Supporting information

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Materials and Methods

Preparation of RNA oligonucleotides.

RNA oligonucleotides were synthesized on a 0.2 μmol scale on an H-8 SE DNA synthesizer (Gene World, Tokyo, Japan) using 2′-*O*-TOM-protected β-cyanoethyl phosphoramidites. To synthesize the RNA sequence containing a phosphorothioate (PS) group at the 3′ end, the first nucleotide was added on a 3′-phosphate CPG column (Glen Research, Sterling, VA), followed by sulfurization with a sulfurizing reagent (3H-1,2-Benzodithiole-3-one-1,1-dioxide, Glen Research), and synthesis continued according to the normal protocol. To synthesize an RNA sequence containing a 5′-Amino-dT modification, 5′-Amino-dT phosphoramidite (Glen Research) was used in the last coupling step. The oligonucleotides were treated with AMA reagent (1:1 mixture of 40% aqueous methylamine and 28% ammonium hydroxide) for 10 min at 65 °C and concentrated *in vacuo*. Tetra-*n*-butylammonium fluoride (1 M solution in tetrahydrofuran) was added to the concentrated residue, and incubated for 10 min at 55 °C and then at room temperature overnight. The reaction was quenched by the addition of 1 M Tris-HCl (pH 7.2) and desalted with a NAP-25 column (GE Healthcare, Amersham, England). Deprotected oligonucleotides were purified by 20% denaturing PAGE (7.5 M urea, 1×TBE), and then isolated by the crush and soak method[1] (elution buffer: 0.2 M NaCl, 10 mM EDTA, pH 8.0). Desalted RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol. Oligonucleotides containing 3′-PS or 5′-Amino-dT were used in the subsequent reactions without PAGE purification.

Synthesis of caged PS-RNA (CPS-RNA)

The reactions were performed in a 200 μL reaction volume, containing 200 μM of the 3′-PS oligonucleotide, 4 mM of diphenyl disulfide (Tokyo Chemical Industry, Tokyo, Japan), and 2 mM of 1-fluoro-2,4-dinitrobenzene (Wako Pure Chemical Industries, Osaka, Japan), dissolved in 60% dimethyl sulfoxide containing 50 mM Tris-HCl (pH 7.2). The reaction mixture was incubated at room temperature for 2 h and purified by reversed-phase HPLC, using a Hydrosphere C18 column (4.6 × 250 mm; YMC, Kyoto, Japan). Eluent A was 5% acetonitrile (MeCN) in 50 mM triethylammonium acetate (TEAA) buffer (pH 7.0), and eluent B was 100% MeCN. The concentration of eluent B was increased from 0–40% over 20 min, at a flow rate of 1.0 mL/min.

Synthesis of iodoacetyl RNA (IAc-RNA)

Reactions were performed in a 200 μL reaction volume, containing 200 μM of the 5′-Amino dT oligonucleotide, 8 mM of *N*-succinimidyl iodoacetate (Tokyo Chemical Industry) dissolved in *N*,*N*-dimethylformamide, and 50 mM sodium tetraborate (pH 8.5). The reaction mixture was incubated at room temperature for 1 h and purified by reversed-phase HPLC, as above.

Synthesis of acetyl RNA (Ac-RNA)

Reactions were performed in a 100 μL reaction volume, containing 100 μM of the 5′-Amino dT oligonucleotide, 5 mM of sodium acetate, and 5 mM of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Wako Pure Chemical Industries) dissolved in methanol. The reaction mixture was incubated at room temperature for 2 h and purified by reversed-phase HPLC, as above.

Synthesis of ligated siRNA product from IBR-RNA in test tube

Reactions were performed in a 20 μL reaction volume, containing 100 μM of both CPS-RNA and IAc-RNA, 500 μM of DTT, and 35 mM Tris-borate containing 5 mM $MgCl_2$ (pH 8.0). The reaction mixture was incubated at room temperature for 2 h and purified by reversed-phase HPLC, as above.

Buildup reaction of ligated siRNA from IBR-RNA triggered by GSH in test tube (Figures 3, S6, S10)

Reactions were performed in a 20 μL reaction volume, containing 500 nM (lane 4, 5 in Figure 3, S6, S10) or 2 μM (lane 6, 7 in Figure 3, S6, S10) RNAs, and 50 μM GSH in Hank's Balanced Salt Solution (HBSS) with 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, pH 7.4 (HBSS, Gibco, Carlsbad, CA). The mixture was incubated at 37 °C for 30 min. Loading buffer (95% formamide containing 0.05% bromophenol blue) was added and incubated at 90 °C for 5 min to denature the dsRNA. The samples were analyzed by 20% denaturing PAGE (7.5 M urea, 1×TBE) and stained with SYBR Green II (LONZA).

PAGE analysis of the Dicer cleavage reactions of RNAs

Annealed RNAs (2 μM) were incubated with 0.1 units/μL of recombinant human Turbo Dicer (Genlantis, San Diego, CA) at 37 °C. At time points 1, 6, and 12 h, aliquots (6 μL) were taken from the mixture and the reaction was stopped by mixing with 1.0 μL of Dicer stop solution. The samples were analyzed by 15% native PAGE and stained with SYBR Green I (LONZA, Basel, Switzerland), and then visualized by scanning on a BioRad Molecular Imager FX (BioRad, Hercules, CA). siRNA Ladder Marker (Takara Bio, Otsu, Japan) was used as the size marker for dsRNA.

