MS ID#: RNA/2010/024885

Supplemental Materials and Methods, Figures, and Tables for "Precursor miR-886, a novel non-coding RNA repressed in cancer, associates with PKR and modulates its activity."

Supplemental Materials and Methods

Analysis by nano-LC-MS/MS

Eluted proteins from the immobilized pre-miR-886 were concentrated by precipitation with Trichloroacetate and were separated by NuPAGE® 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). After separation, the gel was stained with GelCode® Blue Stain Reagent (Thermo scientific, Rockford, IL). In-gel digestion was carried out with 12.5 ng/μl sequencing grade modified trypsin (Promega, Madison, WI) in 50mM NH_4HCO_3 buffer (pH 7.8) at 37°C for overnight. Produced tryptic peptides were extracted with 5% formic acid in 50% acetonitrile solution at room temperature for 20 min. The supernatants were collected and dried by SpeedVac. The samples were purified and concentrated in 0.1% formic acid using C18 ZipTips (Millipore, MA) before MS analysis.

The tryptic peptides were loaded onto a fused silica microcapillary column (12 cm x 75 μm) packed with C18 reversed phase resin (5 μm, 200 Å). Peptide separation was conducted with a series of step gradients composed of initial isobaric flow for 5 min with 3%

solvent B (0.1% formic acid in acetonitrile), then linear gradient from 3% to 35% for 30 min, followed by linear gradient to 40% for 5 min. At the end of each running, 90% of solvent B was eluted for 10 min with the flow rate of 250 nL/min. The % gradient of solvent B was against solvent A (0.1% formic acid in H_2O).

The column was directly connected to LTQ linear ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nano-electrospray ion source. Each full MS scan was followed by five MS/MS scan corresponding from the most intense to the fifth intense peaks of full MS scan. Repeat count of peaks for dynamic exclusion was 1, and its repeat duration was 30s. The dynamic exclusion duration was set for 180s and width of exclusion mass was ± 1.5 Da. The list size of dynamic exclusion was 50.

Database search and validation

The acquired LC-ESI-MS/MS fragment spectra were searched in the BioWorksBrowser™ (version Rev. 3.3.1 SP1, Thermo Fisher Scientific Inc., CA) with the SEQUEST search engine against the non-redundant human database in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The search conditions were trypsin enzyme specificity, a permissible level for two missed cleavages, peptide tolerance of ±2 amu, a mass error of ±1 amu on fragment ions and fixed modifications of carbamidomethylation of cysteine (+57 Da) and oxidation of methionine (+16 Da) residues.

Supplemental Figure and Table Legends

Figure S1. Expression of pre-miR-886 and vtRNA1-1 in various cell lines

Northern hybridization of pre-miR-886, vtRNA1-1, and tRNA-Ala in the immortalized lung cell line CRL2741 and the indicated cancer cell lines: T47D (breast), MCF-7 (breast), MDA-MB-231 (breast), MDA-MB435 (breast or melanoma), HeLa (cervix), C33A (cervix), and HCT116 (colon). The leftmost lane is the cytoplasmic RNA of CRL2741 and the others are total cellular RNA.

Figure S2. The stem-loop hairpin structure and Northern hybridization of pre-miR-886 is atypical

A. Northern hybridization of miR-886-5p (left panel) and -886-3p (right panel) from the indicated cell lines. All other descriptions are the same as in Fig 1D and 2E.

B. Northern hybridization of miR-205 as an example of a typical miRNA. All other descriptions are the same as in Fig 1D.

C. The stem-loop structures of miR-886 and -205 (adapted from the miRbase: http://www.mirbase.org/). The mature miRNA portions are in pink. The structure of pre-miR-886 was reclaimed from Fig 1A for direct comparison to that of miR-205.

Figure S3. Schematic diagram of 5'- and 3'-RACE

A. Schematic of 5'-RACE. Thick solid line, wavy line, and arrows indicate the 5'-linker oligoribonucleotide, pre-miR-886 (or precursor tRNA-Ala), and PCR primers (sequences in Supplemental Table 2), respectively.

B. Schematic of 3'-RACE. All descriptions are the same as for panel A, unless otherwise mentioned below. The 3'-RACE protocol was adapted and modified from the original protocol of small RNA cloning (Lau et al. 2001). A 3'-linker oligonucleotide (5'-p-CTGTAGGCACCATCAATx-3') was ligated to object RNA by T4 RNA Ligase 1 (New England Biolabs Inc.). The 3'-linker was 5'-phosphorylated and 3'-blocked (by x = DMT-O-C3 –CPG), and so could be ligated only to the 3'-OH residue of the object RNA. cDNA synthesis was done using a primer specific to the 3'-linker ("mp3-RACE-RT" in Supplemental Table 2).

Figure S4. Pre-miR-886 is inefficiently processed to mature miRNAs, albeit degraded by Dicer.

