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SIDT2 mediates gymnosis, the uptake of naked single-stranded oligonucleotides into living cells

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ABSTRACT

Single-stranded oligonucleotides (ssOligos) are efficiently taken up by living cells without the use of transfection reagents. This phenomenon called ‘gymnosis’ enables the sequence-specific silencing of target genes in various types of cells. Several antisense ssOligos are used for the treatment of human diseases. However, the molecular mechanism underlying the uptake of naked ssOligos into cells remains to be elucidated. Here, we show that systemic RNA interference deficient-1 (SID-1) transmembrane family 2 (SIDT2), a mammalian ortholog of the *Caenorhabditis elegans* double-stranded RNA channel SID-1, mediates gymnosis. We show that the uptake of naked ssOligos into cells is significantly downregulated by knockdown of SIDT2. Furthermore, knockdown of SIDT2 inhibited the effect of antisense RNA mediated by gymnosis. Overexpression of SIDT2 enhanced the uptake of naked ssOligos into cells, while a single amino acid mutation in SIDT2 abolished this effect. Our findings highlight the mechanism of extra- and intracellular RNA transport and may contribute to the further development of nucleic acid-based therapies.

Abbreviations: exRNA, extracellular RNA; miRNA, microRNA; miR-16, miRNA-16; miR-19a, miRNA-19a; MEFs, mouse embryonic fibroblasts; qPCR, quantitative reverse transcription PCR; sgRNA, small guide RNA; SID-1, systemic RNA interference defective-1; SIDT1, SID1 transmembrane family, member 1; SIDT2, SID1 transmembrane family, member 2; siRNA, small interference RNA; ssOligos, single-stranded oligonucleotides; ssRNA, single-stranded RNA

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Introduction

Nucleic acid based drugs, which could be applied to the treatment of various diseases,¹ include antisense oligonucleotides,² aptamers,^{3,4} small interference RNA (siRNA),^{5,6} antisense oligonucleotides targeting microRNAs (miRNAs),^{7,8} miRNA mimics,⁹ decoy oligonucleotides,¹⁰ and CpG oligonucleotides.¹¹ Currently, antisense oligonucleotides, including RNA and/or DNA, unassisted by transfection reagents are clinically used for the treatment of homozygous familial hypercholesterolemia^{12–14} and cytomegalovirus retinitis.^{15,16}

Recently, Stein et al. reported that single-stranded oligonucleotides (ssOligos) can be efficiently taken up by living cells without the use of transfection reagents.¹⁷ The uptake of ssOligos without the use of a transfection reagent (naked ssOligos) is called ‘gymnosis’ (nakedness in Greek).¹⁷ These authors showed that naked antisense oligonucleotides can induce sequence-specific silencing of target genes in various types of cells. Small guide RNAs (sgRNAs) are a class of artificially designed single-stranded RNAs (ssRNAs) that can induce gene silencing by the tRNA maturase tRNase Z^L-dependent mechanism.^{18–24} Multiple papers, including ours, reported that naked

2'-O-methylated sgRNAs targeting various miRNAs and mRNAs can be taken up by different types of living cells without the use of a transfection reagent and reduce target RNA levels.^{25–28} Iizuka et al. reported that the uptake of naked sgRNA is not mediated by clathrin or caveolin-dependent endocytosis; however, the molecular mechanism of naked ssRNA uptake into living cells remains unclear.²⁸

The *Caenorhabditis elegans* systemic RNA interference deficient-1 (SID-1) protein mediates the uptake of double-stranded RNA (dsRNA) into cells.¹⁸ SID-1 functions as a bidirectional dsRNA transporter, but does not transport ssRNA.²⁹ SID-1 transmembrane family 1 and 2 (SIDT1 and SIDT2) are mammalian orthologs of SID-1.^{30,31} SIDT1 localizes to the plasma membrane and mediates the bidirectional transport of dsRNA in human cells.^{30,32} Whether SIDT1 transports ssRNA has not been elucidated. Considering that SIDT1 is expressed in restricted types of cells such as dendritic cells and lymphocytes,^{30,32,33} it is unlikely to account for the ubiquity of gymnosis. By contrast, SIDT2 is ubiquitously expressed in many types of cells.^{31,33–35} In addition, we recently found that SIDT2 on the lysosomal membrane mediates the uptake of ssRNA into

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lysosomes,³⁶ indicating that SIDT2 can transport ssRNA. This led us to hypothesize that SIDT2 is involved in gymnosis. In the present study, we explored potential mechanisms underlying gymnosis and investigated whether the uptake of naked ssOligos is mediated by SIDT2.

Results

Naked ssOligos can be taken up by living HeLa cells

We examined whether small ssOligos can be taken up by living HeLa cells without the use of a transfection reagent. Fully 2'-O-methylated and 5'-Alexa568-labeled 15-nt oligonucleotides containing 5'- and 3'-phosphates (Alexa568-ssOligos) were synthesized. HeLa cells were cultured in the presence or absence of 500 nM of naked Alexa568-ssOligos for 6 and 24 hours and analyzed by confocal fluorescence microscopy. The results showed that naked Alexa568-ssOligos were efficiently taken up by HeLa cells without the use of a transfection reagent (Fig. 1A). Furthermore, time-course experiments of the uptake of naked ssOligos into living HeLa cells performed by culturing cells in the presence

of 500 nM of naked Alexa568-ssOligos for 0, 2, 4, 6 or 8 hours followed by confocal fluorescence microscope analysis showed that the oligonucleotides were taken up by cells in a time-dependent manner (Fig. 1B). We next investigated whether naked antisense oligonucleotide targeting microRNA-16 (miR-16) can inhibit the endogenous miR-16 expression levels in HeLa cells. Previously, we reported that naked antisense oligonucleotides targeting miR-16 can reduce miR-16 expression levels in 293 cells.²⁵ Cells were incubated with or without 20 nM of naked antisense oligonucleotides targeting miR-16 or negative control oligonucleotide for 6 hours, and the endogenous miR-16 expression levels were analyzed by quantitative reverse transcription PCR (qPCR). We observed that naked antisense oligonucleotides targeting miR-16 reduced the endogenous miR-16 expression levels, while negative control oligonucleotides (poly-A) did not (Fig. 1C and D).

SIDT2 partly localizes to the plasma membrane

In HeLa cells, *SIDT1* mRNA expression was not detected, whereas expression of *SIDT2* mRNA was detected (Fig. 2A).

