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Gene-silencing by the tRNA maturase tRNase Z^L under the direction of small guide RNA

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Running title: Gene-silencing by tRNase Z^L and sgRNA

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We have been developing a unique system for downregulation of a gene expression through cutting a specific mRNA by the long form of tRNA 3' processing endoribonuclease (tRNase Z^L) under the direction of small guide RNA (sgRNA). However, the efficacy of this system and the involvement of tRNase Z^L in the living cells were not clear. Here we show, by targeting the exogenous luciferase gene, that the efficacy of the sgRNA/tRNase Z^L method can become comparable to that of the RNA interference technology and that the gene-silencing is due to tRNase Z^L directed by sgRNA not due to a simple antisense effect. We also show that tRNase Z^L together with sgRNA can downregulate expression of the endogenous human genes Bcl-2 *and* GSK-3β *by degrading their mRNAs. Furthermore, we demonstrate that a gene expression in the livers of postnatal mice can be inhibited by an only 7-nucleotide sgRNA. These data suggest that sgRNA might be utilized as therapeutic agents to treat diseases such as cancers and AIDS.*

Keywords: *gene-silencing; RNAi; sgRNA; tRNase Z; tRNase Z^L*

Introduction

The tRNAs are transcribed as larger precursor molecules, which subsequently undergo various processing steps such as removal of 5′ and 3′ extra sequences to generate mature tRNAs.[1] tRNase Z is one of the tRNA-maturing enzymes, which removes a 3′ trailer from pre-tRNA.[2–4] Most tRNase Zs cleave pre-tRNAs immediately downstream of a discriminator nucleotide, onto which the CCA residues are added to produce mature tRNA, while tRNase Z from *Thermotoga maritima* exceptionally cleaves pre-tRNAs containing the CCA sequence precisely after the A residue to create the mature 3′-termini.[5]

tRNase Zs can be divided into two groups: a short form (tRNase Z^S) that consists of 300–400 amino acids and a long form (tRNase Z^L) that contains 800–900 amino acids.[6] Bacteria and archaea genomes contain a tRNase Z^S gene only, while eukaryotic genomes encode either only tRNase Z^L or both forms. Interestingly, the human tRNase Z^L is encoded by a prostate cancer-associated gene on chromosome 17, and the human tRNase Z^S gene is on chromosome 18. The C-terminal half region of tRNase Z^L has high similarity to the whole region of tRNase Z^S, and these regions contain a well-conserved histidine motif, which has been shown to be essential for the tRNase Z activity.[5,7]

We have demonstrated *in vitro* that mammalian tRNase Z^L can cleave any target RNA at any desired site by recognizing a pre-tRNA-like or micro-pre-tRNA-like complex formed between the target RNA and artificial small guide RNA (sgRNA).[8–11] sgRNA is divided into four categories, 5′-half-tRNA,[9] 14-nucleotide (nt) linear RNA,[12] RNA heptamer,[10,11] and hook RNA.[13] For example, with the aid of appropriate 5′-half-tRNAs, the two partial HIV-1 RNA targets ENV and GAG were cleaved site-specifically by tRNase Z^L.[9] A 14-nt linear RNA guided target RNA cleavage by tRNase Z^L through forming a 14-base-pair (bp) double-stranded RNA.[12] Amazingly, tRNase Z^L can also recognize a 12-bp hairpin RNA complex between a separate target RNA and an RNA heptamer, and cleave the target.[10,11] In this case, however, the target sites are restricted to immediate downstream regions of stable hairpin structures resembling the T stem/loop. This is advantageous because a heptamer can direct efficient RNA cleavage with a specificity of roughly 12-nt sequence and not merely a 7-nt sequence due to the need for a stable hairpin structure. In contrast, tRNase Z^S can process pre-tRNAs but not these pre-tRNA-like or micro-pre-tRNA-like complexes *in vitro*.[4,6]

We have shown the efficacy of this RNA targeting method in the living cells to some degree by introducing sgRNAs either as their expression plasmids or as

2′-*O*-methyl RNAs.[14,15] The expression of exogenous reporter genes for *Escherichia coli* chloramphenicol acetyltransferase (CAT) and firefly luciferase were downregulated by appropriately designed sgRNAs in mammalian culture cells,[14] and the HIV-1 expression in Jurkat cells was almost completely suppressed by a 5′-half-tRNA-type sgRNA up to 18 days.[15] However, the involvement of tRNase Z^L in this gene-silencing remained unproven.

In this paper, we show that the efficacy of the sgRNA/tRNase Z^L method can become comparable to that of the RNA interference (RNAi) technology and that the gene-silencing is due to tRNase Z^L directed by sgRNA not due to a simple antisense effect. We also demonstrate that tRNase Z^L together with sgRNA can downregulate expression of the endogenous human genes *Bcl-2* and *GSK-3β* and that a gene expression in the livers of postnatal mice can be inhibited by an only 7-nt sgRNA. The present study suggests that the sgRNA/tRNase Z^L technology might surpass the RNAi technology and that sgRNA might be utilized as therapeutic agents to treat diseases such as cancers and AIDS.

Results and discussion

The efficacy of sgRNA can become comparable to that of siRNA

To assess the efficacy of the sgRNA/tRNase Z^L technology more strictly, we compared the gene-silencing level by small interfering RNA (siRNA) with those by sgRNAs by targeting the exogenous firefly luciferase gene. The two types of sgRNA 5′-half-tRNA and 14-nt linear RNA were designed at each of three arbitrary sites (Figure 1). Each 5′-half-tRNA consists of 7- and 5-nt sequences complementary to a sequence 5′ to the desired cleavage site and the D arm of the human pre-$tRNA^{Arg}$ in between.[9] Each 5′-half-tRNA and the target luciferase mRNA can form a pre-tRNA-like structure through 7 and 5 base-pairings corresponding to the acceptor and anticodon stems, respectively. Each linear type sgRNA is a 14-nt sequence complementary to a sequence 5′ to the desired cleavage site, and can form a 14-bp double-stranded RNA with the target RNA, which roughly corresponds to a combination of the acceptor and T stems.[12] An siRNA, siRLuc, targeting downstream to one of the sites, which has been shown to work very efficiently,[16] was used as a positive control, and an unrelated 14-nt linear RNA, sgGSKL2 (5′-CUUAGUCCAAGGAU-3′), was as a negative control.

We co-transfected human kidney 293 cells with the modified luciferase expression plasmid p5LucWT and the reference plasmid pTKβ together with 0.4 μM of siRNA or each 2′-*O*-methyl sgRNA. The plasmid p5LucWT contains a modified luciferase gene that produces an mRNA containing the target site of the heptamer-type

sgRNA Hep1 in the 5′ untranslated region,[14] and the plasmid pTKβ possesses a β-galactosidase gene. All these sgRNAs except for sgLucL1 downregulated the luciferase activity very efficiently to 3.2–13.6% (Figure 2a). The suppression levels by sgLucH2 and sgLucH3 were 3.7 and 3.2%, respectively, and comparable to that by siRLuc (3.0%). Although this result was the first to show that linear type sgRNAs indeed work in the cells, the 5′-half-tRNA type was generally more effective than the 14-nt linear RNA type (Figure 2a). Luciferase mRNA was hardly detected in northern analysis for representative transfectants with sgLucH1 or sgLucH3, suggesting that the downregulation of luciferase activity is due to mRNA degradation not due to translational suppression (Figure 2b).

We also examined how the gene-silencing level would be affected by changing the sgRNA amount using the least efficient 5′-half-tRNA sgLucH1. sgLucH1 was introduced into 293 cells in the range of 0.2 to 4.0 μM. When its amount was increased to more than 1.0 μM, even the least effective sgLucH1 became comparable to siRLuc (Figure 2c). Taken together, these results suggest that gene-silencing efficiency of sgRNA would generally become comparable to that of siRNA as an sgRNA amount is increased.

tRNase Z^L is responsible for the gene-silencing by sgRNA

Although these and previous results[14,15] indicated that appropriate sgRNAs can downregulate targeted gene expressions in the cells, no direct evidence existed demonstrating that the downregulation is due to specific mRNA cleavage by endogenous tRNase Z^L not due to a simple antisense effect or other different mechanisms. To clarify this issue, we generated two types of stable 293 cell line which produce more tRNase Z^L or less tRNase Z^L, and examined silencing levels for the luciferase gene in these transfectants. If tRNase Z^L is the responsible enzyme, the downregulation level should change depending on the tRNase Z^L amount. The 293 cells containing a tRNase Z^L cDNA controlled by a human cytomegalovirus promoter produced ~2-fold more tRNase Z^L, while the cells, in which the tRNase Z^L gene was knocked down by shRNA (Supplementary Table 1), produced the ~4-fold less enzyme (Figure 3a). The tRNase Z^S levels were not affected in these cell lines (Figure 3a). As controls, we also generated another two types of stable 293 cell line which produce more tRNase Z^S or less tRNase Z^S together with the unaffected level of tRNase Z^L (Figure 3a). These cell lines were created in the same way as above.