A. ^{32}P-labeled, *in vitro* transcribed pre-miR-886 and pre-miR-23a were prepared and transfected into 293T cells. Total RNA was prepared 24 hrs after transfection and subjected to 15% denaturing polyacrylamide gel electrophoresis. EtBr staining is shown for equal loading. The blot was overexposed to visualize mature miRNAs derived from the transfected synthetic

pre-miRNAs (bottom panel). Each signal was quantified as described in Fig 3D (numbers at the bottom).

B and C. Pre-miR-886 measurement by Northern (panel B) and qRT-PCR measurement of Dicer mRNA (panel C), after suppression of Dicer by siRNAs (Stealth RNAi™ siRNAs purchased from Invitrogen). siRNA treatment was done as described in Fig 3D with siGFP as a negative control duplex, quantitation of pre-miR-886 was done as Fig 2E, and qRT-PCR data were plotted as in Fig 1E.

Figure S5. Pre-miR-886 does not generate functional mature miR-886-5p and -886-3p

A. A heatmap view of hierarchical clustering of miRNA target genes in the probe sets with log fold change > 1.5, from our mRNA array data (HGU133 Plus 2.0) of CRL2741 and H1299 cells. Targets of miR-886-5p and -886-3p were predicted using the miBridge method (Lee et al. 2009), including G:U matches in the 5'UTR interaction due to the small number of targets. When hypergeometric distribution p-values were calculated, none of the miR-886-5p or -886-3p targets (either with or without G:U matches in the 5'UTR interaction) has p-value less than 0.1, indicating no significant target depletion.

B. Luciferase assays were performed as described in (Lee and Dutta 2007). pcDNA3.1-Zeo(+)-Pp was a derivative of pcDNA3.1-Zeo(+) (Invitrogen Corp.) harboring the open reading frame of firefly (*Photinus pyralis*) luciferase. A sequence perfectly complementary to

miR-886-3p and -886-5p was cloned downstream of the luciferase open reading frame, to construct miR-886-3p sensor and -886-5p sensor, respectively. Each sensor plasmid was co-transfected with pRL-SV40 (Promega) expressing Renilla luciferase, using Lipofectamine™ 2000 reagent (Invitrogen Corp.) per the manufacturer's instructions. Luciferase assays were performed 24 hrs after transfection of the plasmids, using a Dual-Luciferase® Reporter Assay System (Promega). Each value of the firefly luciferase (*Pp*) was first normalized to the Renilla luciferase value (*Rr*), and then the *Pp*/*Rr* value of the sensor plasmid was again normalized to that of the vector control (pcDNA3.1-Zeo(+)-Pp), yielding the relative luciferase activity of the sensor plasmid (y-axis).

If endogenous functional mature miR-886-5p (or -886-3p) existed, they would have repressed the luciferase activity from the sensor plasmid, and this repression would have been de-repressed by a synthetic inhibitor of the miRNA (Anti-miR™ miRNA inhibitor from Applied Biosystems/Ambion). Before sensor plasmid transfection into the indicated cell lines, they were transfected with the miRNA inhibitor against miR-886-5p or -886-3p (black bar) or a negative control inhibitor (plain bar, set as 1 in each cell line).

The relative luciferase activity of the miR-886-5p sensor was not increased by its inhibitor, indicating that there was no endogenous functional miR-886-5p in the cells. In the case of miR-886-3p, we observed higher sensor activity in cells treated with its inhibitor; however, we observed no difference between cell lines whether they expressed pre-miR-886 or not. We

cannot rule out the possibility that all of these cell lines express miR-886-3p at a level that is similar among them and undetectable by Northern, regardless of pre-miR-886. This situation is unlikely, and even if it were true, it was pre-miR-886, not miR-886-3p that was decreased in cancer (Fig 1D-F) and exhibited biological effects (Fig 5 and 6).

Figure S6. Subcellular fractionation and localization of pre-miR-886 and vtRNA1-1 in CRL2741 cells

A. Western blotting (top two panels) and Northern hybridization (bottom panels) after subcellular fractionation of CRL2741 cells. All other descriptions are the same as in Fig 4D.

B. *In situ* detection of vtRNA1-1 (green color), with DAPI staining (blue color) and ACTB RNA detection (red color). Probes are complementary to nt 26 – 69 of vtRNA1-1 (nt coordinates according to the sequence in Fig 4A) as a probe. All other descriptions are the same as Fig 2C.

Figure S7. Expression of pre-miR-886 and PKR exhibits anti-correlation in oral cell lines.

Western blotting of total PKR and phospho-PKR (top panel) and qRT-PCR of pre-miR-886 (bottom panel; reclaimed from Fig 1E for direct comparison with PKR) in the oral cell lines used in Fig 1E.

Supplemental Table 1. List of proteins that physically interact with pre-miR-886

The 49 proteins were tabulated according to the PANTHER (Protein ANalysis THrough Evolutionary Relationships: http://www.pantherdb.org/) protein classification.