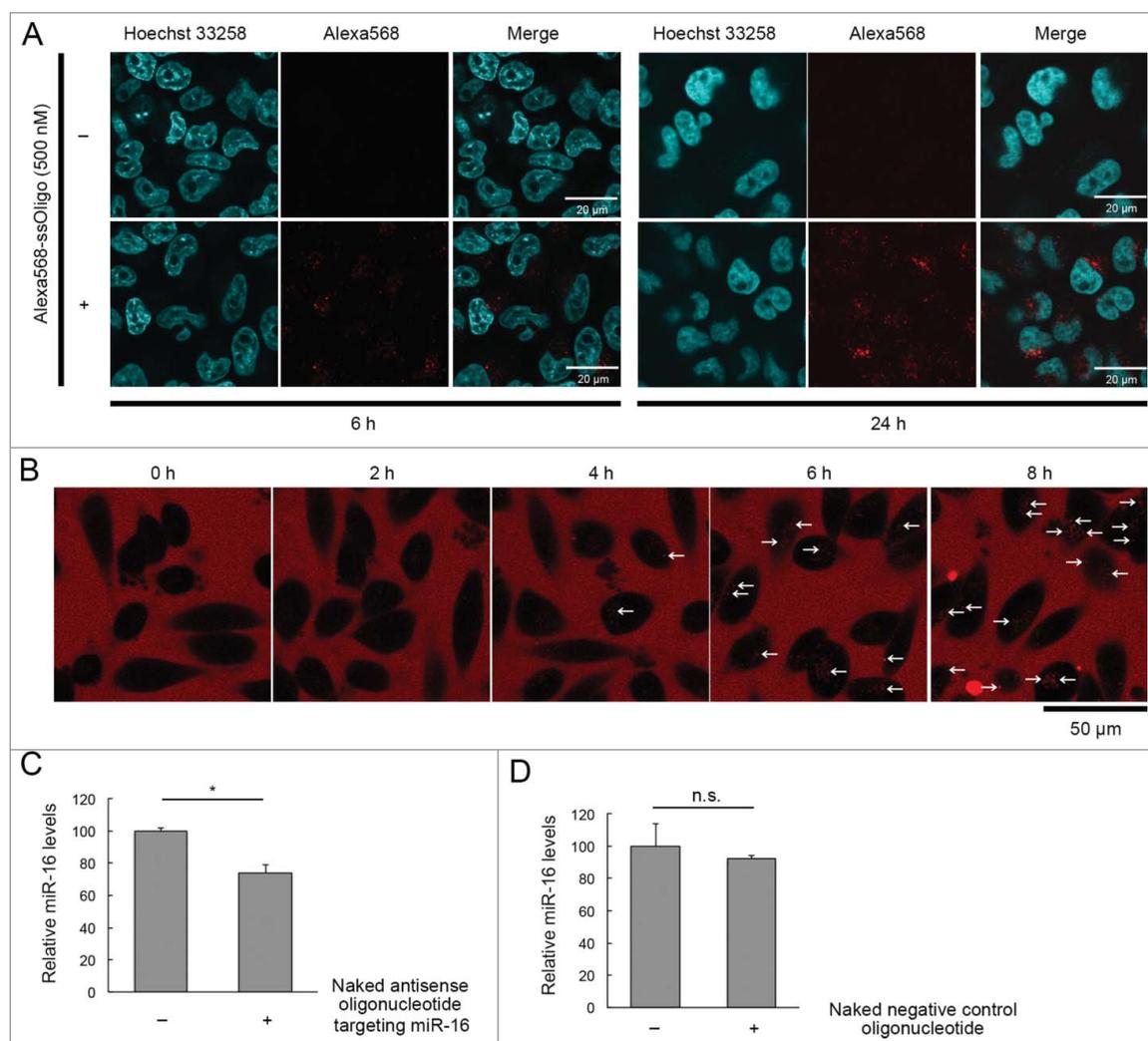


Figure 1. Uptake of naked ssOligos by HeLa cells. (A) Confocal imaging of HeLa cells. HeLa cells were incubated with or without 500 nM of the naked 15-nt 5'-Alexa568-labeled oligonucleotides (Alexa568-ssOligos) for 6 and 24 hours. Cells were washed with PBS and stained with Hoechst 33258. (B) HeLa cells were incubated with 500 nM of Alexa568-ssOligos for 0, 2, 4, 6 and 8 hours, and analyzed by confocal microscopy. Arrows indicate the 5'-Alexa568-labeled oligonucleotides in cells. (C and D) HeLa cells were incubated with or without 20 nM of naked antisense oligonucleotides targeting miR-16 (C) or naked negative control oligonucleotides (D) for 6 hours, and analyzed by qPCR analysis, miR-16 levels were normalized against β -actin mRNA levels. Error bars indicate s.d. (n = 3). *, $P < 0.05$, n.s., not significant.

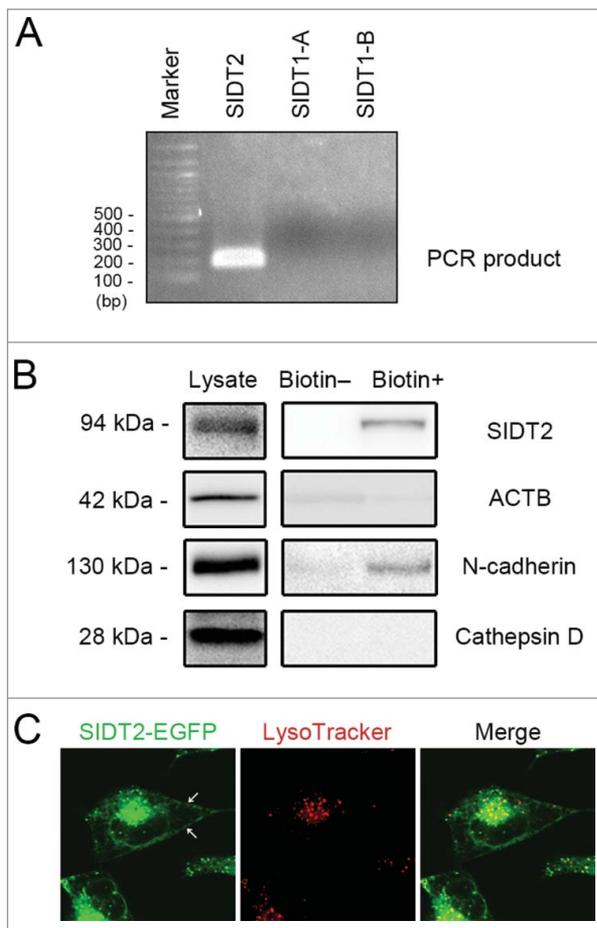


Figure 2. Localization of SIDT2 to the plasma membrane. (A) mRNA levels in HeLa cells were analyzed by RT-PCR. Expression of SIDT2 mRNA was detected. In contrast, *SIDT1* mRNA was not detected in HeLa cells. (B) HeLa cells were incubated with (+) or without (-) biotin, and a biotin-streptavidin pull-down assay was performed to purify cell surface proteins as described in the Materials and Methods section. Proteins were analyzed by western blotting using anti-SIDT2 (Abnova), anti- β -actin (ACTB), anti-N-cadherin and anti-cathepsin D antibodies. (C) HeLa cells expressing EGFP-tagged SIDT2 were incubated with LysoTracker Red. Fluorescence images were visualized using a confocal laser-scanning microscope. Arrows indicate plasma membrane localization.