The 5′-half-tRNA sgLucH1 downregulated the luciferase gene expression to ~14% in normal 293 cells, while its expression level in the cells producing more tRNase

Z^L went down to ~2% in accordance with the expressed tRNase Z^L level (Figure 3b). On the other hand, the silencing level by sgLucH1 was relieved ~7 times in the 293 cells producing ~4-fold less tRNase Z^L (Figure 3b). The downregulation level of luciferase activity by sgLucH1 was not changed very much (relieved only ~1.5-fold) in the 293 cells producing more tRNase Z^S or less tRNase Z^S. This observation is consistent with the *in vitro* observation that tRNase Z^S cannot cleave pre-tRNA-like complexes.[6,12]

In the same fashion, the downregulation level of the luciferase activity by the linear type sgRNA sgLucL1 was enhanced ~3-fold in the tRNase Z^L-overproducing cells, and was relieved ~2.5-fold in the tRNase Z^L-knockdown cells (Figure 3c). The downregulation level was enhanced only slightly (~1.3-fold) in the cell lines producing altered levels of tRNase Z^S. In contrast, the silencing level by siRLuc was not affected significantly in these modified 293 cell lines (Figure 3d). From these results, we concluded that the gene-silencing is attributed to tRNase Z^L directed by sgRNA.

To see how longer linear type RNAs, which cannot guide RNA cleavage by tRNase Z^L *in vitro*,[12] affect gene expressions, we tested the 21- and 28-nt linear type RNAs for luciferase gene-silencing (Figure 4a). A plasmid that can express the linear type RNA from a mouse U6 promoter was co-transfected into the 293 cells together

with the reporter and reference plasmids. Consistent with the *in vitro* observation, the 21- and 28-nt linear type RNAs did not downregulate the luciferase activity, while the sgRNAs sgLucH1 and sgLucL1 did so (Figure 4b). This result supports the involvement of tRNase Z^L in gene-silencing by sgRNA. Under different conditions or by targeting different sites, however, even longer linear type RNA could repress a gene expression to some degree by a simple antisense effect as previously observed using a 25-nt linear type RNA targeting the CAT mRNA.[14]

Downregulation of the Bcl-2 expression

To further confirm the efficacy of the sgRNA/tRNase Z^L technology, we targeted an endogenous gene, *Bcl-2*.[17] Although we have shown that a heptamer type sgRNA targeting mouse Bcl-2 mRNA causes a reduction in Sarcoma 180 cell viability, there was no direct evidence showing degradation of the Bcl-2 mRNA because neither Bcl-2 mRNA nor protein amount was checked.[14] We designed the 5′-half-tRNA sgBclH and the 14-nt linear RNA sgBclL to target human Bcl-2 mRNA (Figure 5a) and generated stable 293 cell lines that produce either of these sgRNAs from a human H1 promoter.

The Bcl-2 mRNA and protein levels were reduced intensively in both transfectants (Figure 5b, c), indicating that the Bcl-2 downregulation is due to

degradation of its mRNA not due to translational suppression. As a result, apoptosis of both 293 transfectants was induced by removing sera from culture media more efficiently (~2-fold by sgBclH and ~1.5-fold by sgBclL) than normal 293 cells (Figure 5d). The increase in apoptosis rate was also observed in four stable transfectants producing one of four different sgRNAs targeting two other sites of Bcl-2 mRNA (data not shown). These results indicate that sgRNA generated from an exogenous plasmid also functions to downregulate an endogenous gene expression.

GSK-3β *silencing*

We also targeted another endogenous human gene, *GSK-3β*, which encodes an evolutionarily conserved serine-threonine kinase involved in several cellular signaling pathways.[18] The 5′-half-tRNA sgGSKH and the 14-nt linear RNA sgGSKL for GSK-3β mRNA targeting were designed (Figure 6a), and stable 293 transfectants that produce either of these sgRNAs from the H1 promoter were generated. In both transfectants, the GSK-3β protein level was drastically decreased while the mRNA level was decreased to only ~43% (Figure 6b, c). This suggests that the decrease in the protein amount would be not only due to degradation of its mRNA but also partly due to translational suppression. Because suppression of the GSK-3β protein level leads to upregulation of a

Lef1/Tcf transcriptional factor, we can monitor the GSK-3β protein level by using the luciferase expression plasmid TOPflash that contains the Lef1/Tcf binding sites. By co-transfecting TOPflash with 2′-*O*-methyl sgGSKH or sgGSKL into normal 293 cells, the luciferase activity was augmented to ~140–150 % (Figure 6d), corroborating the efficacy of these sgRNAs in silencing the endogenous *GSK-3β* gene.

Gene-silencing in postnatal mice

To see whether sgRNAs can function to inhibit gene expressions in organs of postnatal mice, we introduced an sgRNA expression plasmid together with the CAT expression plasmid pcDNA3/CAT into mice through the hydrodynamics-gene-transfer method.[19,20] The sgRNA expression plasmid psgCAT generates the sgRNA sgCAT from the $tRNA^{Arg}$ promoter, which is designed to direct the CAT mRNA cleavage at 222 nucleotides downstream from the initiation codon (Figure 7a).[14] The negative control plasmid psgCATM expresses a similar sgRNA, which, however, contains seven nt substitutions and virtually cannot bind to the CAT mRNA. The CAT protein level in the livers of mice injected with psgCAT was reduced ~4-fold compared with that with psgCATM (Figure 7b). This result closely parallels what we observed in the transfection experiment with MDCK cells,[14] and indicates that sgRNA indeed works to suppress a

gene expression in postnatal mice.

Gene-silencing by an RNA heptamer in mice

We also examined whether heptamer-type sgRNA can function to knock down a specific mRNA in animals by testing a 2′-*O*-methyl RNA heptamer that targets the luciferase mRNA for reduction of its activity. The heptamer Hep1, Hep2 or Bclhep was injected into mice together with the modified luciferase expression plasmid p5LucWT. Hep1 is complementary to the 7-nt target sequence in the 5′ untranslated region of the luciferase mRNA and can form a perfect 12-bp stem-loop complex with the modified luciferase mRNA, whereas Hep2 contains three nt substitutions (Figure 7a), and Bclhep has an unrelated sequence (5′-GGGGGCA-3′). As expected from the observations in the cell culture experiment,[14] Hep1 injection into mice downregulated the luciferase activity ~4-fold relative to Hep2 or Bclhep injection (Figure 7c), indicating the efficacy of heptamer-type sgRNA in postnatal mice.

To examine whether the downregulation by the RNA heptamer is also through mRNA cleavage by tRNase Z^L, we compared the downregulation level in normal 293 cells with those in modified 293 cells producing more tRNase Z^L or less tRNase Z^L. The downregulation level was augmented ~8-fold in the tRNase Z^L-overproducing cell line

and relieved ~2.3-fold in the tRNase Z^L-knockdown cell line (Figure 7d). In contrast, the downregulation level was not changed significantly in the 293 cells producing more tRNase Z^S or less tRNase Z^S. These results confirm the involvement of tRNase Z^L in the heptamer-directed gene-silencing.

The small size of heptamer RNA would provide some advantages; heptamers can be taken up readily by living cells without the use of stimulating reagents such as cationic liposomes, and are much easier and cheaper to synthesize than long oligomers such as siRNA.

Another role of tRNase Z^L

Here we convincingly demonstrated that, under the direction of sgRNA, tRNase Z^L can downregulate a gene expression by eliminating its mRNA. This, together with the fact that tRNase Z^L has the extra N-terminal region that is dispensable for pre-tRNA processing,[4,6] implies an additional role of this enzyme. There exist a huge number of miRNAs that are believed to work as translational regulators,[21] and, curiously, their length is ~21–23 nt, which is similar to the length of hook-type sgRNA. These observations suggest that some of the miRNAs could work as hook RNA that directs cellular RNA cleavage by tRNase Z^L.