Supplemental Table 2. Primers used in this study

Supplemental References

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294**(5543): 858-862.

Lee, I., Ajay, S.S., Yook, J.I., Kim, H.S., Hong, S.H., Kim, N.H., Dhanasekaran, S.M., Chinnaiyan, A.M., and Athey, B.D. 2009. New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res* **19**(7): 1175-1183.

Lee, Y.S. and Dutta, A. 2007. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* **21**(9): 1025-1030.

Fig S1

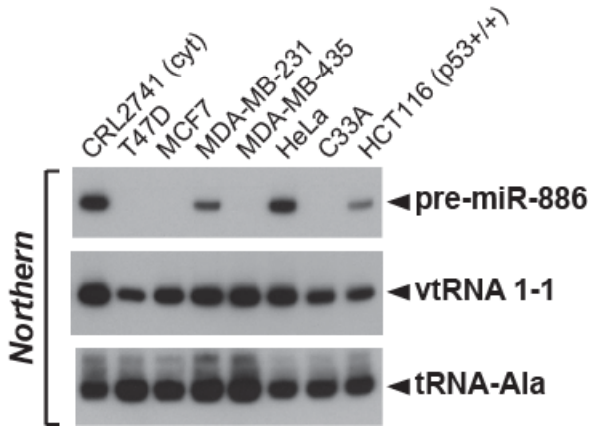
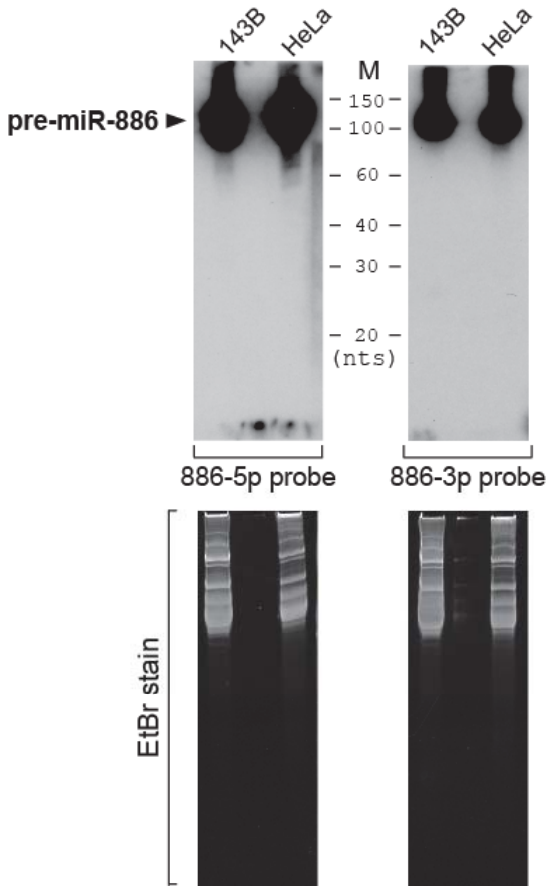
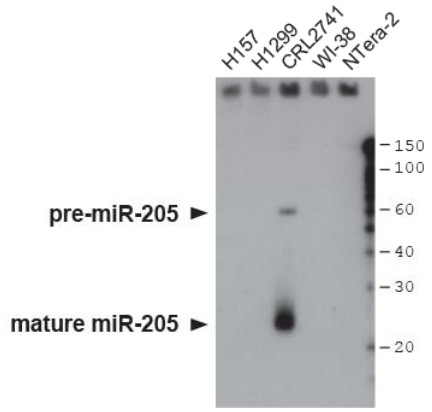

CRL2741 (cyt)
T47D
MCF7
MDA-MB-231
MDA-MB-435
HeLa
C33A
HCT116 (p53+/+)
Northern
pre-miR-886
vtRNA 1-1
tRNA-Ala

Fig S2

A



B



C

Homo sapiens miR-886 stem-loop

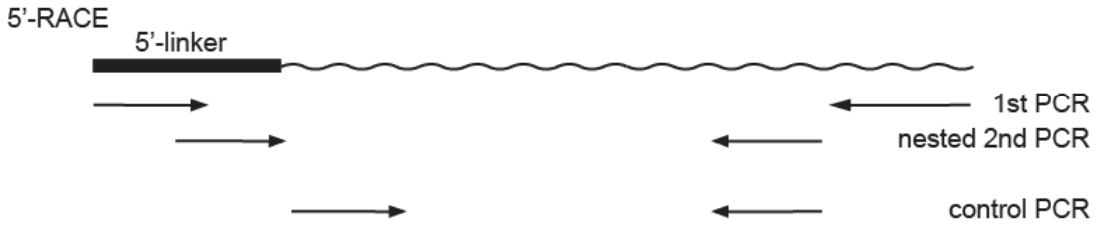
```
--cacuccuaccc       aguu   u aa    -  a -cu     u  - ------    uuu
            gggucgg    agc c  gcgg uu c   ccuca gc c      ggac   c
            |||||||    ||| |  |||| || |   ||||| || |      ||||   u
            cccaguc    ucg g  cgcc ag g   ggggu cg g      ccug   a
aauaacguauuuu       --au   u gg    c  a cuu     -  u ucucua    ucu
```