Similar results were obtained when we used 293FT (human embryonic kidney cells), HL60 (human promyelocytic leukemia cells), Neuro2a (mouse neuroblastoma cells), and mouse embryonic fibroblasts (data not shown), suggesting that SIDT1 does not account for ubiquitousness of gymnosis. SIDT2 mainly localizes to lysosomes.^{31,33-36} However, it is possible that a small portion of SIDT2 localizes to the plasma membrane. To test whether endogenous SIDT2 exists in the plasma membrane, we biotinylated cell surface proteins in HeLa cells, which express endogenous SIDT2 proteins,³⁶ and performed a biotin-streptavidin pull-down assay followed by western blot analysis. β -actin, an established intracellular protein, and cathepsin D, an intralysosomal protein were not biotinylated, while N-cadherin, a plasma membrane-integrated protein was biotinylated (Fig. 2B), confirming that plasma membrane proteins were selectively biotinylated. Endogenous SIDT2 was detected in biotinylated cell surface proteins (Fig. 2B), indicating that a portion of endogenous SIDT2 localizes to the plasma membrane. Moreover, using a C-terminal EGFP-tag, we also

observed that SIDT2 mainly localized to lysosomes and partly to the plasma membrane (Fig. 2C).

SIDT2 knockdown reduces the uptake of naked ssOligos by cells

To investigate whether the uptake of naked ssOligos is mediated by SIDT2, we assessed the effect of SIDT2 knockdown on gymnosis. HeLa cells were transfected with siRNA targeting SIDT2 or control siRNA, and incubated for 72 hours (Fig. 3A-C). Then, cells were cultured in the presence or absence of 1 μ M or 500 nM of Alexa568-ssOligos for 6 hours and analyzed by confocal microscopy. When 1 μ M and 500 nM of naked Alexa568-ssOligos were added to the culture media, the Alexa568 intensity was decreased to 60% and 64% in SIDT2 knockdown cells, respectively, compared with control cells (Fig. 3D and E). Similar results were obtained when we used another siRNA against SIDT2 (Fig. 3F), or with another cell line, wild-type (WT) mouse embryonic fibroblasts (MEFs) (Fig. S1). These results indicated that SIDT2 mediates uptake of ssOligos by cells.

Knockdown of SIDT2 inhibits the antisense effect induced by gymnosis

We next investigated whether the antisense effect induced by gymnosis is mediated by SIDT2. Therefore, we examined the effect of SIDT2 knockdown on the antisense effect induced by naked antisense oligonucleotides targeting miR-16. Cells were transfected with siRNA against SIDT2 or control siRNA, and cultured for 72 hours (Fig. 4A). Then, cells were incubated with or without 20 nM of naked antisense oligonucleotides targeting miR-16 for 6 hours, and the endogenous miR-16 expression levels were analyzed by qPCR. The results showed that HeLa cells transfected with siRNA targeting SIDT2 (#1 and #2) inhibited the antisense effect induced by gymnosis, compared with negative control siRNA (Fig. 4B and C). These results indicate that SIDT2 mediates the antisense effect induced by gymnosis.

Overexpression of SIDT2 enhances the uptake of naked ssRNA into cells

The effect of SIDT2 overexpression on the uptake of naked ssOligos into living cells was examined next. Cells were transfected with empty vector, mouse SIDT2 plasmid or human SIDT2 plasmid, and cultured for 48 hours (Fig. 5A and B). Then, cells were incubated with or without 500 nM of naked Alexa568-ssOligos for 24 hours and analyzed by confocal fluorescence microscopy (Fig. 5A). The Alexa568 intensities in cells overexpressing mouse or human SIDT2 were increased to 186% and 175%, respectively, compared with control cells with empty vector (Fig. 5C), showing that overexpression of SIDT2 enhances the uptake of ssRNA into cells.

Mutant SIDT2 does not enhance the uptake of naked ssOligos into cells

Overexpression of missense mutant SID-1 (A173T or S536I), which is found in a systemic RNAi-defective mutant