Over the RNAi technology

As we showed, the efficacy of the sgRNA/tRNase Z^L technology can become comparable to that of the RNAi technology. In addition, sgRNA has some advantages over siRNA. sgRNA would never trigger interferon response and a subsequent translational shutdown.[22] A potential off-target effect of sgRNA would be much less serious than that of siRNA because the length of an sgRNA sequence complementary to target RNA is only 7–14 nt, much smaller than siRNAs' length of ~21–23 nt.[22] sgRNA, especially the 14-nt linear type, could be used to cleave and eliminate even miRNA to elucidate each miRNA's function, while siRNA could not judging from the RNAi mechanism. These considerations suggest that the sgRNA/tRNase Z^L technology might partly take over the siRNAs' job, and that sgRNA might be utilized as therapeutic agents to treat diseases such as cancers and AIDS.

Materials and methods

RNA

The 5′-phosphorylated 2′-*O*-methyl RNAs and the siRNA siRLuc were synthesized with a DNA/RNA synthesizer and purified by high-performance liquid chromatography

(Nippon Bioservice).

Plasmid construction

The tRNase Z^L and tRNase Z^S expression plasmids were generated by cloning their cDNAs[4] into pcDNA4/TO/myc-His (Invitrogen). The shRNA expression plasmids for silencing the tRNase Z^L and tRNase Z^S genes and the sgRNA expression plasmids for silencing *Bcl-2* and *GSK-3β* genes were constructed by inserting annealed synthetic oligonucleotides (Supplementary Table 1) between *Bam*H I and *Hin*d III sites of pRNATin-H1.2/Neo (GenScript). Plasmids for expression of sgLucH1, sgLucL1, LucL21, and LucL28 were constructed by inserting annealed synthetic DNA oligonucleotides (Supplementary Table 1) between *Apa* I and *Eco*R I sites of pSilencer 1.0-U6 (Ambion). The primary transcripts from pRNATin-H1.2/Neo and pSilencer 1.0-U6 were designed to include a 3′ uridine stretch corresponding to the RNA polymerase III terminator.

Cell culture

The human kidney 293 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (MP Biomedicals) and 1% penicillin-streptomycin (Invitrogen) at

37°C in a 5% CO_2 humidified incubator.

Transfection

The 293 cells were transfected with plasmids or with plasmids and 2′-*O*-methyl sgRNA using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. To generate stable transfectants that possess a gene controlled by a tetracycline-inducible promoter, we co-transfected the 293 cells with a plasmid from pcDNA4/TO/myc-His or from pRNATin-H1.2/Neo together with the tetracycline repressor expression plasmid pcDNA6/TR (Invitrogen). Stable transfectants were selected and cultured in the presence of 2 μg/ml of blasticidin (Invitrogen) and 50 μg/ml of zeocin (Invitrogen) or 500 μg/ml of geneticin (MP Biomedicals). Although the gene expression from the tetracycline-inducible promoter was observed even in the absence of tetracycline, it was added at 3 μg/ml to culture media to ensure the expression.

Luciferase assays and CAT assays

These assays were performed basically as described before.[14] In culture cell experiments, the modified luciferase expression plasmid p5LucWT[14] and the luciferase plasmid TOPflash (Upstate Biotechnology) were used as reporters, and the β-galactosidase

expression plasmid pTKβ (Clontech) was as a reference.

Northern analysis

Total RNA (20 μg per lane), extracted from 293 cells with ISOGEN (Nippon Gene), was separated by formaldehyde/1% agarose gel electrophoresis, and transferred to a nitrocellulose membrane. Hybridization with digoxigenin-labeled RNA probes and detection were performed according to the manufacturer's protocol (Roche Diagnostics).

Real-time reverse transcriptase-PCR

cDNA synthesis for 1 μg of total RNA was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time PCR for the cDNA was performed using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit according to the manufacturer's protocol (Roche Diagnostics). The primers for amplifying a 120-bp fragment of GSK-3β cDNA were 5′-TGCATTAAGCACCTGCGC-3′ and 5′-AGCAGGACAGCCACTCTCC-3′,[23] and the primers for amplifying a 187-bp fragment of glyceroaldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA were 5′-ACCCACTCCTCCACCTTTG-3′ and 5′-CTCTTGTGCTCTTGCTGGG-3′.

Western analysis

Whole cell extracts, dissolved in a buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT), were separated by SDS/10% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-Bcl-2 or anti-GSK-3β antibodies (BD biosciences) or antibodies raised to a human tRNase Z^L peptide (amino acid 812–826) or recombinant human tRNase Z^S using the ECL Western Blotting Detection System (Amersham Biosciences).

Measurement of cell numbers

The living cell number was measured using TetraColor ONE (Seikagaku) according to the manufacturer's protocol.

Hydrodynamics-based transfection

Four-week-old JcI:ICR female mice (18–20 g) were co-injected through the hydrodynamics-based gene transfer method[19,20] with a 1.5-ml buffer containing 5 μg of pcDNA3/CAT (Invitrogen), 5 μg of pGL3-control (Promega) (or p5LucWT for luciferase targeting), and 5 μg of psgCAT or psgCATM, (or 3 nmol of the 2′-*O*-methyl

RNA heptamer Hep1, Hep2 or Bclhep for luciferase targeting). The CAT protein amounts and the luciferase activities in mouse liver extracts were measured 8 h after injection. These experiments were carried out in accordance with the guidelines on the care and use of laboratory animals issued by Hokkaido University.

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Note: Supplementary information is available at the Gene Therapy's website.

References

1 Mörl M, Marchfelder A. The final cut. The importance of tRNA 3′-processing. *EMBO Rep* 2001; **2:** 17–20.

2 Nashimoto M. Distribution of both lengths and 5′ terminal nucleotides of mammalian pre-tRNA 3′ trailers reflects properties of 3′ processing endoribonuclease. *Nucleic Acids Res* 1997; **25:** 1148–1155.

3 Schiffer S, Rosch S, Marchfelder A. Assigning a function to a conserved group of proteins: the tRNA 3′-processing enzymes. *EMBO J* 2002; **21:** 2769–2777.

4 Takaku H, Minagawa A, Masamichi T, Nashimoto M. A candidate prostate cancer susceptibility gene encodes tRNA 3′ processing endoriobonuclease. *Nucleic Acids Res* 2003; **31:** 2272–2278.

5 Minagawa A, Takaku H, Takagi M, Nashimoto M. A novel endonucleolytic mechanism to generate the CCA 3′ termini of tRNA molecules in *Thermotoga maritima*. *J Biol Chem* 2004; **279:** 15688–15697.

6 Takaku H, Minagawa A, Takagi M, Nashimoto M. The N-terminal half-domain of the long form of tRNase Z is required for the RNase 65 activity. *Nucleic Acids Res* 2004; **32:** 4429–4438.

7 Zareen N, Yan H, Hopkinson A, Levinger L. Residues in the conserved His domain

of fruit fly tRNase Z that function in catalysis are not involved in substrate recognition or binding. *J Mol Biol* 2005; **350:** 189–199.

8 Nashimoto M. Conversion of mammalian tRNA 3′ processing endoribonuclease to four-base-recognizing RNA cutters. *Nucleic Acids Res* 1995; **23:** 3642–3647.

9 Nashimoto M. Specific cleavage of target RNAs from HIV-1 with 5′ half tRNA by mammalian tRNA 3′ processing endoribonuclease. *RNA* 1996; **2:** 2523–2524.

10 Nashimoto M, Geary S, Tamura M, Kasper R. RNA heptamers that directs RNA cleavage by mammalian tRNA 3′ processing endoribonuclease. *Nucleic Acids Res* 1998; **26:** 2565–2571.

11 Nashimoto M. Anomalous RNA substrates for mammalian tRNA 3′ processing endoribonuclease. *FEBS Letters* 2000; **472:** 179–186.

12 Shibata HS, Takaku H, Takagi M, Nashimoto M. The T loop structure is dispensable for substrate recognition by tRNase ZL. *J Biol Chem* 2005; **280:** 22326–22334.

13 Takaku H, Minagawa A, Takagi M, Nashimoto M. A novel four-base-recognizing RNA cutter that can remove the single 3′ terminal nucleotides from RNA molecules. *Nucleic Acids Res* 2004; **32:** *e*91.