Homo sapiens miR-205 stem-loop

```
aaagaucc    acaa       gcuu      uc           c        ucuca
        ucag    uccaugu    cucuug  cuucauuccac ggagucug      u
        ||||    |||||||    ||||||  |||||||||||| ||||||||      a
        aguc    agguacg    gaggac  gaagugaggug cuuuagac      c
------ac    ---g       ----      uu           a        caacc
```

Fig S3

A


5'-RACE
5'-linker
1st PCR
nested 2nd PCR
control PCR

B

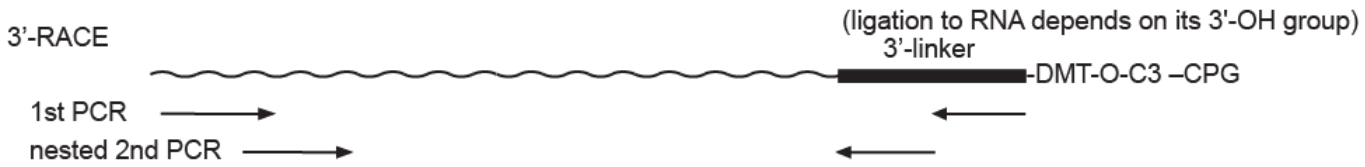

3'-RACE
(ligation to RNA depends on its 3'-OH group)
3'-linker
-DMT-O-C3 –CPG
1st PCR
nested 2nd PCR

Fig S4

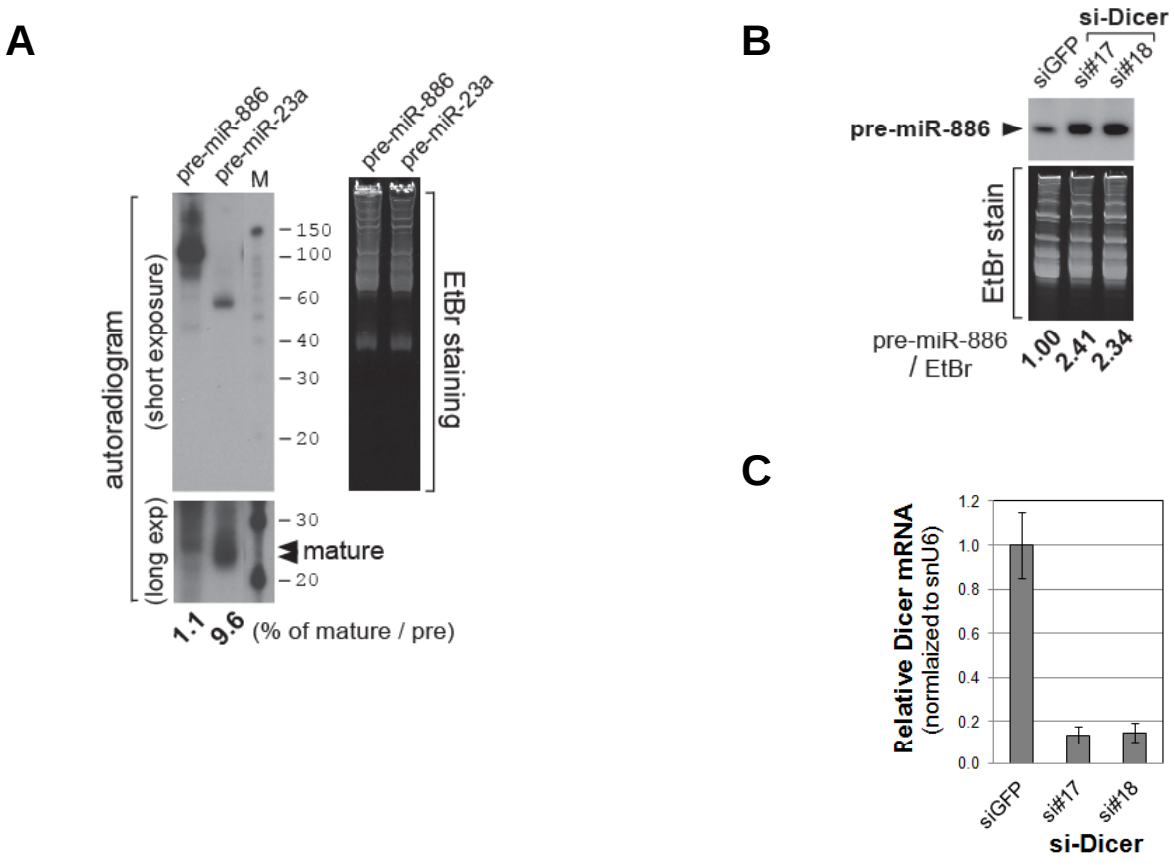
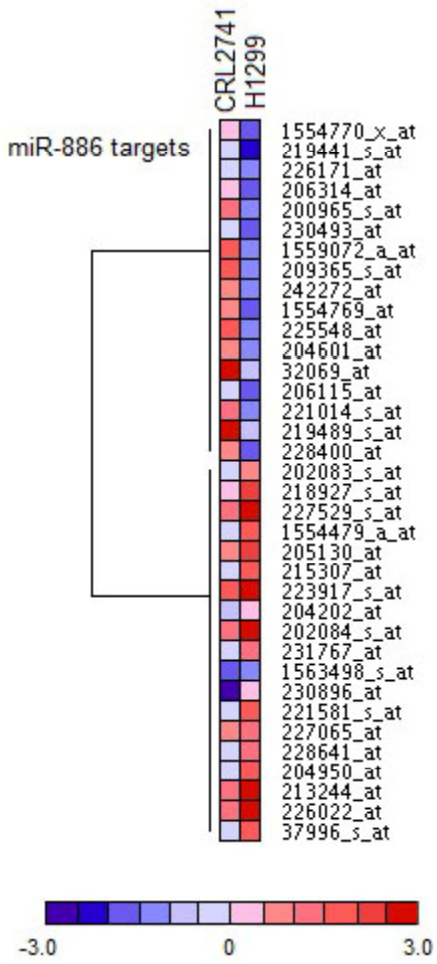