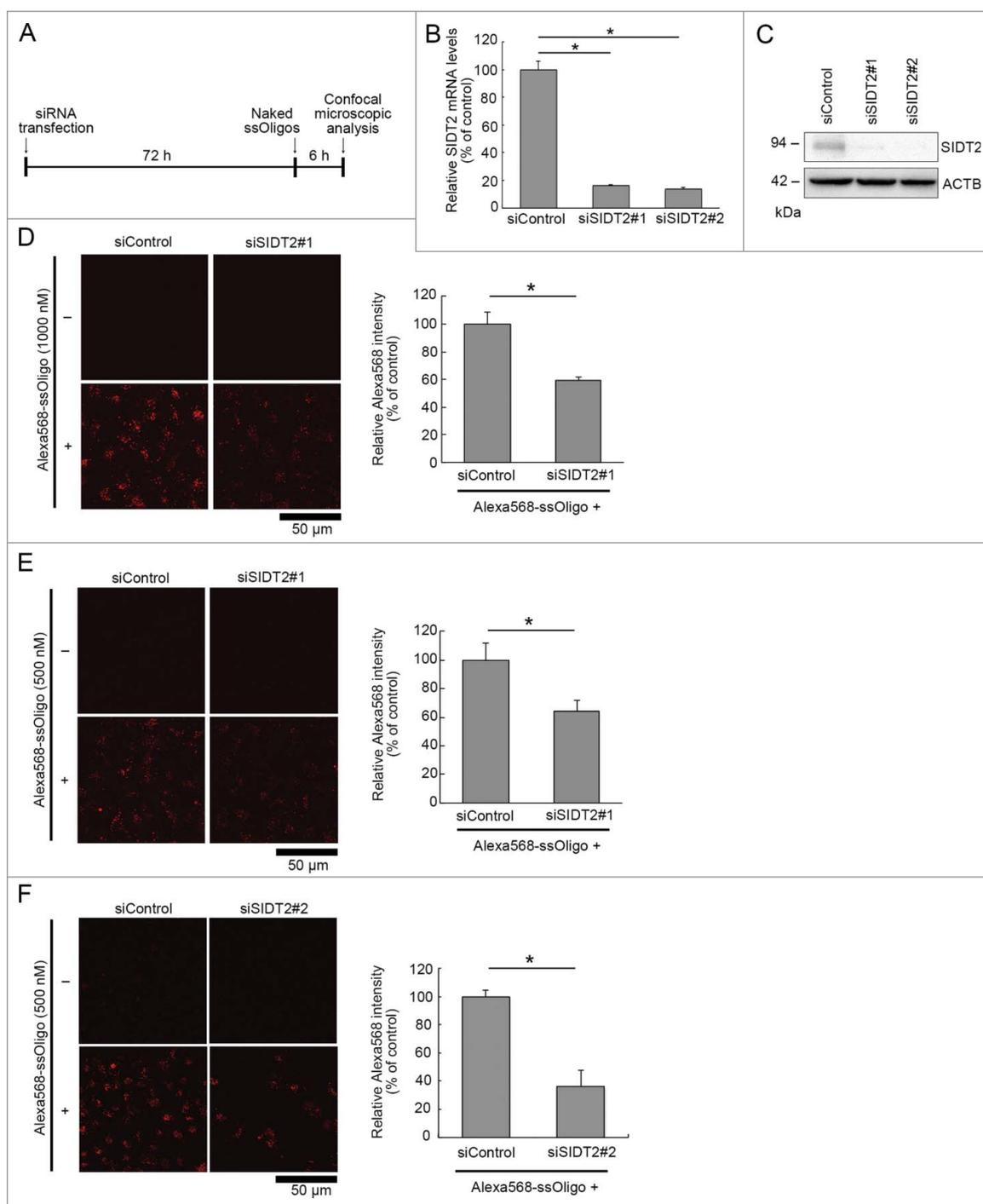


Figure 3. Knockdown of SIDT2 reduces the uptake of naked ssOligos. (A) Experimental paradigm for the confocal microscopic analysis. (B and C) HeLa cells were transfected with 10 nM of control siRNA (siControl) or siRNA against SIDT2 (siSIDT2#1 or #2). At 72 hours after transfection, *SIDT2* mRNA levels (B) and SIDT2 protein levels (C) were analyzed. In the qRT-PCR analysis, *SIDT2* mRNA levels were normalized against β -actin mRNA levels (B). In the western blot analysis, anti-SIDT2 (custom antibody) and anti- β -actin antibodies were used (C). Error bars indicate s.d. ($n = 3$). *, $P < 0.05$. (D–F) HeLa cells were transfected with siControl or siSIDT2#1 (D and E) and siControl or siSIDT2#2 (F). Seventy-two hours after transfection, cells were washed with PBS, and incubated with or without 1000 nM (D) or 500 nM (E and F) of the naked Alexa568-ssOligos for 6 hours. Cells were washed with PBS again, and analyzed by confocal microscopy. Quantification of fluorescence intensity was performed with ImageJ. Error bars indicate s.d. ($n = 3$). *, $P < 0.05$.

C. elegans,²⁹ cannot enhance the uptake of dsRNA into cells.^{29,37} The Ala-173 and Ser-536 of SID-1 correspond to Phe-154 and Ser-564 of mouse SIDT2, respectively. Therefore, we hypothesized that mutant SIDT2 containing a mutation on Phe-154 or Ser-536 could not mediate gymnosis. To test this hypothesis, cells were transfected with

empty vector, WT SIDT2 plasmid or mutant SIDT2 (F154T or S564A) plasmids (Fig. 6A), and the effect of the mutations on the uptake of naked Alexa568-ssOligos was assessed. The Alexa568 intensities in cells overexpressing SIDT2 mutants were not significantly increased compared with that of control cells (Fig. 6B), indicating that both

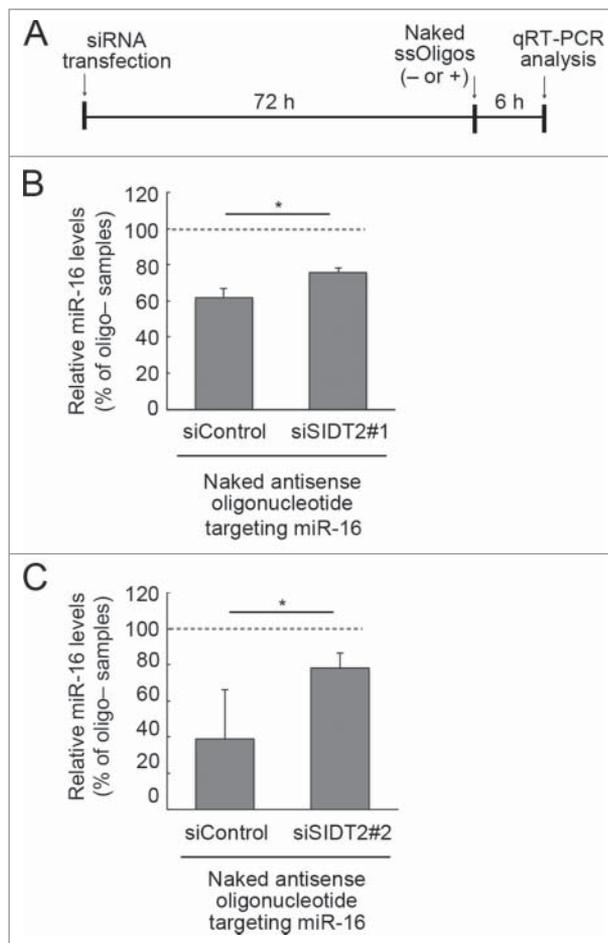


Figure 4. Knockdown of SIDT2 inhibits the antisense effect induced by gymnosis. (A) Experimental paradigm for the qRT-PCR analysis. (B and C) HeLa cells were transfected with siControl or siSIDT2#1 (B) and siControl or siSIDT2#2 (C). Seventy-two hours after transfection, cells were washed with PBS, incubated with (oligo+) or without (oligo-) 20 nM of the naked antisense oligonucleotides targeting miR-16 for 6 hours, and RNA levels were analyzed by qPCR. The miR-16 levels were normalized against β -actin mRNA levels. The miR-16 levels were expressed as percentages compared to the levels of oligo- samples (expressed as 100%). Error bars indicate s.d. ($n = 6$). *, $P < 0.05$.