Cell culture

HeLa cells stably expressing luciferase (which was kindly gifted from Prof. H. Harashima at Hokkaido University) were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries) supplemented with 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel). Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 70% confluency, cells were plated in 96-well plates (4×10^3 cells/100 μL per well) for measurement of RNAi activity, and 24-well plates (4×10^4 cells/300 μL per well) for analysis of the intracellular buildup reaction.

T98G cells (RIKEN Cell bank) were cultured in RPMI-1640 (Wako Pure Chemical Industries) supplemented with 10% FCS. Cells were regularly passaged to maintain exponential growth. One day prior to transfection at 70% confluency, cells were plated in 48-well plates (3×10^4 cells/150 μL per well) for analysis of the immune response induced by RNA.

Measurements of RNAi activity

RNAs were transfected into HeLa cells stably expressing luciferase with GeneSilencer (Genlantis), following the manufacturer's method for adherent cell lines. Immediately prior to transfection, the culture medium was replaced with 50 μL of HBSS serum-free buffer (Gibco) to avoid the reaction between cysteine in the medium and protecting disulfide groups, and 2.5, 5.0, or 10 pmol of RNA formulated into liposomes (50 μL) were added to each well. Six hours post-transfection, wells were washed with PBS and 100 μL of DMEM supplemented with 10% FCS was added to each well. After 16 h, luciferase expression was measured with the Luciferase Assay System

(Promega, Fitchburg, WI) on a Mithras LB 940 plate reader (Berthold, Bad Wildbad, Germany) and protein was quantified using a bicinchoninic acid (BCA protein) assay, to adjust luciferase activity, with a Pierce® BCA Protein Assay Kit (Themo Fisher Scientific, Rockford, IL).

PAGE analysis of the ligation reactions in HeLa cells stably expressing luciferase

RNAs (30 pmol) formulated into liposomes with HBSS (300 μL, Gibco) were transfected into HeLa cells stably expressing luciferase using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as described by the manufacturer's method for adherent cell lines. Six hours post-transfection, 300 μL of DMEM supplemented with 20% FCS was added to each well. After 18 h, wells were washed with PBS and total RNAs were isolated with ISOGEN (Nippon Gene, Tokyo, Japan), as described by the manufacturer. Seven micrograms of total RNA were used for 15% denaturing PAGE analysis (5.6 M urea, 25% formamide, and 1×TBE) of the ligation reaction.

Semi-quantitative RT–PCR analysis of immune response induced by RNA

RNAs (15 or 75 pmol) formulated into liposomes with HBSS (150 μL, Gibco) were transfected into T98G cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's method for adherent cell lines. Four hours post-transfection, 150 μL of DMEM supplemented with 20% FCS was added to each well. After 20 h, wells were washed with PBS and total RNA was extracted from T98G cell lysates using ISOGEN (Nippon Gene), following the manufacturer's method. Ten nanograms of each total RNA were reverse transcribed and amplified in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using a One Step PrimeScript® RT-PCR Kit (Takara Bio) and gene-specific primers. β-actin was used as the internal control. Samples were assayed in triplicate and the data were analyzed according to the comparative threshold cycle (Ct) method, where the amount of target, normalized to an endogenous reference and relative to an experimental control, is given by $2^{-\Delta\Delta Ct}$. Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The ΔCt value is determined by subtracting the mean Ct value of β-actin from the mean Ct value of IFN-β. The ΔΔCt value involves subtraction of the ΔCt experimental control value.

Amplification parameters were 42 °C for 5 min, followed by 40 cycles of 42 °C for 10 s, 95 °C for 5 s, and 60 °C for 30 s.

The primer sequences for each gene were:

IFN-β forward: 5′–GTTCGTGTTGTCAACATGACCA–3′
reverse: 5′–TCAATTGCCACAGGAGCTTCT–3′

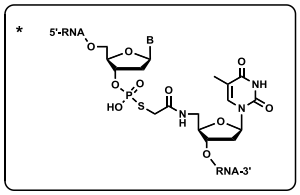
β-actin forward: 5′–TGGCACCCAGCACAATGAA–3′
reverse: 5′–CTAAGTCATAGTCCGCCTAGAAGCA–3′

Reference

1) Sambrook J, Russe, D. W., in Molecular Cloning: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory Press, NY, Vol. 2 (2001).

Table S1. Sequences and MALDI-TOF MS analysis of RNAs used in the main text.

Name	Sequences	Mw (M + H) calcd	Mw (M + H) found
siRNA-n-1s	5'-CCUCAUAGAACUGCCUGCGUGAGAU-3'	7958.8	7957.3
siRNA-n-1as	5'-AUCUCACGCAGGCAGUUCUAUGAGG-3'	7998.8	7997.7
siRNA-scr-1s	5'-AUCGUUGCACGGACUGACAUCUAGG-3'	7958.8	7957.6
siRNA-scr-1as	5'-CCUAGAUGUCAGUCCGUGCAACGAU-3'	7998.8	7997.6
siRNA-lig-1s	5'-CCUCAUAGAACUGCCUGCdG*dTGAGAU-3'	8013.9	8013.8
siRNA-lig-1as	5'-AUCUCACGCAGGCAGUUdC*dTAUGAGG-3'	8053.9	8055.0
Caged PS-RNA (1)	5'-CCUCAUAGAACUGCCUGCdG-O-P(=O)(OH)-S-S-Ph	6185.8	6186.6
Caged PS-RNA (2)	5'-AUCUCACGCAGGCAGUUdC-O-P(=O)(OH)-S-S-Ph	5880.6	5880.3
Iodoacetyl RNA (3)	I-CH2-C(=O)-NHdTGAGAU-3'	2064.2	2064.8
Iodoacetyl RNA (4)	I-CH2-C(=O)-NHdTAUGAGG-3'	2409.3	2410.0
Acetyl RNA (5)	H3C-C(=O)-NHdTGAGAU-3'	1938.3	1939.6
Acetyl RNA (6)	H3C-C(=O)-NHdTAUGAGG-3'	2283.4	2283.8
segmented siRNA-1	5'-CCUCAUAGAACUGCCUGCG-3'	5997.6	5698.1
segmented siRNA-2	5'-AUCUCACGCAGGCAGUUC-3'	5692.5	5693.4
segmented siRNA-3	5'-UGAGAU-3'	1899.3	1898.9
segmented siRNA-4	5'-UAUGAGG-3'	2244.3	2243.7