A
pre-miR-886
pre-miR-23a
M
pre-miR-886
pre-miR-23a
150
100
60
40
30
20
EtBr staining
autoradiogram
(short exposure)
(long exp)
30
mature
20
1.1
9.6
(% of mature / pre)
B
si-Dicer
siGFP
si#17
si#18
pre-miR-886
EtBr stain
pre-miR-886
/ EtBr
1.00
2.41
2.34
C
Relative Dicer mRNA
(normlaized to snU6)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siGFP
si#17
si#18
si-Dicer

Fig S5

A


CRL2741
H1299
miR-886 targets
1554770_x_at
219441_s_at
226171_at
206314_at
200965_s_at
230493_at
1559072_a_at
209365_s_at
242272_at
1554769_at
225548_at
204601_at
32069_at
206115_at
221014_s_at
219489_s_at
228400_at
202083_s_at
218927_s_at
227529_s_at
1554479_a_at
205130_at
215307_at
223917_s_at
204202_at
202084_s_at
231767_at
1563498_s_at
230896_at
221581_s_at
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37996_s_at
-3.0
0
3.0

B

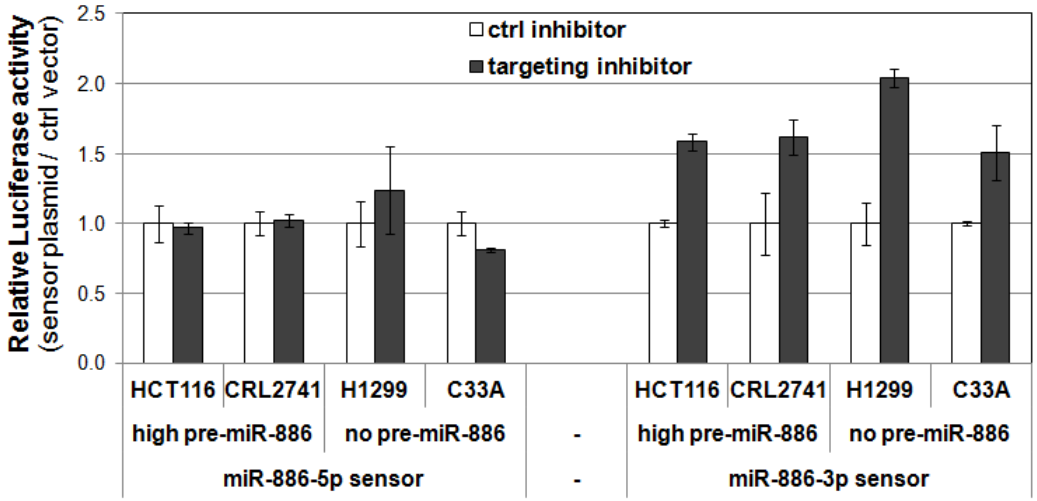
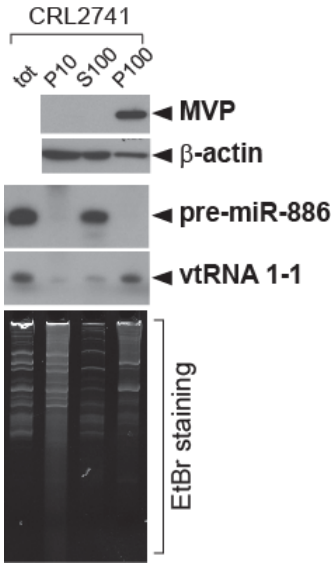