Phe-154 and Ser-564 of SIDT2 are essential for SIDT2-mediated gymnosis. The subcellular localization of both mutants was shown to be comparable with that of WT SIDT2, namely WT and mutant SIDT2 mainly localized to lysosomes and partly to the plasma membrane (Fig. 1B and Fig. 6C).

SIDT2 does not enhance nonselective type endocytosis

As a control experiment, we investigated whether overexpression of SIDT2 enhances endocytosis using Rhodamine B and Rhodamine B-dextran as endocytotic markers.^{38,39} Cells were transfected with empty vector, mouse SIDT2 plasmid or human SIDT2 plasmid, and cultured for 48 hours. Then, cells were incubated with or without 500 nM of Rhodamine B or Rhodamine B-dextran for 24 hours, and analyzed by confocal fluorescence microscopy. The Rhodamine B and Rhodamine B-dextran intensities in cells transfected with mouse SIDT2 plasmid or human SIDT2 plasmid were not changed compared with that in control cells with empty vector (Fig. 7A–C). These

results indicated that SIDT2 does not enhance nonselective type endocytosis.

Discussion

In the present study, we showed that the uptake of naked ssOligos into living cells is decreased in SIDT2 knockdown cells (Fig. 3D–F), and knockdown of SIDT2 inhibited the antisense effect induced by naked oligonucleotides (Fig. 4B and C). These results indicate that gymnosis is partly mediated by SIDT2.

In contrast to the ubiquitous expression of SIDT2, SIDT1 is not expressed in the cell lines tested. Overexpression of SIDT1 has been reported to enhance the uptake of dsRNA into cells.³² Although the ability of SIDT1 to transport ssRNA remains unknown, it is possible that SIDT1 also mediates gymnosis in SIDT1-expressing cells.

Knockdown of SIDT2 did not completely inhibit the uptake of Alexa568-ssOligos (Fig. 3), indicating the possible existence of SIDT2-independent pathway(s) for the uptake of extracellular oligonucleotides. Oligonucleotides can be taken up via clathrin-, caveolin- or dynamin-dependent endocytotic pathways,⁴⁰ and these pathways could be the SIDT2-independent pathways.

We previously reported that SIDT2 primarily localizes to lysosomes and mediates RNautophagy/DNautophagy,^{36,41} an autophagic pathway in which RNA and DNA are directly taken up by lysosomes.^{42–45} In the present study, we showed that a portion of SIDT2 localizes to the plasma membrane, suggesting that plasma membrane SIDT2 mediates gymnosis. The localization of SIDT2 seems reasonable, considering that even a lysosomal marker protein, LAMP2, partly localizes to plasma membranes.⁴⁶ In general, the extracellular domains of plasma membrane proteins topologically correspond to luminal regions of lysosomal membrane proteins. Therefore, from the point of view of topology of SIDT2, RNA translocation occurs in opposite directions during RNautophagy and gymnosis, and our results indicate that SIDT2 can bidirectionally transport RNA. This notion is consistent with other studies showing that SID-1 and SIDT1 mediate the bidirectional translocation of RNA on the plasma membrane.^{30,32,47} Whether SIDT2 also mediates the export of RNA from the cell is currently unknown.

The mechanism by which SIDT2 translocates RNA into living cells is currently unclear. Given that SID-1 is a putative bidirectional RNA channel,²⁹ it is likely that SIDT2 also function as an RNA channel during gymnosis. Our results showing that SIDT2 can bidirectionally transport RNA support this idea. However, the possibility that SIDT2 mediates an endocytosis-like pathway cannot be completely excluded. Although we showed that overexpression of SIDT2 does not enhance the uptake of Rhodamine B or Rhodamine B dextran into living cells (Fig. 7), gymnosis could be explained by SIDT2-mediated RNA-specific endocytosis. In this scenario, a portion of RNAs taken up by cells would be released from endosomes to the cytosol. Further exploration of this issue would be of interest.

SIDT2 is a member of the putative transmembrane hydrolase superfamily.⁴⁸ Ser-564 of SIDT2 is a conserved residue in the superfamily⁴⁸ and is thought to be important for the putative hydrolase activity of SIDT2. We observed that

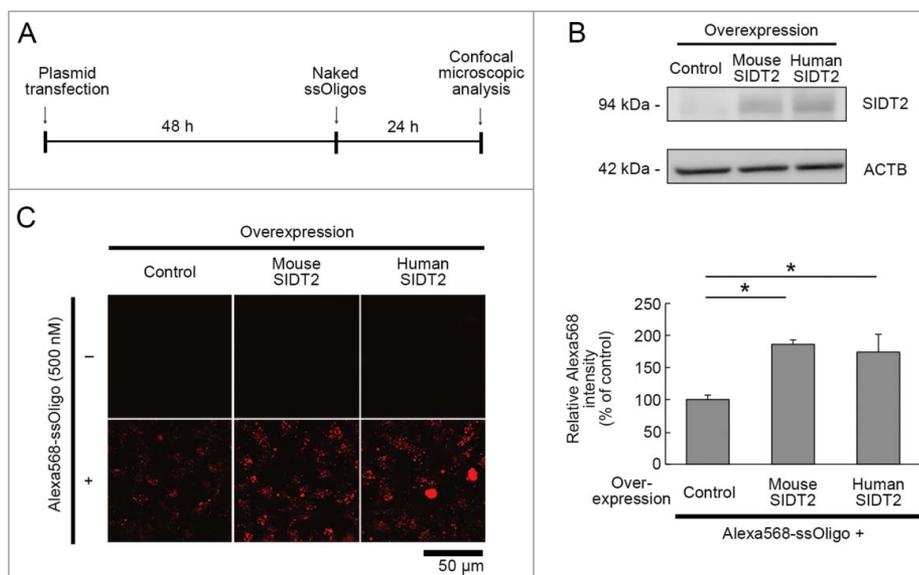


Figure 5. Effect of overexpression of SIDT2 on the uptake of naked ssRNA. (A) Experimental paradigm for the confocal microscope analysis. (B) HeLa cells were transfected with empty vector, pCI-neo/mouse SIDT2 or pCI-neo/human SIDT2 plasmids. β -actin was used as a loading control. Overexpression of SIDT2 was confirmed by western blot analysis using an anti-SIDT2 antibody (Abnova). (C) HeLa cells were transfected as indicated and cultured for 48 hours. Cells were washed with PBS, incubated with or without 500 nM of the naked Alexa568-ssOligos, washed with PBS again, and analyzed by confocal microscopy. Quantification of fluorescence intensity was performed. Error bars indicate s.d. ($n = 3$). *, $P < 0.05$.