s and *as* as the last letters of names denote sense and antisense, respectively.

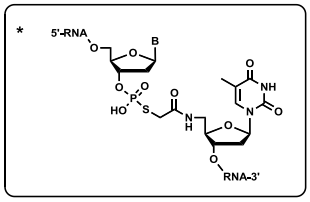
Segmented siRNA-1, 2, 3, and 4 denotes the fragments of segmented siRNA shown in Figure 2F.

siRNA targeting GL3 luciferase (siRNA-n-1) corresponded to coding region nucleotides 910–934, relative to the first nucleotide of the start codon.

Mass data were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Fremont, CA) at RIKEN BSI RRC, by positive mode using 3-hydroxypicolinic acid (HPA) as a matrix.

Table S2. Sequences and MALDI-TOF MS analysis of RNAs used in supporting experiments.

Name	Sequences	Mw (M + H) calcd	Mw (M + H) found
siRNA-n-2s	5'-CUUACGCUGAGUACUUCGAUU-3'	6611.2	6614.4
siRNA-n-2as	5'-UCGAAGUACUCAGCGUAAGUU-3'	6697.2	6700.2
siRNA-scr-2s	5'-UAUGUCCGAUACUGUCAGCUU-3'	6611.2	6609.0
siRNA-scr-2as	5'-GCUGACAGUAUCGGACAUAUU-3'	6697.2	6694.9
siRNA-lig-2s	5'-CUUACGCUGAGUACdT*dTCGAUU-3'	6780.1	6685.9
siRNA-lig-2as	5'-UCGAAGUACUCAGCdG*dTAAGUU-3'	6752.2	6760.1
Caged PS-RNA (7)	5'-CUUACGCUGAGUACdT-O-P(=O)(OH)-S-S-Ph	4915.1	4914.0
Caged PS-RNA (8)	5'-UCGAAGUACUCAGCdG-O-P(=O)(OH)-S-S-Ph	4963.1	4962.2
Iodoacetyl RNA (9)	I-CH2-C(=O)-NHdTCGAUU-3'	2001.2	2001.7
Iodoacetyl RNA (10)	I-CH2-C(=O)-NHdTAAGUU-3'	2025.2	2025.6
siRNA-lig-3s	5'-CCUCAUAGAACUGCdC*dTGCGUGAGAU-3'	8013.9	8011.6
siRNA-lig-3as	5'-AUCUCACGCAGGCAdG*dTUCUAUGAGG-3'	8053.9	8053.8
Caged PS-RNA (11)	5'-CCUCAUAGAACUGCdC-O-P(=O)(OH)-S-S-Ph	4884.1	4886.0
Caged PS-RNA (12)	5'-AUCUCACGCAGGCAdG-O-P(=O)(OH)-S-S-Ph	4963.1	4964.3
Iodoacetyl RNA (13)	I-CH2-C(=O)-NHdTGCGUGAGAU	3366.9	3368.0
Iodoacetyl RNA (14)	I-CH2-C(=O)-NHdTCUAUGAGG	3327.9	3329.3



s and *as* as the last letters of names denote sense and antisense, respectively.

siRNA targeting GL3 luciferase (siRNA-n-2) corresponded to coding region nucleotides 153–173, relative to the first nucleotide of the start codon.

Mass data were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Fremont, CA) at RIKEN BSI RRC, by positive mode using 3-hydroxypicolinic acid (HPA) as a matrix.