14 Tamura M, Nashimoto C, Miyake N, Daikuhara Y, Ochi K, Nashimoto M. Intracellular mRNA cleavage by 3′ tRNase under the direction of 2′-*O*-methyl RNA

heptamers. *Nucleic Acids Res* 2003; **31:** 4354–4360.

15 HabuY, Miyano-Kurosaki N, Kitano M, Endo Y, Yukita M, Ohira S, Takaku H, Nashimoto M, Takaku H. Inhibition of HIV-1 gene expression by retroviral vector-mediated small-guide RNAs that direct specific RNA cleavage by tRNase ZL. *Nucleic Acids Res* 2005; **33:** 235–243.

16 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411:** 494–498.

17 Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol* 2003; **4:** 721–729.

18 Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; **303:** 1483–1487.

19 Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* 1999; **6:** 1258–1266.

20 Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Therapy* 1999; **10:** 1735–1737.

21 McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs.

Nature Rev Genet 2002; **3:** 737–747.

22 Appasani K. *RNA interference technology: from basic science to drug development.* Cambridge University Press: Cambridge, 2005.

23 Cui J, Zhou X, LiuY, Tang Z, Romeih M. Wnt signaling in hepatocellular carcinoma: analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes. *J Gastroenterol Hepatol* 2003; **18:** 280–287.

Figure legends

Figure 1 sgRNAs targeting the luciferase mRNA. sgRNA and siRNA (siRLuc) target sites on the luciferase mRNA and plausible secondary structures of sgRNA/target complexes are shown with expected cleavage sites. The target sequence is numbered starting with the luciferase initiation codon.

Figure 2 The efficacy of sgRNA can become comparable to that of siRNA. (**a**, **c**) The 293 cells were transiently co-transfected with each 2′-*O*-methyl sgRNA or siRNA together with the luciferase reporter and β-galactosidase reference plasmids (0.2 μg/ml each). Luciferase activity (Luc) is normalized against β-galactosidase activity (Gal). Data represent the means ± s.d. of three independent experiments. (**b**) Northern analysis for transcripts from representative transfectants.

Figure 3 The gene-silencing effect by sgRNA depends on the tRNase Z^L amount. (**a**) Detection of tRNase Z^L and tRNase Z^S in normal, tRNase Z^L over-expressing (+tRNase Z^L), tRNase Z^L knockdown (-tRNase Z^L), tRNase Z^S over-expressing (+tRNase Z^S), and tRNase Z^S knockdown (-tRNase Z^S) 293 cells by western analysis. (**b**–**d**) Each 293 cell line was transiently co-transfected with each 2′-*O*-methyl sgRNA

(0.4 μM) or siRLuc (0.01 μM) together with the reporter and reference plasmids (0.2 μg/ml each). Luciferase activity is normalized against β-galactosidase activity. Relative percentages to the normalized luciferase activity in each cell line transfected with the control sgGSKL2 are shown. Data represent the means ± s.d. of three independent experiments.

Figure 4 The longer linear type RNA cannot function as sgRNA. (**a**) A partial structure of the complex of luciferase mRNA with LucL21 or LucL28. The target sequence is numbered starting with the luciferase initiation codon. (**b**) The 293 cells were transiently co-transfected with each small RNA expression plasmid (0.2 μg/ml) together with the luciferase reporter and β-galactosidase reference plasmids (0.2 μg/ml each). Luciferase activity shown is normalized against β-galactosidase activity. Data represent the means ± s.d. of three independent experiments.

Figure 5 Downregulation of the endogenous human gene *Bcl-2* by sgRNA. (**a**) A partial structure of the complex of the human Bcl-2 mRNA with sgBclH or sgBclL. An arrow denotes an expected cleavage site. The target sequence is numbered starting with the Bcl-2 initiation codon. (**b**, **c**) Detection of Bcl-2 mRNA and protein in normal 293 cells

and stable 293 transfectants containing an sgBclH or sgBclL expression system by northern (**b**) and western (**c**) analyses, respectively. (**d**) Cell viability after serum removal was measured in each cell line. Data represent the means ± s.d. of three independent experiments.

Figure 6 Silencing of Human *GSK-3β* by sgRNA. (**a**) A partial structure of the complex of the human GSK-3β mRNA with sgGSKH or sgGSKL. An arrow denotes an expected cleavage site. The target sequence is numbered starting with the GSK-3β initiation codon. (**b**, **c**) Detection of GSK-3β mRNA and protein in normal 293 cells and stable 293 cell lines containing an sgGSKH or sgGSKL expression system by real-time PCR (**b**) and western (**c**) analyses, respectively. The amount of GSK-3β mRNA is normalized against that of GAPDH mRNA. Data represent the means ± s.d. of three independent experiments. (**d**) Normal 293 cells were transiently co-transfected with each 2′-*O*-methyl sgRNA (0.4 μM) together with the reporter and reference plasmids (0.2 μg/ml each). Luciferase activity is normalized against β-galactosidase activity. Data represent the means ± s.d. of three independent experiments.

Figure 7 sgRNA-directed knockdown of the CAT or luciferase mRNA in postnatal mice.

(**a**) Plausible secondary structures of the sgCAT/CAT mRNA complex and the Hep1/luciferase mRNA complex. Arrows crossing the mRNAs indicate the expected tRNase Z^L cleavage sites.[10,11] The substituted bases in sgCATM and Hep2 are shown by arrows. (**b**, **c**) Relative CAT protein levels (**b**) and relative luciferase activities (**c**) in mouse liver extracts. Data represent the means ± s.d. of three independent experiments, and were significant at $p < 0.01$ by the Student's *t*-test. (**d**) Each 293 cell line (described in the legend to Figure 3) was transiently co-transfected with each 2′-*O*-methyl Hep1 (3 μM) together with the reporter and reference plasmids (0.2 μg/ml each). Luciferase activity is normalized against β-galactosidase activity. Relative percentages to the normalized luciferase activity in each cell line transfected with the control sgGSKL2 are shown. Data represent the means ± s.d. of three independent experiments.

