let-7d (loop label)**	0.3927 (ns)

Table S5. Statistical significance associated with **Fig. 2C** between chemiluminescence produced by cells transfected with chloroalkane-labeled pre-miR-21 and pre-let-7d sequences and cells transfected with varying amounts of unlabeled pre-miR-21 and pre-let-7d in cytoplasmic RiPCA (ns = not significant). Statistical significance determined by one-way ANOVA Dunnett's multiple comparison test (n = 8, except 12.5, for which n = 4). One data point from the pre-let-7d [33.4] data set was identified as an outlier using the ROUT method where Q = 1% (Prism) and eliminated from the data set and statistical analysis.

[Unlabeled RNA] (nM)	pre-miR-21	pre-let-7d
8.3	0.2845 (ns)	0.5244 (ns)
12.5	0.9738 (ns)	>0.9999 (ns)
16.7	0.9554 (ns)	0.3660 (ns)
33.4	0.9690 (ns)	0.0004 (***)

Table S6. Statistical significance associated with **Fig. 3B** between signal-to-background (S/B) produced by various pre-miRNA-Cl sequences and pre-miR-21 with each LgBiT fusion in cytoplasmic RiPCA (ns = not significant). Statistical significance determined by two-way ANOVA Tukey's multiple comparison test (n = 8).

Sequence	Lin28A	CSD	ZKD
pre-miR-34a	0.7456 (ns)	>0.9999 (ns)	0.9922 (ns)
pre-let-7a-1	0.1464 (ns)	0.8381 (ns)	0.5962 (ns)
pre-let-7d	<0.0001 (****)	<0.0001 (****)	0.0152 (*)
pre-let-7g	<0.0001 (****)	0.2826 (ns)	0.4645 (ns)

Table S7. Statistical significance associated with **Fig. 4** between signal-to-background (S/B) produced by various pre-miRNA-Cl sequences and pre-miR-21 with Lin28B-LgBiT in cytoplasmic RiPCA (ns = not significant). Statistical significance determined by one-way ANOVA Sidak's multiple comparison test (n = 8).

Sequence	Lin28B
pre-miR-34a	0.8874 (ns)
pre-let-7a-1	0.9929 (ns)
pre-let-7d	<0.0001 (****)
pre-let-7g	<0.0001 (****)

Table S8. Statistical significance associated with **Fig. 6** between signal-to-background (S/B) produced by various pre-miRNA-Cl sequences and pre-miR-21 with Lin28A- or Lin28B-LgBiT fusion in nuc-RiPCA (ns = not significant). Statistical significance determined by two-way ANOVA Tukey's multiple comparison test (n = 8).

Sequence	Lin28A	Lin28B
pre-miR-34a	0.8330 (ns)	0.9073 (ns)
pre-let-7a-1	0.9970 (ns)	0.9995 (ns)
pre-let-7d	<0.0001 (****)	<0.0001 (****)
pre-let-7g	<0.0001 (****)	<0.0001 (****)

I. References

1. Song, J. M.; Menon, A.; Mitchell, D. C.; Johnson, O. T.; Garner, A. L., High-Throughput Chemical Probing of Full-Length Protein-Protein Interactions. *ACS Comb Sci* **2017,** *19* (12), 763-769.
2. Sherman, E. J.; Lorenz, D. A.; Garner, A. L., Click Chemistry-Mediated Complementation Assay for RNA-Protein Interactions. *ACS Comb Sci* **2019,** *21* (7), 522-527.