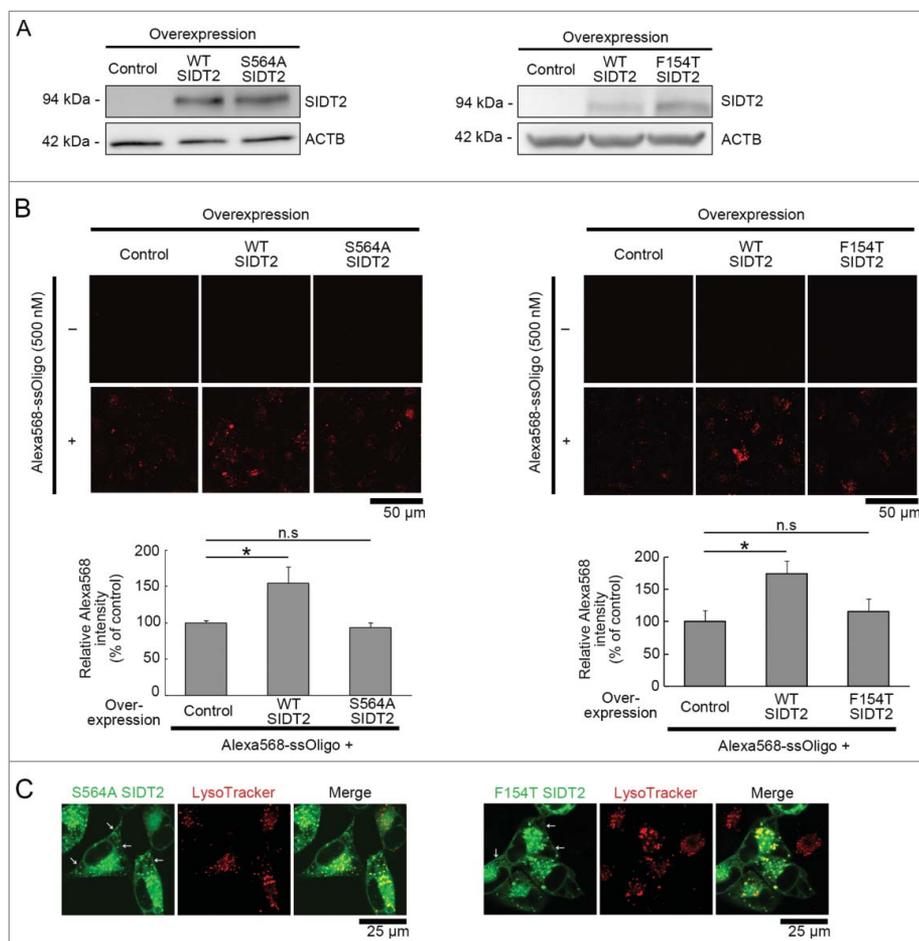


Figure 6. Effect of mutations of SIDT2 on the uptake of naked ssOligos. (A) HeLa cells were transfected with empty vector, pCI-neo/WT SIDT2, pCI-neo/F154T SIDT2, or pCI-neo/S564A SIDT2 plasmids. Overexpression of SIDT2 variants was confirmed by western blot analysis using an anti-SIDT2 antibody (Abnova). β -actin was used as a loading control. (B) HeLa cells were transfected as indicated and cultured for 48 hours. Cells were washed with PBS, incubated with or without 500 nM of the naked Alexa568-ssOligos, washed with PBS again, and analyzed by confocal microscopy. Quantification of fluorescence intensity was performed. Error bars indicate s.d. ($n = 3$, $D: n = 6$). *, $P < 0.05$. n.s., not significant. (C) HeLa cells expressing EGFP-tagged SIDT2 (S564A or F154T) were incubated with LysoTracker Red. Fluorescence images were acquired using a confocal laser-scanning microscope. Arrows indicate plasma membrane localization.

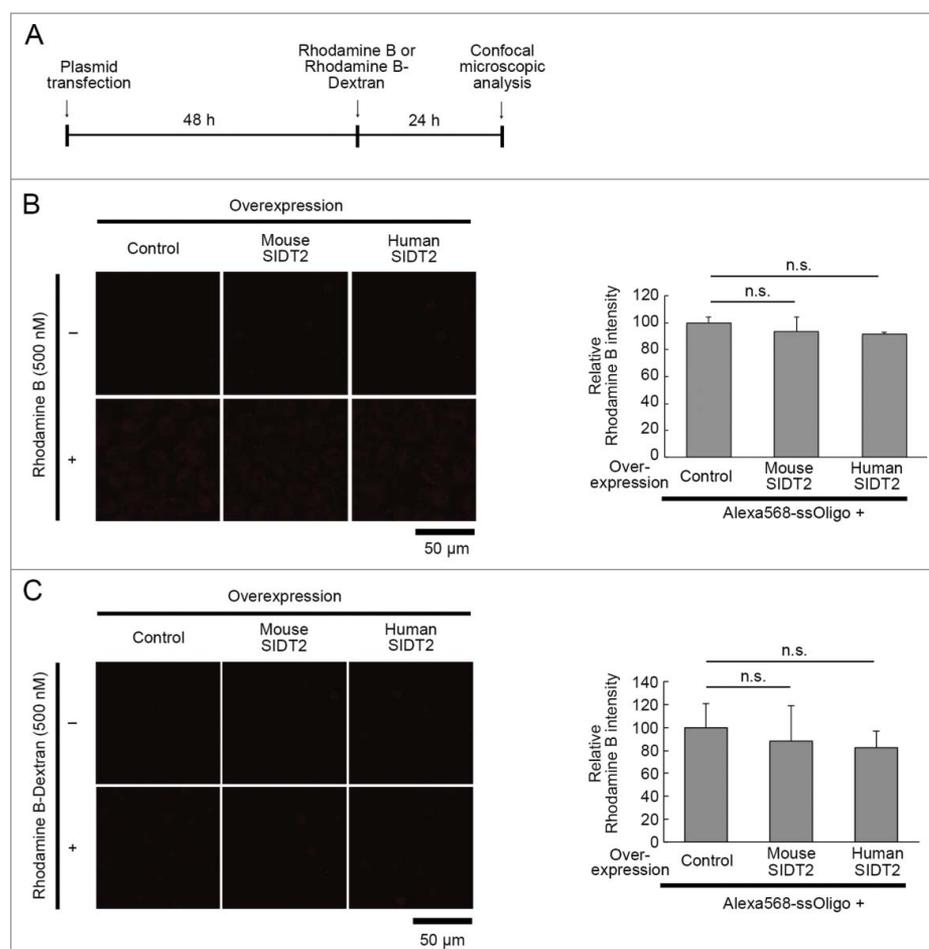


Figure 7. Effect of overexpression of SIDT2 on endocytosis. (A) Experimental paradigm for the confocal microscope analysis. (B and C) HeLa cells were transfected with empty vector, pCI-neo/mouse SIDT2 or pCI-neo/human SIDT2 plasmids, and cultured for 48 hours. Cells were washed with PBS, and incubated with or without 500 nM of Rhodamine B (B) or Rhodamine B isothiocyanate-dextran (C). Cells were washed with PBS again, and analyzed by confocal microscopy. Quantification of fluorescence intensity was performed. Error bars indicate s.d. ($n = 3$). *, n.s., not significant.