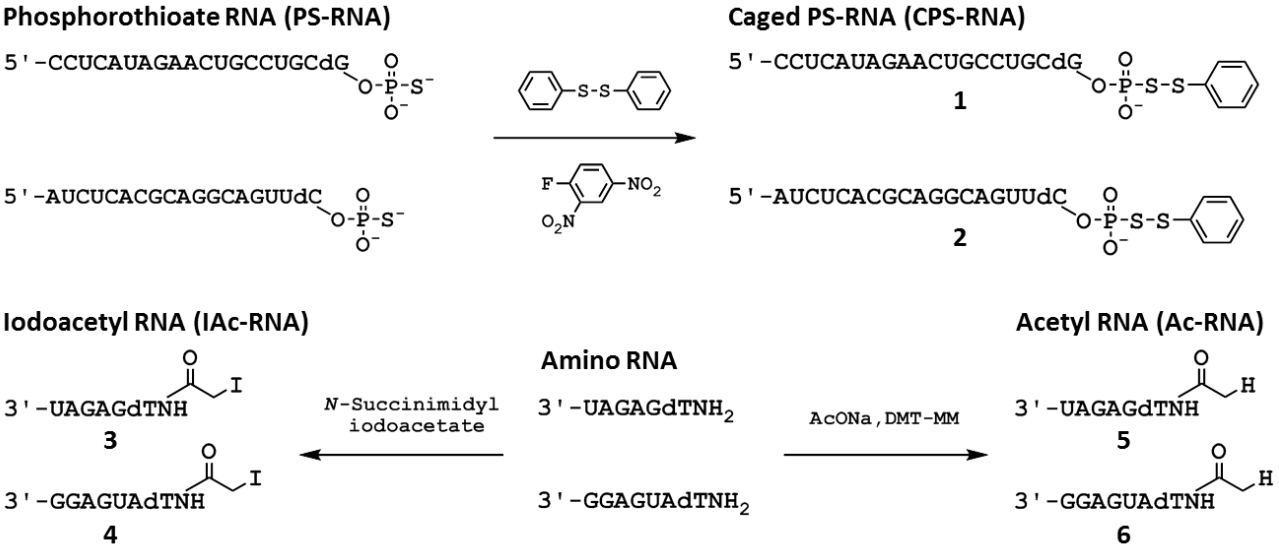


Figure S1. Synthesis of CPS-RNA (**1**, **2**), IAc-RNA (**3**, **4**), and Ac-RNA (**5**, **6**).

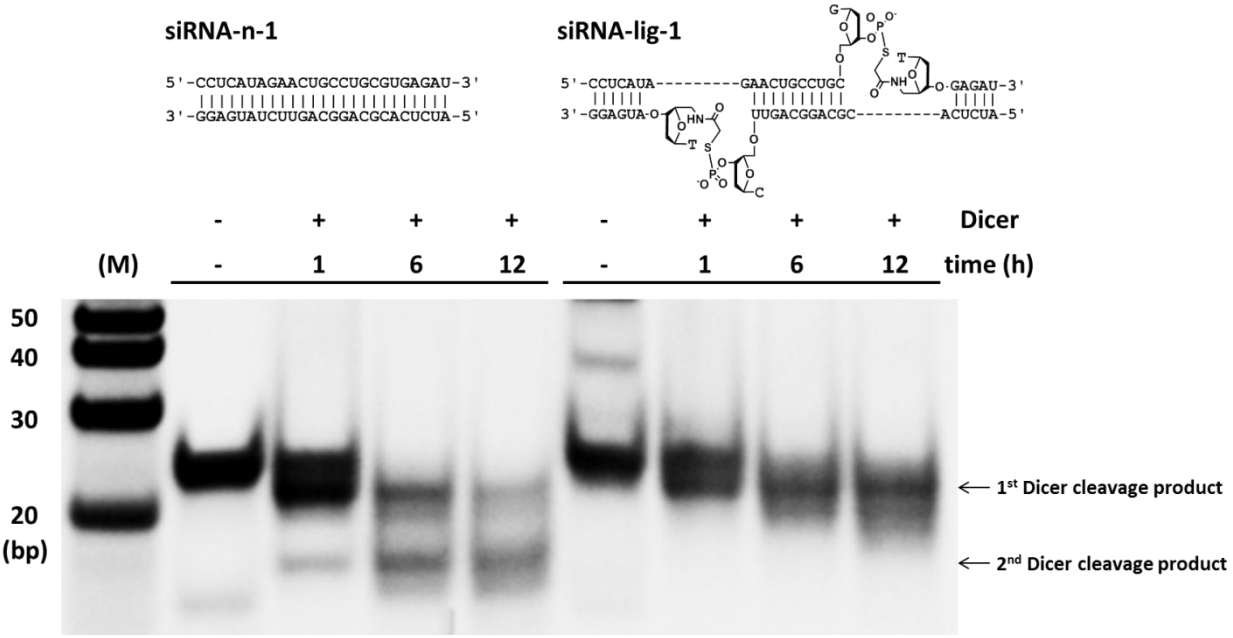


Figure S2. PAGE analysis of the Dicer cleavage reactions of RNAs. Annealed siRNA-n-1 or siRNA-lig-1 (2 μM) was incubated with recombinant human Dicer in the reaction buffer at 37°C. At time points of 1, 6, and 12 h, aliquots (6 μL) were taken from the mixture and analyzed by 15% native PAGE. The gel was stained with SYBR Green I.

Dicer cleavage of siRNA-n-1 produced two RNA products of different lengths on PAGE analysis. The first band is slightly smaller than siRNA-n-1 (~20 bp), corresponding to the active siRNA species. The second band, which is smaller than 20 bp, might be produced by further digestion of the first band product. In contrast, dicer cleavage of siRNA-lig-1 showed only the first band. Thus, it appears that the unnatural backbone of siRNA-lig-1 prevented further digestion of the first band product.