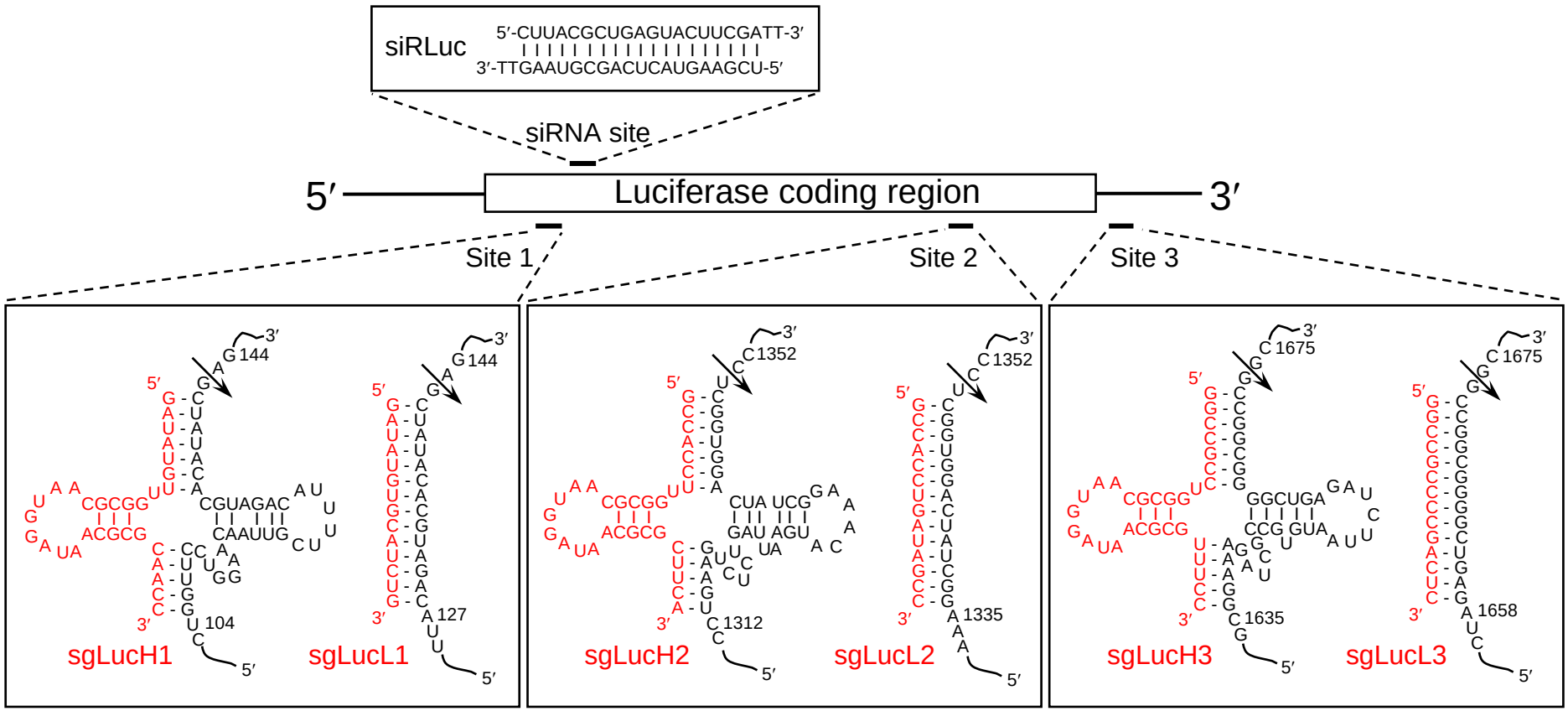

siRLuc
5′-CUUACGCUGAGUACUUCGATT-3′
3′-TTGAAUGCGACUCAUGAAGCU-5′
siRNA site
5′
Luciferase coding region
3′
Site 1
Site 2
Site 3
sgLucH1
sgLucL1
sgLucH2
sgLucL2
sgLucH3
sgLucL3

Figure 1

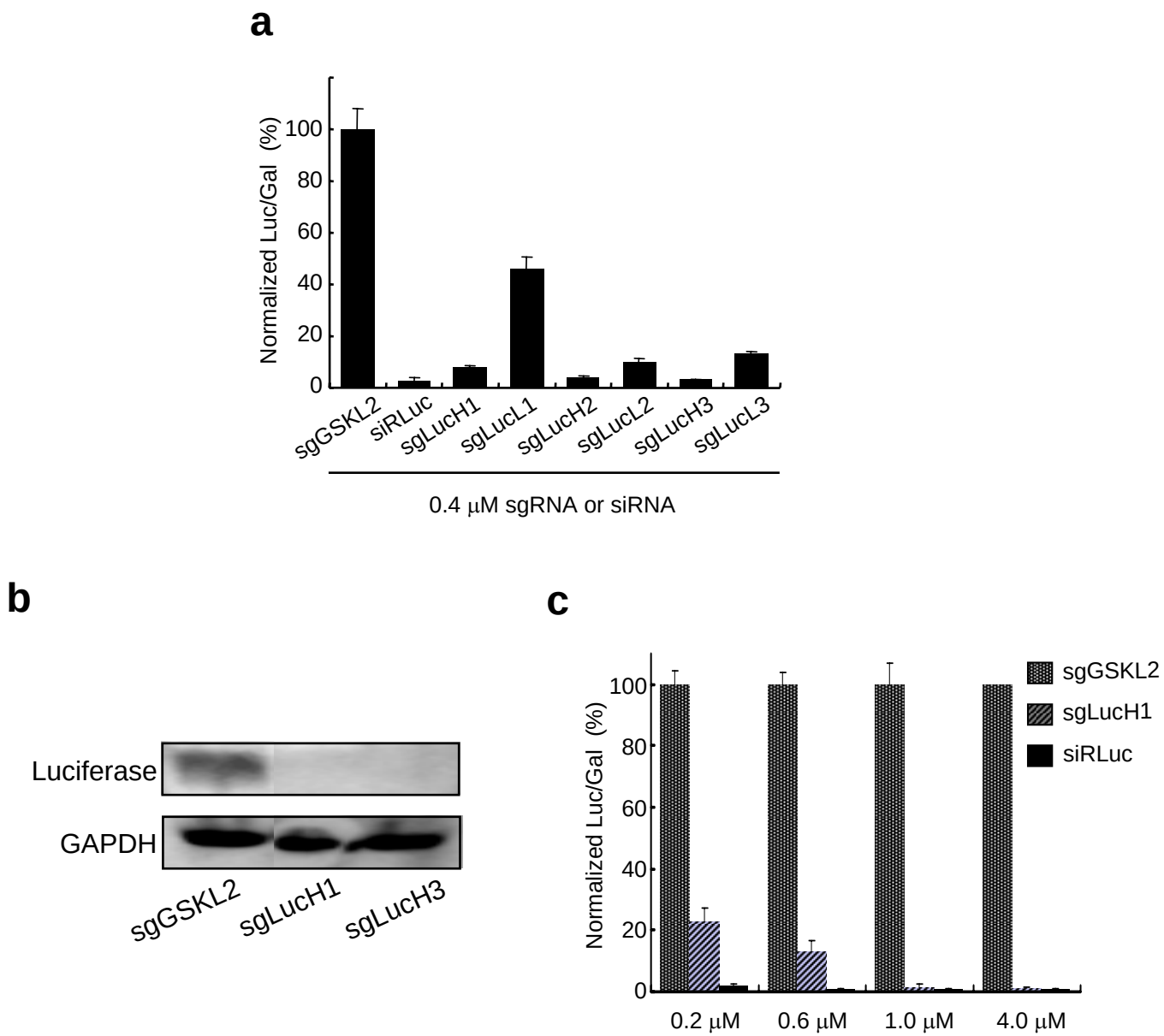

a
Normalized Luc/Gal (%)
100
80
60
40
20
0
sgGSKL2
siRLuc
sgLucH1
sgLucL1
sgLucH2
sgLucL2
sgLucH3
sgLucL3
0.4 μM sgRNA or siRNA
b
Luciferase
GAPDH
sgGSKL2
sgLucH1
sgLucH3
c
Normalized Luc/Gal (%)
100
80
60
40
20
0
sgGSKL2
sgLucH1
siRLuc
0.2 μM
0.6 μM
1.0 μM
4.0 μM