overexpression of SIDT2 enhanced the uptake of naked ssOligos into living cells (Fig. 5C), whereas overexpression of the S564A mutant of SIDT2 did not (Fig. 6B). This indicated that S564 is essential for RNA translocation by SIDT2 during gymnosis. We previously showed that S564 is also indispensable for RNA translocation by SIDT2 in the process of RNautophagy.³⁶ Identification of the hydrolase substrate of SIDT2 would contribute to clarify the mechanism of RNA translocation via SIDT2.

We also showed that overexpression of the F154T mutant of SIDT2 does not enhance the uptake of naked ssOligos into cells (Fig. 6B). A recent paper reported that the N-terminal extracellular domain of SIDT2 (22-292 amino acids of mouse SIDT2) shows affinity for RNA and the F154T mutation attenuates RNA binding, indicating that the Phe154 residue mediates RNA binding.³⁷ Taken together with these observations, our results suggest that the RNA binding activity of the extracellular domain of SIDT2 is required for SIDT2-mediated gymnosis.

In general, nucleic acid medicines have high specificity.⁴⁹ Some nucleic acid-based drugs may become therapeutic agents for a variety of disorders. However, Sledz et al. reported that dsRNAs such as siRNAs induce an interferon response.⁵⁰ In many cases, the interferon response is associated with cytotoxicity. On the other hand, naked ssRNAs, such as the 7-nt

sgRNA, do not induce interferon- β production and can be taken up by living cells without a transfection reagent.²⁶ Therefore, our study will encourage further development of ssOligos without transfection reagents.

Our findings may contribute to the further development of nucleic acid based drugs such as ssOligos. Naked ssOligos targeting miRNAs specific for the SIDT2 mRNA or the mRNAs of other gymnosis-related genes might be able to reduce the expression of these miRNAs. Consequently, it is expected that gymnosis will be enhanced. However, because SIDT2 mainly localizes to lysosomes, the upregulation of SIDT2 expression should enhance RNautophagy and lead to RNA degradation.^{36,42} It is possible that ssOligos are taken up by lysosomes after the uptake into the cytoplasm. Therefore, the application of SIDT2 to enhance the functional uptake of naked ssOligos is currently difficult.

Recent papers reported that some RNAs are exported from cells and play a role in biological processes, such as cell regulation and cell-to-cell communication.⁵¹ These RNAs are called extracellular RNAs (exRNAs), and some exRNAs have been identified as biomarker candidates in cancer.⁵² At least a portion of exRNAs is found within vesicles such as exosomes.⁵³ However, we showed that naked ssRNA is taken up by living cells via the putative RNA transporter SIDT2. Whether exRNAs exist outside

of extracellular vesicles and whether gymnosis occurs during normal biological processes are interesting issues to be resolved.

Materials and methods

RNA/DNA preparation

The following fully 2'-*O*-methylated, 5'-Alexa568-labeled, 5'- and 3'-phosphate oligonucleotides containing phosphodiester backbones were chemically synthesized and subsequently purified by high-performance liquid chromatography as a custom service by Nippon Bioservice (Saitama, Japan): 15-nt 5'-Alexa568-labeled oligonucleotide, 5'-AAAAAAAAAAAAAA-3'; antisense oligonucleotide targeting miR-16, 5'-CGCCAAUAUUUACGUGCUGCUA-3'; and negative control oligonucleotide, 5'-AAAAAAAAAAAAAAAAAAAAA-3'. The following primers used for real-time reverse-transcription polymerase chain reaction (RT-PCR) were obtained from Fasmac (Kanagawa, Japan) or Hokkaido System Science (Hokkaido, Japan): SIDT2 forward primer, 5'-ATGAGT TCCCTGAAGGCGTG-3'; SIDT2 reverse primer, 5'-TCTTT GCGCTGTACGGTGAT-3'; SIDT1 forward primer A, 5'-AGATTTGGGGAATTTTCCGGCG-3'; SIDT1 reverse primer A, 5'-AGAGGGCGAAGGACCAGTTA-3'; SIDT1 forward primer B, 5'-GTGGCATCTCATCCCATTC-3'; SIDT1 reverse primer B, 5'-GGACATCTGCCTTCCAGGAC-3'; β -actin forward primer, 5'-ACAATGTGGCCGAGGACTTT-3'; β -actin reverse primer, 5'-TGTGTGGACTTGGGAGAGGA-3'; miR-16 reverse transcription primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG-GATACGACCGCCAA-3'; miR-16 forward primer, 5'-CGCGCTAGCAGCACGTAAAT-3'; miR-16 reverse primer, 5'-GTGCAGGGTCCGAGGT-3'.

Plasmids

The plasmids pCI-neo-mSIDT2 [WT or S564A] and pCI-neo-hSIDT2 (WT) for the expression of SIDT2 variants without a tag, and pEGFP-mSIDT2 (WT or S564A) for the expression of SIDT2 with an EGFP tag at the C-terminus were prepared as described previously.³⁶ Plasmids for the expression of the F154T mutant SIDT2 were constructed using the QuikChange Site-Directed Mutagenesis Kit. All resulting constructs were confirmed by sequencing.

RNA interference

For siRNA-mediated knockdown of SIDT2, cells were transfected with 10 nM SIDT2-targeting or control siRNAs. The target sequences were as follows: 5'-GAGGAUGACUACGACA CAU-3' [human SIDT2 siRNA#1³⁶], 5'-CCGCUCCUUUGAACCUGUA-3' [human SIDT2 siRNA#2], and 5'-CAGCACGACUUCUUAAGU-3' [EGFP siRNA⁵⁴]. EGFP siRNA was used as a control.

Cell culture and transfection

WT MEFs were kind gifts from Dr. Noboru Mizushima (The University of Tokyo, Tokyo, Japan).⁵⁵ WT MEFs and HeLa

cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, C11995500BT) supplemented with 10% fetal bovine serum (Sigma-Aldrich, 172012) at 37°C in a 5% CO₂ humidified incubator. Cells were transfected with each siRNA or each plasmid vector using Lipofectamine 3000 reagent (Thermo Fisher Scientific, L3000015) according to the manufacturer's protocol, and cultured for 72 or 48 hours, respectively. Then, transfected cells were washed with phosphate-buffered saline (PBS), and treated with naked oligonucleotides in culture medium.