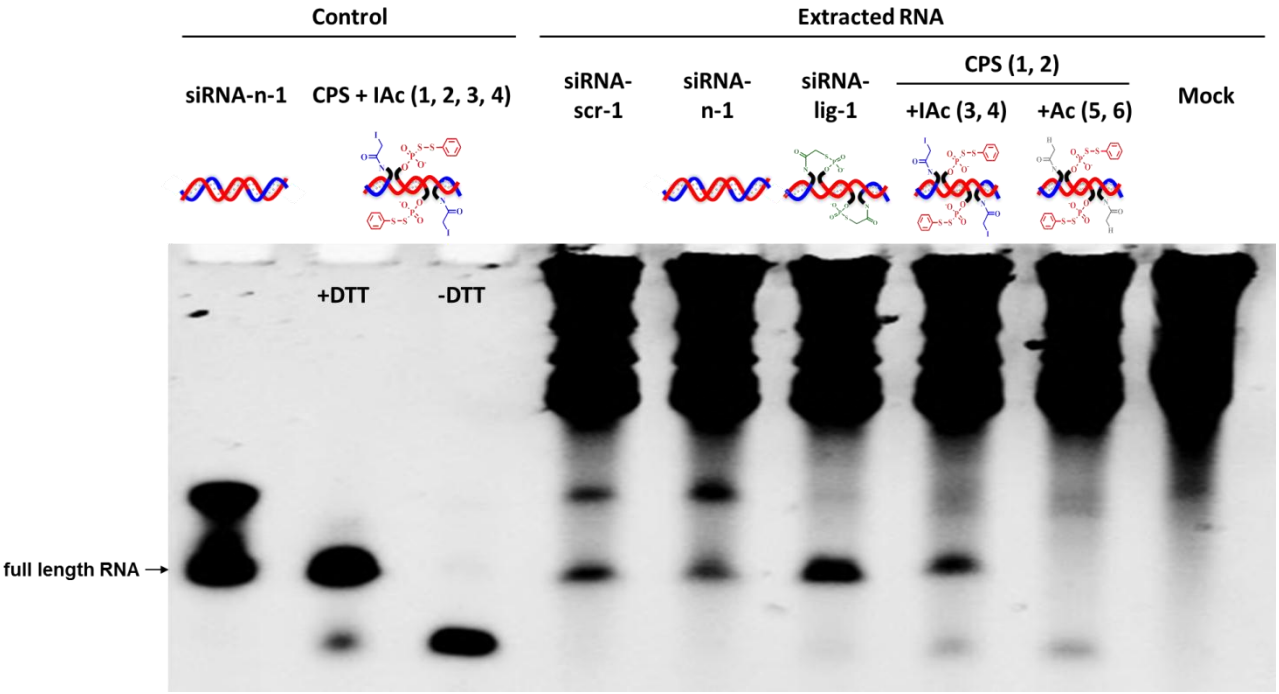


Figure S3. PAGE analysis of the ligation reactions in HeLa cells stably expressing luciferase. A series of RNAs, siRNA-scr-1, siRNA-n-1, siRNA-lig-1, IBR-RNA (CPS-RNAs and IAc-RNAs), or non-reactive IBR-RNA (CPS-RNAs and Ac-RNAs) were transfected into cells using Lipofectamine 2000 (Invitrogen). Lipofectamine was used because of its high RNA loading capacity. Total RNAs were isolated from the cells 24 h post-transfection. Extracted total RNA was analyzed by 15% denaturing PAGE and stained with SYBR Green II. Native siRNA (siRNA-n-1) and IBR-RNA (CPS-RNAs and IAc-RNAs), which were prepared *in vitro*, were also analyzed as gel standards.

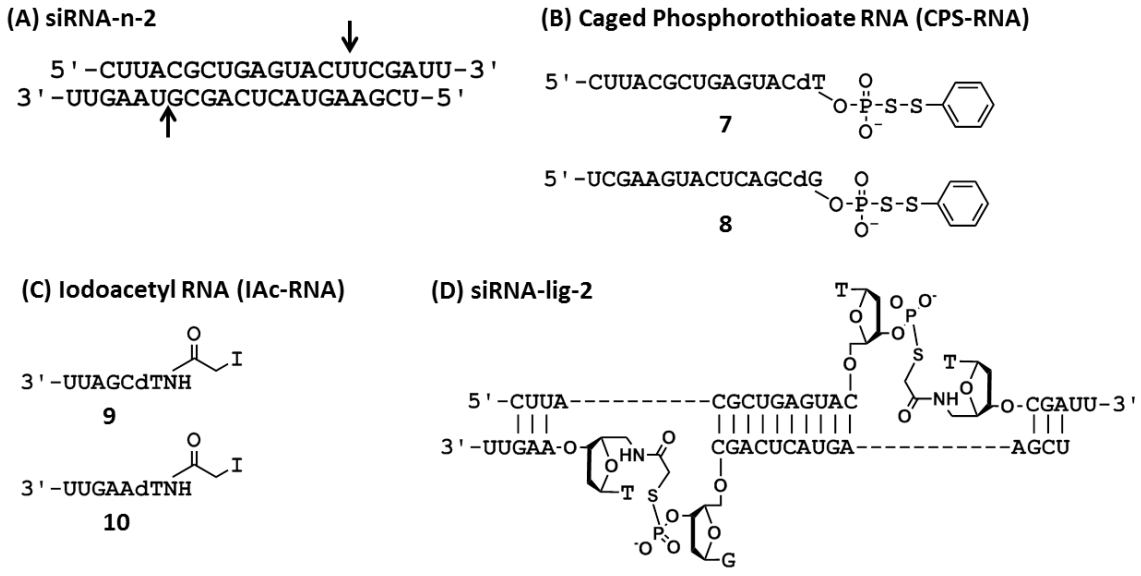


Figure S4. Structures and sequences of IBR-RNA (**7**–**10**). (A) Structure of siRNA-n-2 with arrows showing segmented positions. (B), (C) Segmented RNA sequences and structures of CPS-RNA (B) and IAc-RNA (C). (D) Sequences and structure of siRNA-lig-2.