Figure 2

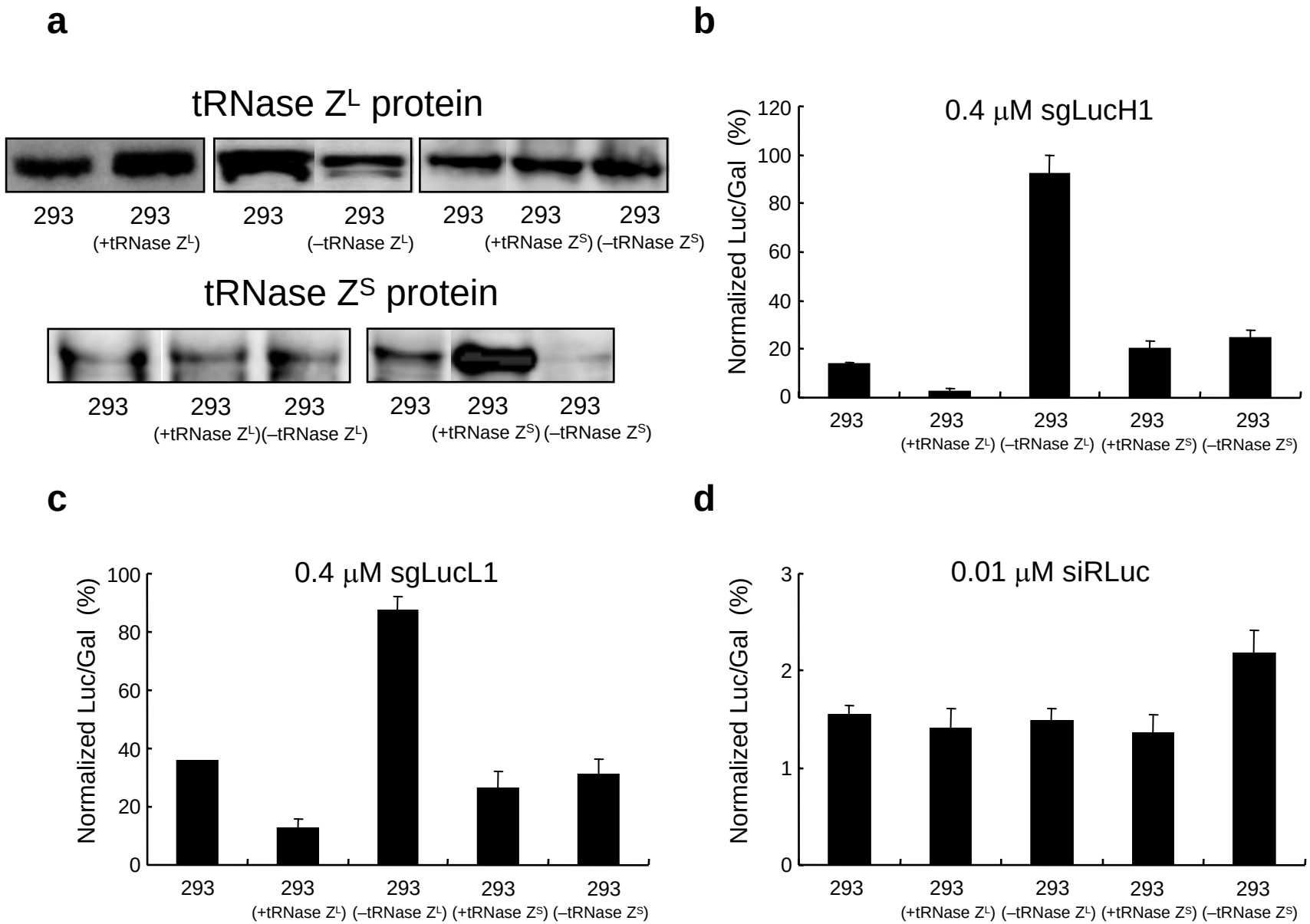

a
tRNase ZL protein
293 293 293 293 293 293 293
(+tRNase ZL) (–tRNase ZL) (+tRNase ZS) (–tRNase ZS)
tRNase ZS protein
293 293 293 293 293 293
(+tRNase ZL)(–tRNase ZL) (+tRNase ZS) (–tRNase ZS)
b
0.4 μM sgLucH1
Normalized Luc/Gal (%)
120
100
80
60
40
20
0
293 293 293 293 293
(+tRNase ZL) (–tRNase ZL) (+tRNase ZS) (–tRNase ZS)
c
0.4 μM sgLucL1
Normalized Luc/Gal (%)
100
80
60
40
20
0
293 293 293 293 293
(+tRNase ZL) (–tRNase ZL) (+tRNase ZS) (–tRNase ZS)
d
0.01 μM siRLuc
Normalized Luc/Gal (%)
3
2
1
0
293 293 293 293 293
(+tRNase ZL) (–tRNase ZL) (+tRNase ZS) (–tRNase ZS)

Figure 3

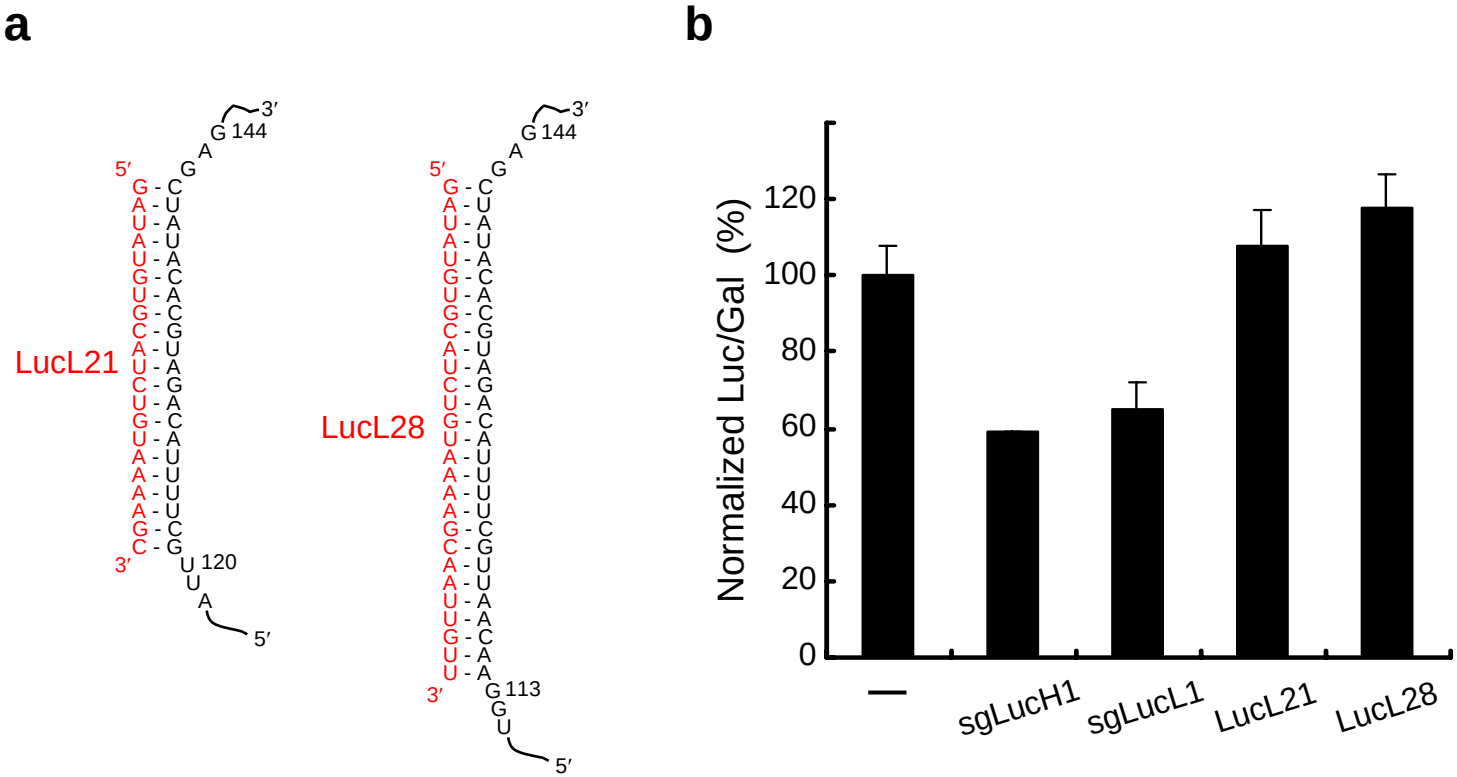

a
LucL21
LucL28
3′
G 144
A
G
5′
G - C
A - U
U - A
A - U
U - A
G - C
U - A
G - C
C - G
A - U
C - G
U - A
G - A
U - C
G - A
U - A
A - U
A - U
A - U
G - C
C - G
3′
U 120
U
A
5′
3′
G 144
A
G
5′
G - C
A - U
U - A
A - U
U - A
G - C
U - A
G - C
C - G
A - U
C - G
U - A
G - A
U - C
G - A
U - A
A - U
A - U
A - U
G - C
C - G
A - U
U - A
A - U
G - C
U - A
3′
G 113
G
U
5′
b
Normalized Luc/Gal (%)
120
100
80
60
40
20
0
—
sgLucH1
sgLucL1
LucL21
LucL28