Relative Luciferase activity
(sensor plasmid / ctrl vector)
2.5
2.0
1.5
1.0
0.5
0.0
ctrl inhibitor
targeting inhibitor
HCT116
CRL2741
H1299
C33A
high pre-miR-886
no pre-miR-886
-
miR-886-5p sensor
miR-886-3p sensor

Fig S6

A


CRL2741
tot
P10
S100
P100
MVP
β-actin
pre-miR-886
vtRNA 1-1
EtBr staining

B

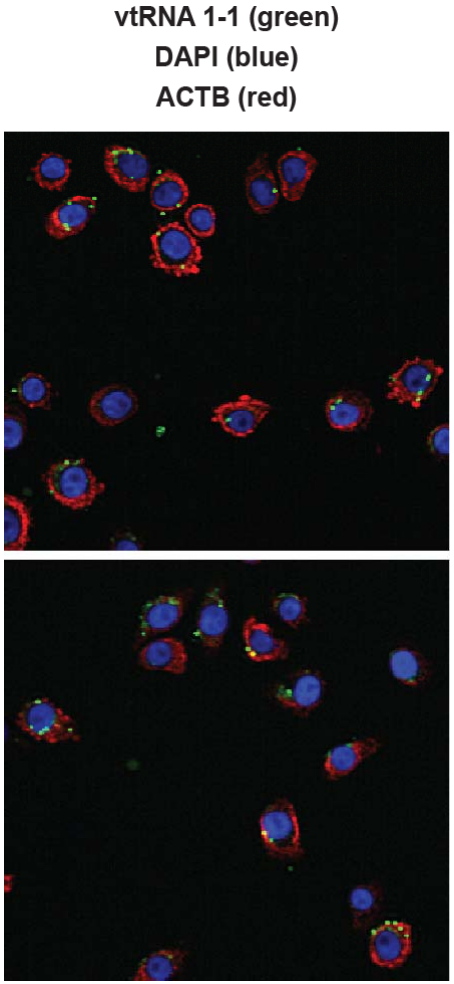
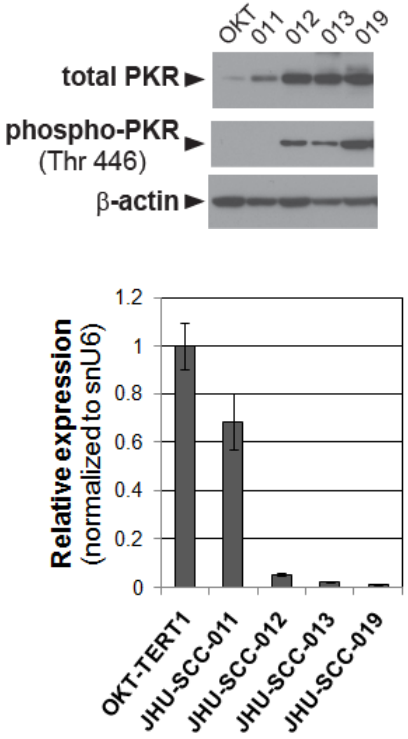

vtRNA 1-1 (green)
DAPI (blue)
ACTB (red)

Fig S7


OKT
011
012
013
019
total PKR
phospho-PKR
(Thr 446)
β-actin
Relative expression
(normalized to snU6)
1.2
1
0.8
0.6
0.4
0.2
0
OKT-TERT1
JHU-SCC-011
JHU-SCC-012
JHU-SCC-013
JHU-SCC-019