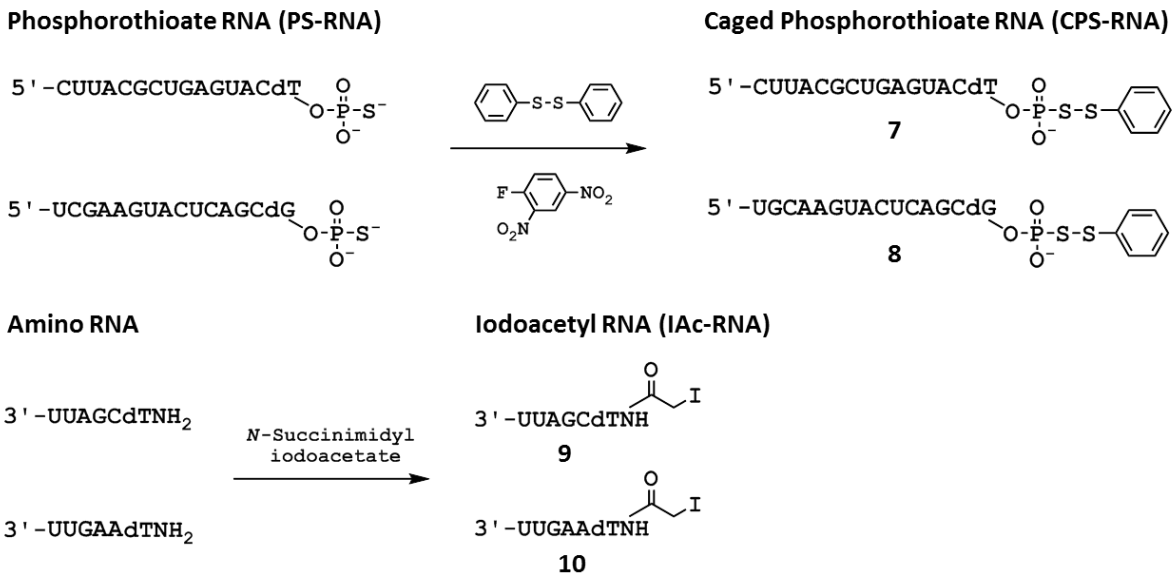


Figure S5. Synthesis of CPS-RNA (**7**, **8**) and IAc-RNA (**9**, **10**). Structures of RNA (**7**, **8**, **9,** and **10**) are also shown in Figure S4.

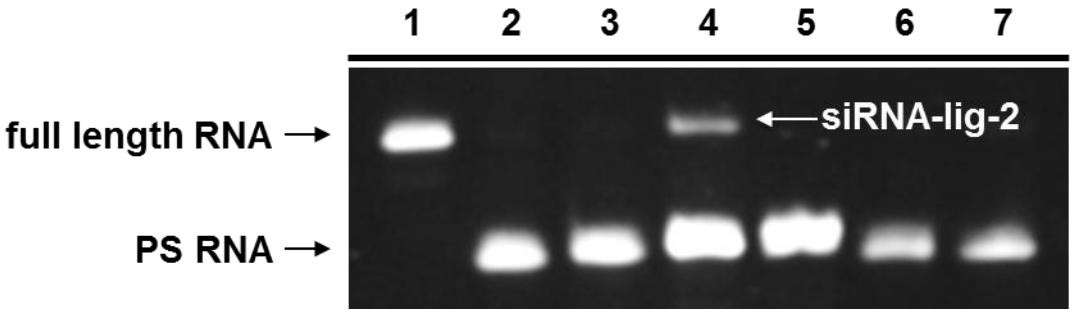


Figure S6. Buildup reaction of ligated siRNA from IBR-RNA triggered by GSH in test tube. Lane 1, 25 mer RNA; lanes 2 and 3, each PS RNA (**7** and **8**); lanes 4 and 5, reaction mixture (**7** + **8** + **9** + **10**) with (lane 4) or without (lane 5) GSH; lanes 6 and 7, reaction mixture (lane 6, **7** + **9** (sense strand) or lane 7, **8** + **10** (antisense strand)) without complementary strand. Reaction mixture was incubated at 37 °C for 30 min and then samples were analyzed by 20% denaturing PAGE. The gel was stained with SYBR Green II.