Figure 4

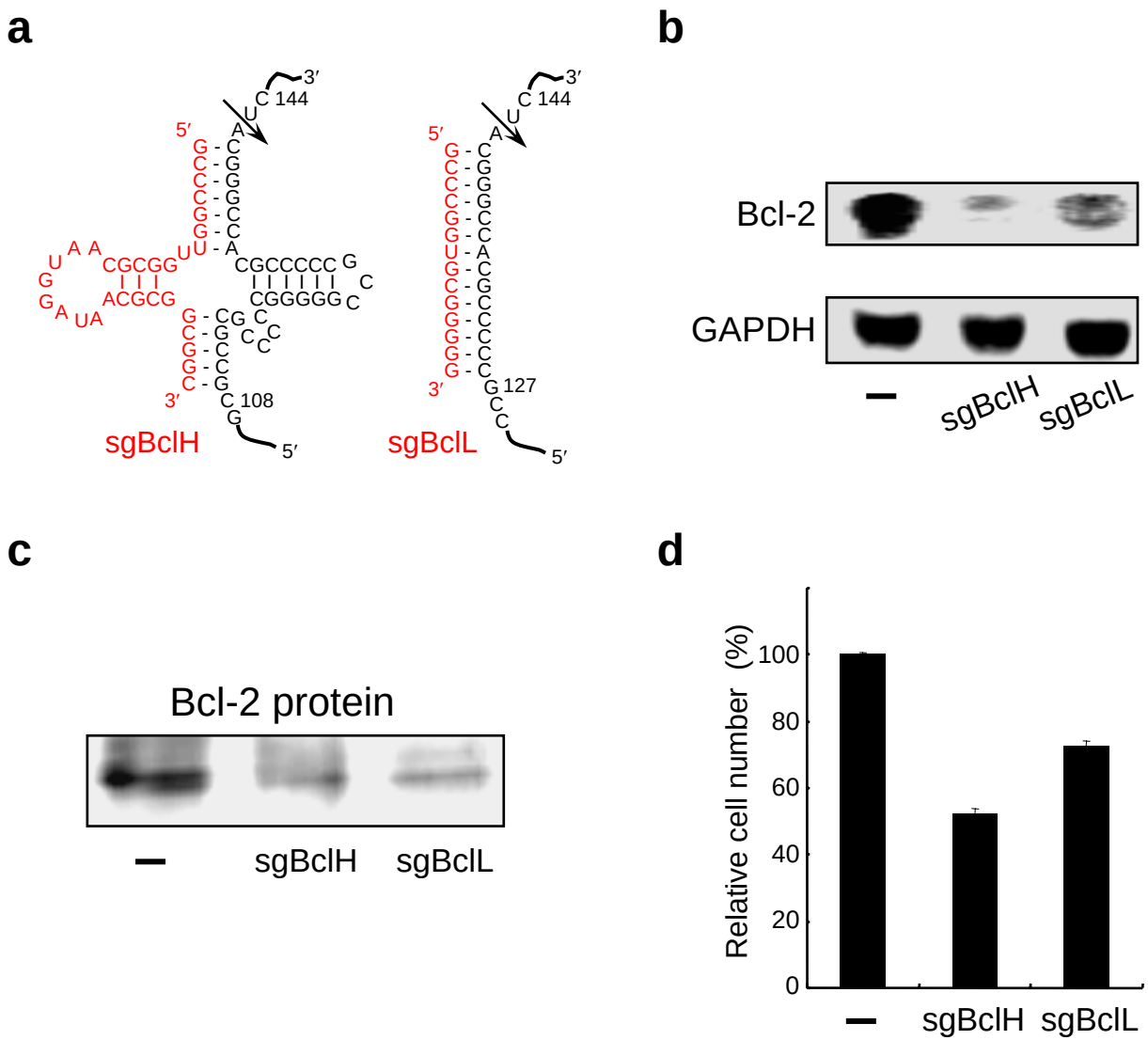

a
3′
C 144
U
A
5′
G - C
C - G
C - G
C - G
G - C
G - C
U - A
A A
U
CGCGG
G
ACGCG
G
A
UA
U
CGCCCCC G
C
CGGGGG C
G - CG C
C - G C C
G - C
G - C
C - G
C 108
G
3′
5′
sgBclH
3′
C 144
U
A
5′
G - C
C - G
C - G
C - G
G - C
G - C
U - A
G - C
C - G
C - G
G - C
G - C
G - C
G 127
C
C
3′
5′
sgBclL
b
Bcl-2
GAPDH
–
sgBclH
sgBclL
c
Bcl-2 protein
–
sgBclH
sgBclL
d
Relative cell number (%)
100
80
60
40
20
0
–
sgBclH
sgBclL

Figure 5

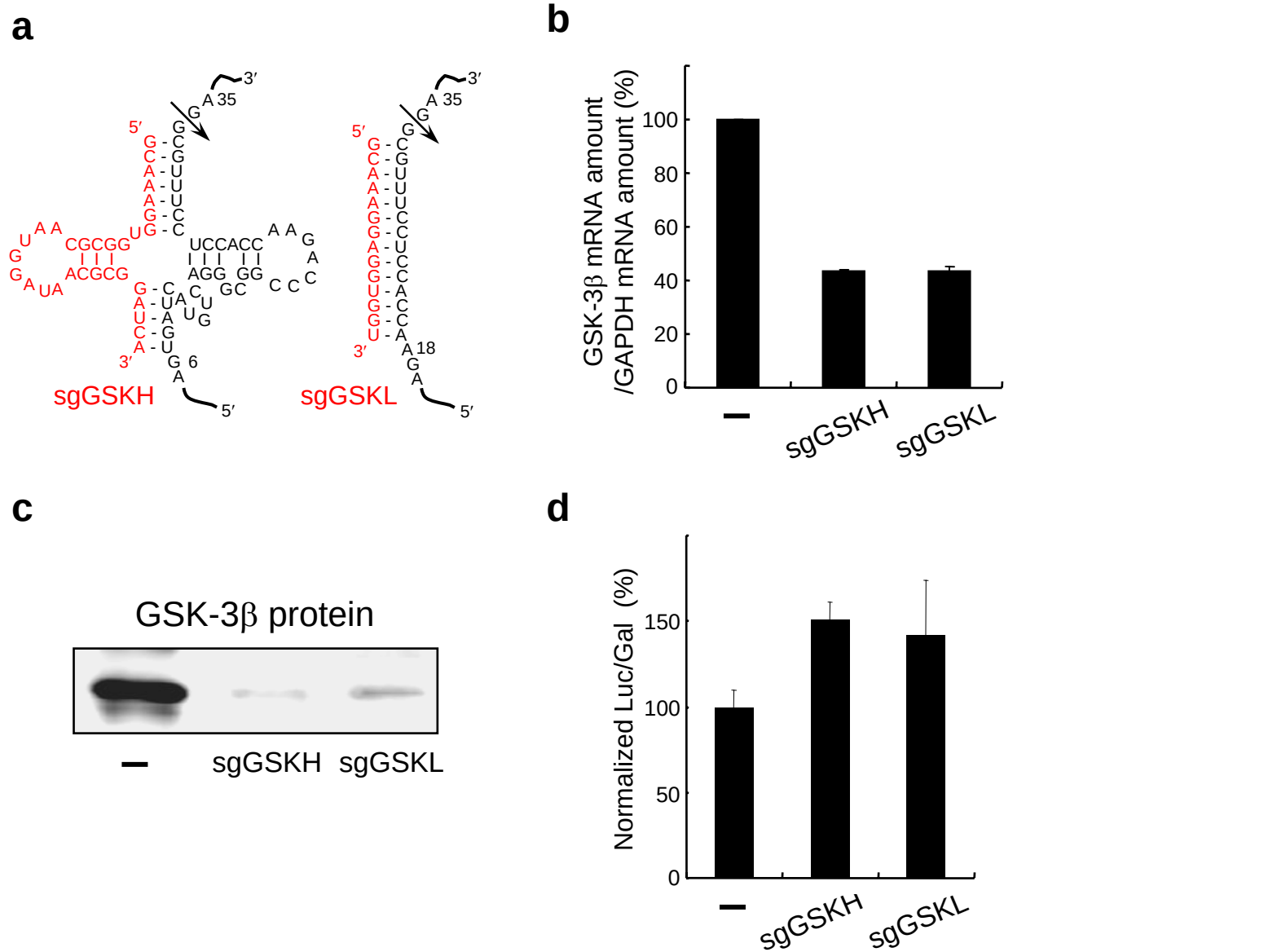
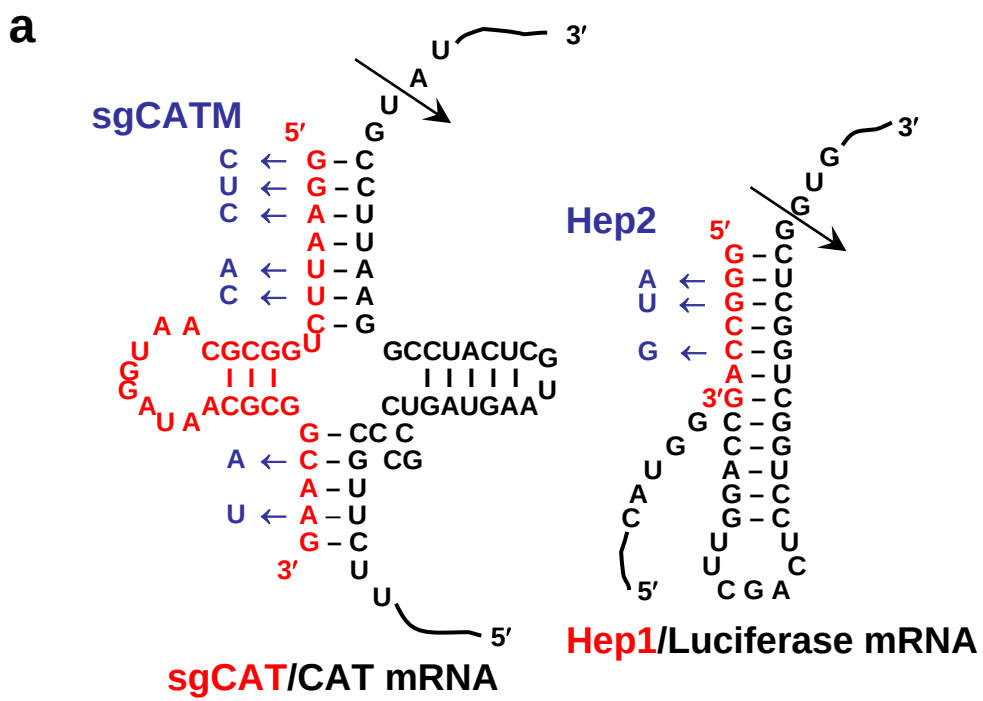
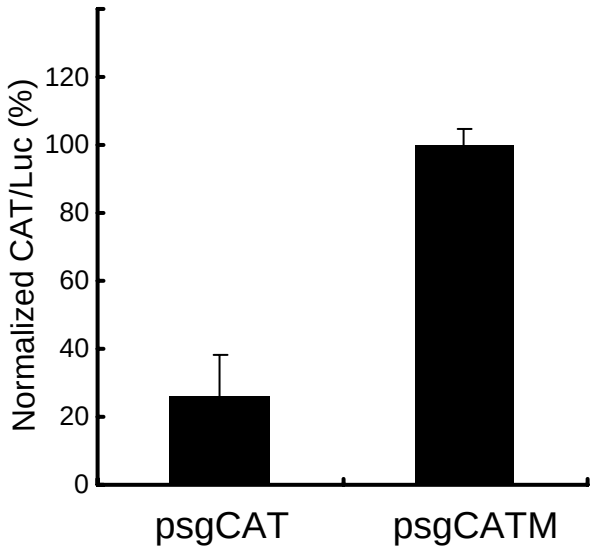
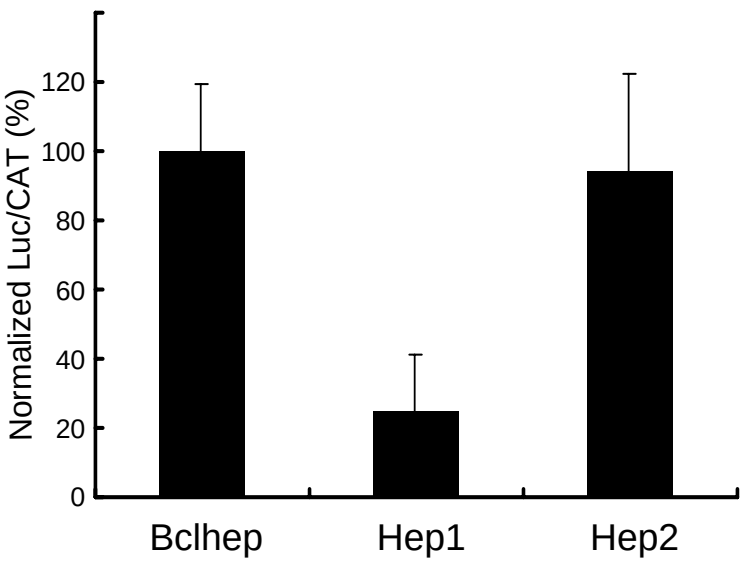