Supplemental Table 1. List of proteins that physically interact with pre-miR-886

GI number	Entrez Gene ID	Description	Protein Class
24415404	23195	midasin [Homo sapiens]	chaperone (PC00072)
41281447	9742	intraflagellar transport protein 140 homolog [Homo sapiens]	cytoskeletal protein (PC00085)
46358428	26160	intraflagellar transport protein 172 homolog [Homo sapiens]	cytoskeletal protein (PC00085)
88999583	4637	myosin light polypeptide 6 isoform 2 [Homo sapiens]	cytoskeletal protein (PC00085)
73858570	710	serpin peptidase inhibitor, clade G, member 1 precursor [Homo sapiens]	enzyme modulator (PC00095)
38348304	340706	von Willebrand factor A domain containing 2 [Homo sapiens]	extracellular matrix protein (PC00102)
21702735	113622	[Protein ADP-ribosylarginine] hydrolase-like protein 1 isoform 1 [Homo sapiens]	hydrolase (PC00121)
63055049	55276	phosphoglucomutase-2 [Homo sapiens]	isomerase (PC00135)
198041692	8675	syntaxin-16 isoform d [Homo sapiens]	membrane traffic protein (PC00150)
166295188	55635	DEP domain-containing protein 1A isoform a [Homo sapiens]	nucleic acid binding (PC00171)
206597515	116985	arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1 isoform d [Homo sapiens]	nucleic acid binding (PC00171)
224028248	4841	non-POU domain-containing octamer-binding protein isoform 2 [Homo sapiens]	nucleic acid binding (PC00171)
77539758	554313	histone H4 [Homo sapiens]	nucleic acid binding (PC00171)
83281438	8669	eukaryotic translation initiation factor 3 subunit J [Homo sapiens]	nucleic acid binding (PC00171)
83367072	8662	eukaryotic translation initiation factor 3 subunit B [Homo sapiens]	nucleic acid binding (PC00171)
4826870	4925	nucleobindin-2 precursor [Homo sapiens]	nucleic acid binding (PC00171)
100913206	1660	ATP-dependent RNA helicase A [Homo sapiens]	nucleic acid binding (PC00171)
4503513	8668	eukaryotic translation initiation factor 3 subunit I [Homo sapiens]	nucleic acid binding (PC00171)
157694499	65986	zinc finger and BTB domain-containing protein 10 isoform a [Homo sapiens]	protease (PC00190)
110825974	9509	a disintegrin and metalloproteinase with thrombospondin motifs 2 isoform 1 preproprotein [Homo sapiens]	protease (PC00190)
4504657	8807	interleukin-18 receptor accessory protein precursor [Homo sapiens]	receptor (PC00197)
40805858	145501	isthmin-2 isoform 1 [Homo sapiens]	signaling molecule (PC00207)
116256354	1284	collagen alpha-2(IV) chain preproprotein [Homo sapiens]	signaling molecule (PC00207)
261337173	4052	latent transforming growth factor beta binding protein 1 isoform 5 precursor [Homo sapiens]	signaling molecule (PC00207)
188528648	7148	tenascin XB isoform 1 precursor [Homo sapiens]	signaling molecule (PC00207)
195972883	5333	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1 isoform 1 [Homo sapiens]	signaling molecule (PC00207)
12056468	3728	junction plakoglobin [Homo sapiens]	storage protein (PC00210)
41152093	84146	zinc finger protein 644 isoform 1 [Homo sapiens]	transcription factor (PC00218)
208431829	5610	interferon-induced, double-stranded RNA-activated protein kinase isoform b [Homo sapiens]	transferase (PC00220)
208431827	5610	interferon-induced, double-stranded RNA-activated protein kinase isoform a [Homo sapiens]	transferase (PC00220)
4503483	1938	elongation factor 2 [Homo sapiens]	transferase (PC00220)
10140845	2046	ephrin receptor EphA8 isoform 1 precursor [Homo sapiens]	transferase (PC00220)
203098098	57619	protein Shroom3 [Homo sapiens]	transporter (PC00227)
8923870	55867	solute carrier family 22 member 11 [Homo sapiens]	transporter (PC00227)
24308382	114823	leukocyte receptor cluster member 8 [Homo sapiens]	transporter (PC00227) cytoskeletal protein (PC00085)
260436922	374897	suprabasin isoform 1 precursor [Homo sapiens]	unclassified
239752152	100290481	PREDICTED: hypothetical protein XP_002348153 [Homo sapiens]	unclassified
193794853	79983	protein POF1B [Homo sapiens]	unclassified
8923936	55850	vesicle transport protein USE1 [Homo sapiens]	unclassified
239749684	256158	PREDICTED: hemicentin 2 [Homo sapiens]	unclassified
291045225	7273	titin isoform N2-A [Homo sapiens]	unclassified
4505821	5304	prolactin-induced protein precursor [Homo sapiens]	unclassified
10047090	23676	small muscle protein, X-linked [Homo sapiens]	unclassified
134254443	80003	pecanex-like protein 2 [Homo sapiens]	unclassified
116805348	27327	trinucleotide repeat-containing gene 6A protein [Homo sapiens]	unclassified
4507357	8407	transgelin-2 [Homo sapiens]	unclassified
239756615	100132364	PREDICTED: hypothetical protein isoform 1 [Homo sapiens]	unclassified
36029914	80124	deubiquitinating protein VCIP135 [Homo sapiens]	unclassified
148806928	57482	hypothetical protein LOC57482 [Homo sapiens]	unclassified