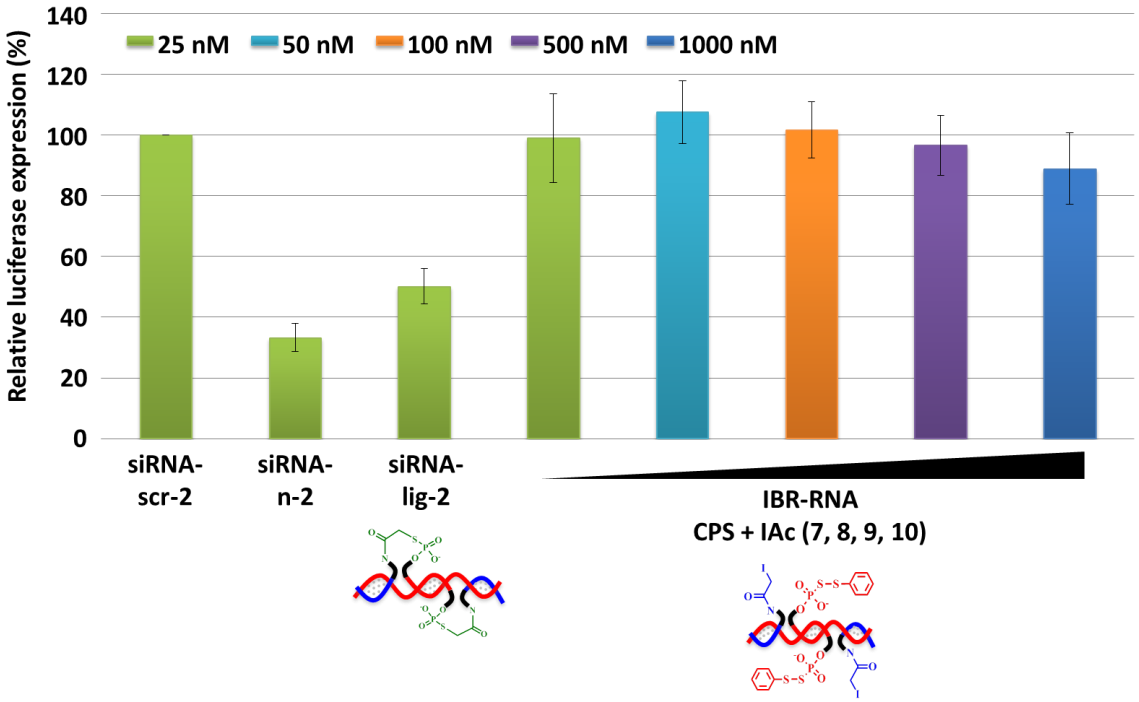


Figure S7. Gene silencing by siRNA-lig-2 and IBR-RNA, a pair of CPS-RNAs (**7**, **8**), and IAc-RNAs (**9**, **10**) in HeLa cells stably expressing luciferase. Luciferase expression was monitored at 24 h post-transfection. The relative expression of luciferase in scramble siRNA (siRNA-scr-2)-transfected cells was defined as 100%. The plotted data are the means ± standard deviation of three independent experiments.

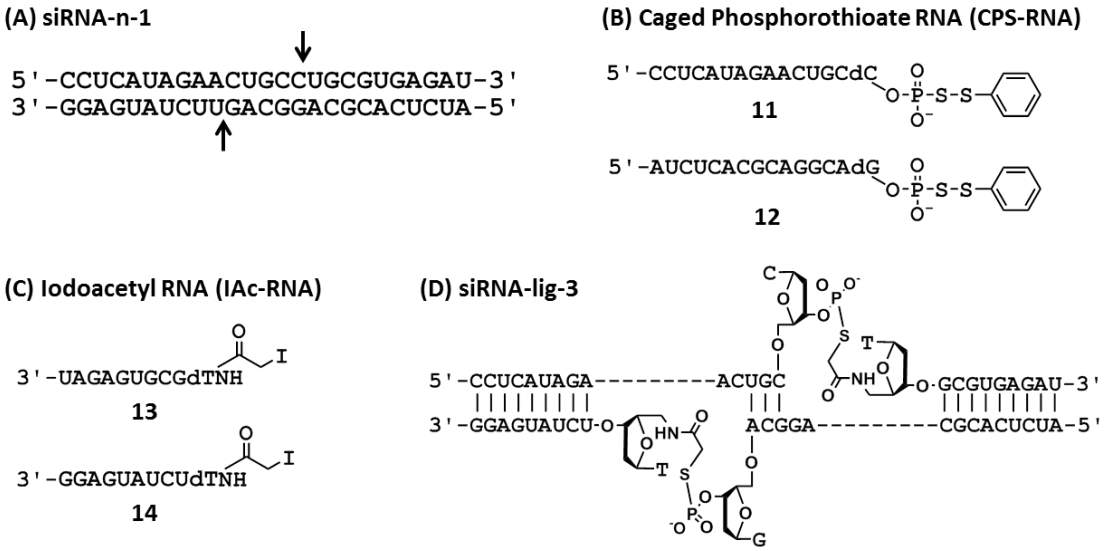


Figure S8. Structures and sequences of IBR-RNA (**11**–**12**). (A) Structure of siRNA-n-1 with arrows showing segmented positions. (B), (C) Segmented RNA sequences and structures of CPS-RNA (B) and IAc-RNA (C). (D) Sequences and structure of siRNA-lig-3.