a
sgGSKH
sgGSKL
b
GSK-3β mRNA amount
/GAPDH mRNA amount (%)
100
80
60
40
20
0
–
sgGSKH
sgGSKL
c
GSK-3β protein
–
sgGSKH
sgGSKL
d
Normalized Luc/Gal (%)
150
100
50
0
–
sgGSKH
sgGSKL

Figure 6


a
sgCATM
5′
3′
5′
sgCAT/CAT mRNA
Hep2
5′
3′
Hep1/Luciferase mRNA


b
Normalized CAT/Luc (%)
120
100
80
60
40
20
0
psgCAT
psgCATM


c
Normalized Luc/CAT (%)
120
100
80
60
40
20
0
Bclhep
Hep1
Hep2

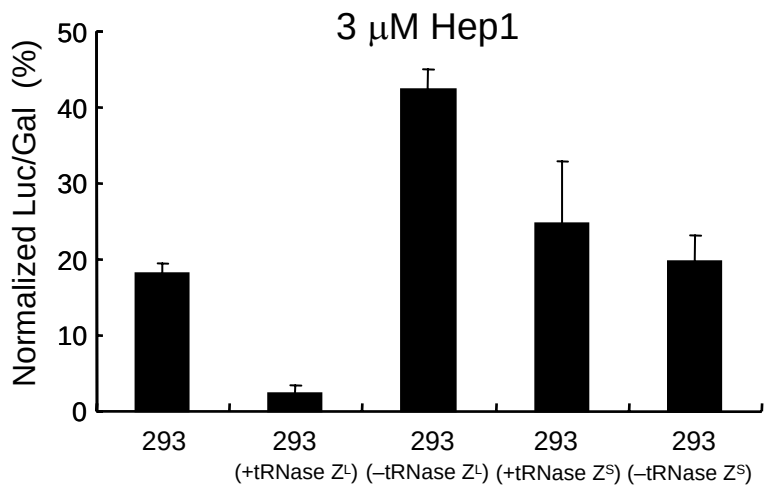

d
3 μM Hep1
Normalized Luc/Gal (%)
50
40
30
20
10
0
293
293
293
293
293
(+tRNase ZL)
(–tRNase ZL)
(+tRNase ZS)
(–tRNase ZS)

Figure 7

Supplementary Table 1. DNA oligonucleotides used to construct plasmids

Name		Sequence
pRNATin-H1.2/Neo		
shRNA(tRNase Z^L)	sense	5'-GATCCCGGCATCCCCACGCTGATGGCTTGATATCCGGCCATCAGCGTGGGGATGCTTTTTTCCAAA-3'
shRNA(tRNase Z^L)	antisense	5'-AGCTTTTGGAAAAAAGCATCCCCACGCTGATGGCCGGATATCAAGCCATCAGCGTGGGGATGCCGG-3'
shRNA(tRNase Z^S)	sense	5'-GATCCCGTTGCTGTGTATCTCAATGGTTGATATCCGCCATTGAGATACACAGCAATTTTTTCCAAA-3'
shRNA(tRNase Z^S)	antisense	5'-AGCTTTTGGAAAAAATTGCTGTGTATCTCAATGGCGGATATCAACCATTGAGATACACAGCAACGG-3'
sgBclH	sense	5'-GATCGCCCGGTTGGCGCAATGGATAACGCGGCGGCTTTTTT-3'
sgBclH	antisense	5'-AGCTAAAAAAGCCGCCGCGTTATCCATTGCGCCAACCGGGC-3'
sgBclL	sense	5'-GATCGCCCGGTGCGGGGGTTTTTT-3'
sgBclL	antisense	5'-AGCTAAAAAACCCCCGCACCGGGC-3'
sgGSKH	sense	5'-GATCGCAAAGGTGGCGCAATGGATAACGCGGATCATTTTTT-3'
sgGSKH	antisense	5'-AGCTAAAAAATGATCCGCGTTATCCATTGCGCCACCTTTGC-3'
sgGSKL	sense	5'-GATCGCAAAGGAGGTGGTTTTTTT-3'
sgGSKL	antisense	5'-AGCTAAAAAAACCACCTCCTTTGC-3'
pSilencer 1.0-U6		
sgLucH1	sense	5'-GATATGTTGGCGCAATGGATAACGCGGAACCTTTTTT-3'
sgLucH1	antisense	5'-AATTAAAAAAGGTTCCGCGTTATCCATTGCGCCAACATATCGGCC-3'
sgLucL1	sense	5'-GATATGTGCATCTGTTTTTT-3'
sgLucL1	antisense	5'-AATTAAAAAACAGATGCACATATCGGCC-3'
LucL21	sense	5'-GATATGTGCATCTGTAAAAGCTTTTTT-3'
LucL21	antisense	5'-AATTAAAAAAGCTTTTACAGATGCACATATCGGCC-3'
LucL28	sense	5'-GATATGTGCATCTGTAAAAGCAATTGTTTTTTTT-3'
LucL28	antisense	5'-AATTAAAAAAAACAATTGCTTTTACAGATGCACATATCGGCC-3'