Supplemental Table 2. Primers used in this study

primer name	sequence	the use in this study
5S rRNA_34-11	GATCGGGCGCGTTCAGGGTGGTAT	Northern probe for 5S rRNA
Dcr 4893-917	TTAACCAGCTGTGGGGAGAGGGCTG	forward primer for qRT-PCR of dicer
Dcr 5429-05	AGCCAGCGATGCAAAGATGGTGTTG	reverse primer for qRT-PCR of dicer
Dsh 431-52	ACATGGAGCCAGACCCTCAGCA	forward primer for qRT-PCR of drosha
Dsh 603-582	GGAAGGGTACAAAGTCTGGTCG	reverse primer for qRT-PCR of drosha
GeneRacer 3'Primer	GCTGTCAACGATACGCTACGTAACG	reverse primer for 1st amplification in 3'-RACE-PCR
let7f1+20-2	TAATGCAGCAAGTCTACTCCTC	reverse primer for qRT-PCR of the primary transcript of let-7f
let7f1-26-3	CCATTCCAGAAGAAAACATTGCTC	forward primer for qRT-PCR of the primary transcript of let-7f
miR-205 as	TCGACAGACTCCGGTGGAATGAAGGAGGCC	Northern probe for miR-205
miR-886 -22-1	GTTTCAGTCGCACACTCCTACC	forward primer for qRT-PCR of the primary transcript of pre-miR-886
miR-886 34-53	TTACCTCCTCATGCCGGACT	forward primer for 2nd amplification in 3'-RACE-PCR of pre-miR-886
miR-886 89-70	GGTCTCGAACCCCAGCACAG	reverse primer for 2nd amplification in 5'-RACE-PCR of pre-miR-886
miR-886-3p as	AAGGGTCAGTAAGCACCCGCG	Northern probe for pre-miR-886
		reverse primer for qRT-PCR of pre-miR-886 and pri-miR-886
		reverse primer for 1st amplification in 5'-RACE-PCR of pre-miR-886
miR-886-5p as	TCGACCGCTTGAGCTAACTCCGACCCGGGCC	Northern probe
miR-886-5p sense	CGGGTCGGAGTTAGCTCAAGCGG	forward primer for qRT-PCR of pre-miR-886
		forward primer for 1st amplification in 3'-RACE-PCR of pre-miR-886
mp3-nested	ATTGATGGTGCCTACAG	reverse primer for 2nd amplification in 3'-RACE-PCR
mp3-RACE-RT	GCTGTCAACGATACGCTACGTAACGATTGATGGTGCCTAC	primer for cDNA synthesis in 3'-RACE-PCR
snoU38b-ext	AGAACTGGACAAAGTTTTCATCAC	Northern probe for snoU38b
snU6 100-79	TGGAACGCTTCACGAATTTGCG	reverse primer for qRT-PCR of snU6
snU6 2-23	TGCTCGCTTCGGCAGCACATAT	forward primer for qRT-PCR of snU6
T7 pre-23a as	GGAAATCCCTGGCAATGTGAT	reverse primer to amplify template DNA for T7 transcription of pre-miR-23a
T7 pre-23a sense	TAATACGACTCACTATAGGGTTCCTGGGGATGGGATTTG	forward primer to amplify template DNA for T7 transcription of pre-miR-23a
T7 pre-886 as	AAAAGGGTCAGTAAGCACCCGCG	reverse primer to amplify template DNA for T7 transcription of pre-miR-886
T7 pre-886 sense	TAATACGACTCACTATAGGGTCGGAGTTAGCTCAAGCGG	forward primer to amplify template DNA for T7 transcription of pre-miR-886
T7 vtRNA1 as	AAAAGGACTGGAGAGCGCCCGCG	reverse primer to amplify template DNA for T7 transcription of vtRNA1-1
T7 vtRNA1 sense	TAATACGACTCACTATAGGGCTGGCTTTAGCTCAGCGGT	forward primer to amplify template DNA for T7 transcription of vtRNA1-1
tAla6-66 rev	CAGCGCTAAAACCTTAATACCTATTGG	reverse primer for 1st amplification in 5'-RACE-PCR of pre-tRNA-Ala
tAlatRF3as-2	TGGAGATGCCGGGGATCGAA	reverse primer for 2nd amplification in 5'-RACE-PCR of pre-tRNA-Ala
		Northern probe for tRNA-Ala
tRF1001 as	AAATAAGAGCACCCGCTTC	Northern probe for pre-tRNA-Ser
vtRNA1 as RI	CCCCGAATTCAAAAGGACTGGAGAGCGCCCG	Northern probe, reverse primer for qRT-PCR of vtRNA1-1
vtRNA2 as RI	CCCCGAATTCAAAAGAGCTGGAAAGCACCCG	Northern probe, reverse primer for qRT-PCR of vtRNA1-2
vtRNA3 as RI	CCCCGAATTCAAGAGGGCTGGAGAGCGCCCG	Northern probe, reverse primer for qRT-PCR of vtRNA1-3
vtRNAall 1-20	GGCTGGCTTTAGCTCAGCGG	forward primer for qRT-PCR of vtRNA1-1, 1-2, 1-3