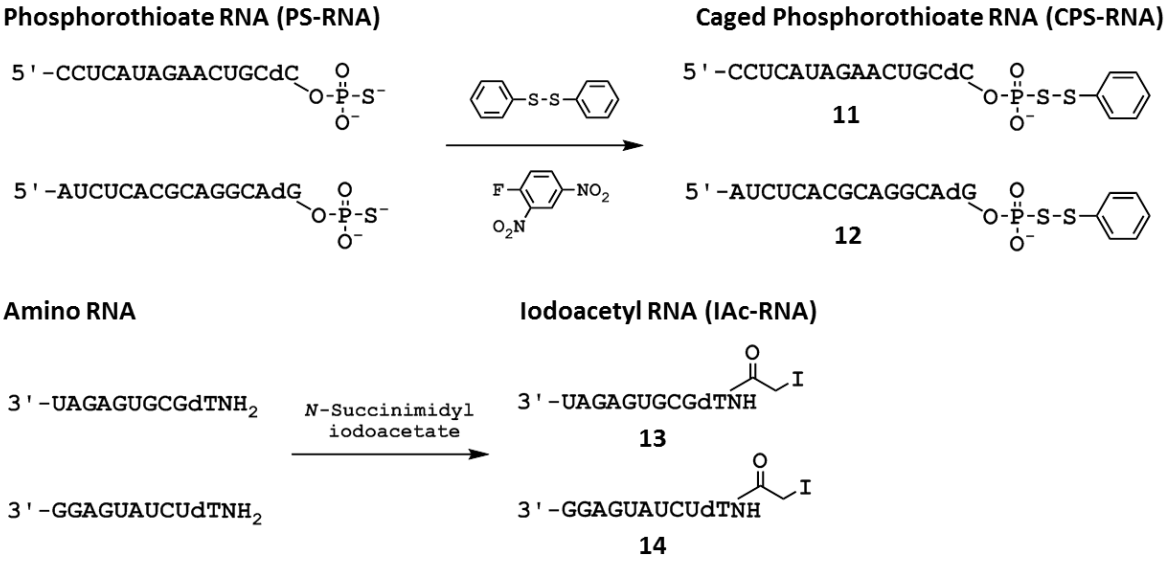


Figure S9. Synthesis of CPS-RNA (**11**, **12**) and IAc-RNA (**13**, **14**). Structures of RNA (**11**, **12**, **13,** and **14**) are also shown in Figure S8.

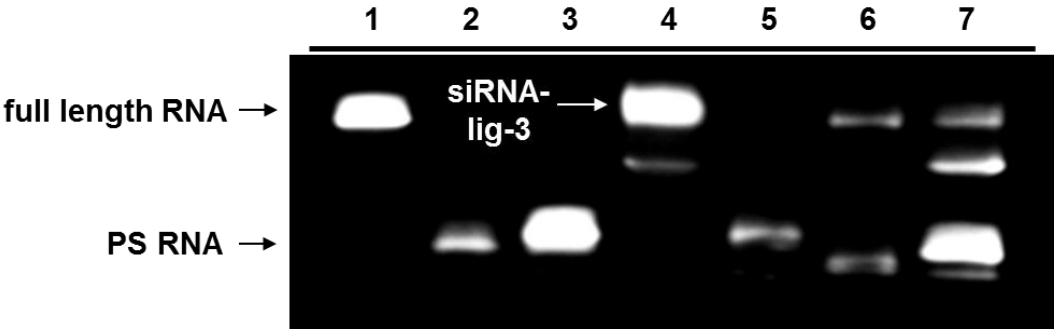


Figure S10. Buildup reaction of ligated siRNA from IBR-RNA triggered by GSH in test tube. Lane 1, 25 mer RNA; lanes 2 and 3, each PS RNA (**11** and **12**); lanes 4 and 5, reaction mixture (**11** + **12** + 1**3** + **14**) with (lane 4) or without (lane 5) GSH; lanes 6 and 7, reaction mixture (lane 6, **11** + **13** or lane 7, **12** + **14**) without complementary strand. Reaction mixture was incubated at 37 °C for 30 min and then samples were analyzed by 20% denaturing PAGE. The gel was stained with SYBR Green II.

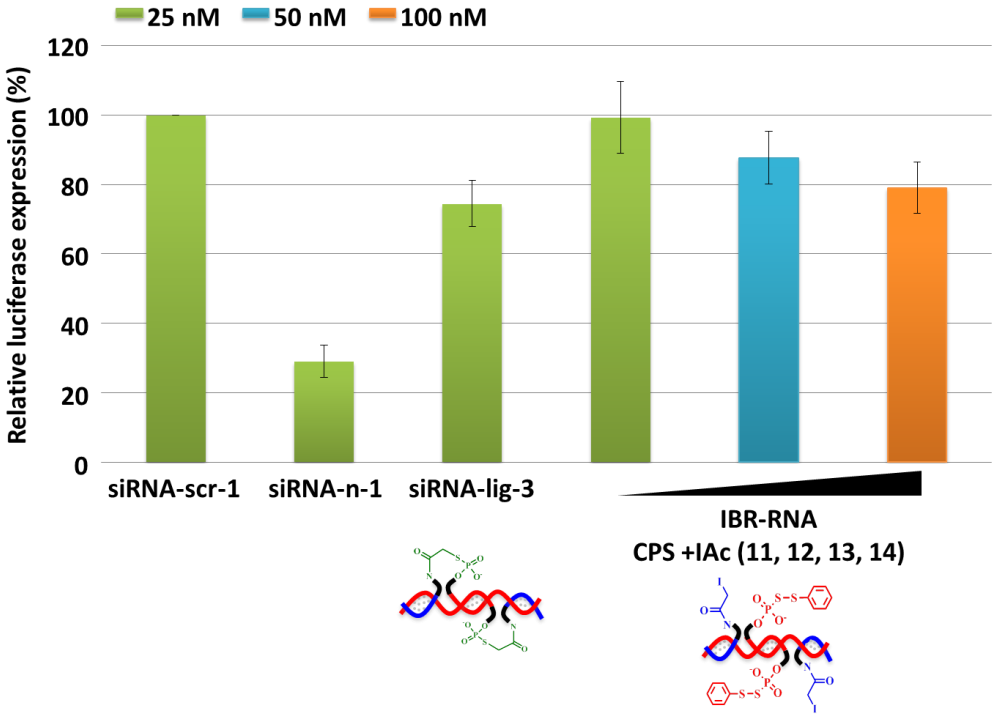


Figure S11. Gene silencing by siRNA-lig-3 and IBR-RNA, a pair of CPS-RNAs (**11**, **12**), and IAc-RNAs (**13**, **14**) in HeLa cells stably expressing luciferase. Luciferase expression was monitored at 24 h post-transfection. The relative expression of luciferase in scramble siRNA (siRNA-scr-1)-transfected cells was defined as 100%. The plotted data are the means ± standard deviation of three independent experiments.