Biotin-streptavidin pull-down assay

HeLa cell surface proteins were biotinylated using EZ-Link™ Sulfo-NHS-Biotin (Thermo Fisher Scientific, 21217) according to the manufacturer's protocol. Then, HeLa cell lysates were prepared in a lysis buffer (50 mM Tris-HCl pH7.5, 1% TritonX-100, 150 mM NaCl, 5 mM EDTA), and biotinylated proteins were pulled down using Streptavidin Sepharose™ High Performance (GE Healthcare, 17-5113-01) according to the manufacturer's protocol. After the beads were washed three times with lysis buffer, proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris, pH 7.8, 3% SDS, 5% glycerol and 0.02% bromophenol blue, 14% 2-mercaptoethanol), and analyzed by western blotting.

Real-Time RT-PCR

Total RNA was extracted from HeLa cells using TRIzol (Thermo Fisher Scientific, 15596018), and cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, RR047A) according to the manufacturer's protocols. The SIDT2 mRNA, β -actin mRNA, and miR-16 were quantified using a CFX96™ Real-Time System (Bio-Rad) with SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, RR0820A).

Western blotting

SDS polyacrylamide gel electrophoresis and western blotting were performed according to standard procedures. The antibodies used were polyclonal antibody against SIDT2 (Abnova, PAB27211), polyclonal anti-SIDT2 antibody raised in a rabbit against synthetic peptides (C+DLDTVQRDKIYVF) containing an amino acid sequence corresponding to the C-terminal region of SIDT2 (custom antibody), monoclonal antibody against β -actin (Sigma-Aldrich, A-4700), monoclonal antibody against N-cadherin (BD Biosciences, 610921), and monoclonal antibody against cathepsin D (BD Biosciences, 610800).

Confocal fluorescence microscopic analysis

Plasmid or siRNA transfected HeLa cells or untransfected cells were prepared in 35 mm glass bottom dishes (IWAKI, 3910-035 or ibidi, 81158). After cells were washed with PBS, cells were incubated with culture medium in the absence or presence of 500 nM or 1 μ M of Alexa568-labeled polyA 15mer, 500 nM of Rhodamine B (Sigma-Aldrich, R6626) or 500 nM of Rhodamine B isothiocyanate-dextran (Sigma-Aldrich, R7623). Then,

cells were washed with PBS unless otherwise indicated. Hoechst 33258 (Dojindo, 343-07961) was used to stain DNA and the cells were analyzed with a FLUOVIEW FV10i or FV1000 confocal microscope (Olympus). Quantification of fluorescence intensity was performed with ImageJ (NIH).

For analysis of intracellular localization, cells were overexpressed with EGFP-tagged SIDT2 (WT or mutants), and incubated with 100 nM LysoTracker Red DND 99 (Thermo Fisher Scientific, L7528) in growth medium for 1 h. Then, fluorescence image acquisition was performed with a FLUOVIEW FV10i or FV1000 confocal microscope.

Statistical analysis

For comparison of two groups, statistical differences were evaluated by Student's *t*-test. We used Tukey's multiple comparison test for the comparison of three groups.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

- Opalinska JB, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov* 2002; 1:503-14; PMID:12120257; <https://doi.org/10.1038/nrd837>
- Mizuno T, Chou MY, Inouye M. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc Natl Acad Sci U S A* 1984; 81:1966-70; PMID:6201848; <https://doi.org/10.1073/pnas.81.7.1966>
- Neves MA, Reinstein O, Saad M, Johnson PE. Defining the secondary structural requirements of a cocaine-binding aptamer by a thermodynamic and mutation study. *Biophys Chem* 2010; 153:9-16; PMID:21035241; <https://doi.org/10.1016/j.bpc.2010.09.009>
- Baugh C, Grate D, Wilson C. 2.8 Å crystal structure of the malachite green aptamer. *J Mol Biol* 2000; 301:117-28; PMID:10926496; <https://doi.org/10.1006/jmbi.2000.3951>
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; 391:806-11; PMID:9486653; <https://doi.org/10.1038/35888>
- 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; 411:494-8; PMID:11373684; <https://doi.org/10.1038/35078107>
- Esau CC. Inhibition of microRNA with antisense oligonucleotides. *Methods* 2008; 44:55-60; PMID:18158133; <https://doi.org/10.1016/j.ymeth.2007.11.001>
- Lennox KA, Owczarzy R, Thomas DM, Walder JA, Behlke MA. Improved performance of anti-miRNA oligonucleotides using a novel non-nucleotide modifier. *Mol Ther Nucleic Acids* 2013; 2:e117; PMID:23982190; <https://doi.org/10.1038/mtna.2013.46>
- Wang Z. The guideline of the design and validation of miRNA mimics. *Methods Mol Biol* 2011; 676:211-23; PMID:20931400; https://doi.org/10.1007/978-1-60761-863-8_15
- Ahmad MZ, Akhter S, Mallik N, Anwar M, Tabassum W, Ahmad FJ. Application of decoy oligonucleotides as novel therapeutic strategy: a contemporary overview. *Curr Drug Discov Technol* 2013; 10:71-84; PMID:22780867; <https://doi.org/10.2174/1570163811310010009>
- Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc Natl Acad Sci U S A* 1997; 94:10833-7; PMID:9380720; <https://doi.org/10.1073/pnas.94.20.10833>
- Merki E, Graham MJ, Mullick AE, Miller ER, Crooke RM, Pitas RE, Witztum JL, Tsimikas S. Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice. *Circulation* 2008; 118:743-53; PMID:18663084; <https://doi.org/10.1161/CIRCULATIONAHA.108.786822>
- El Harchaoui K, Akdim F, Stroes ES, Trip MD, Kastelein JJ. Current and future pharmacologic options for the management of patients unable to achieve low-density lipoprotein-cholesterol goals with statins. *Am J Cardiovasc Drugs* 2008; 8:233-42; PMID:18690757; <https://doi.org/10.2165/00129784-200808040-00003>
- Athyros VG, Kakafika AI, Tziomalos K, Karagiannis A, Mikhailidis DP. Antisense technology for the prevention or the treatment of cardiovascular disease: the next blockbuster? *Expert Opin Investig Drugs* 2008; 17:969-72; PMID:18549334; <https://doi.org/10.1517/13543784.17.7.969>
- Mulamba GB, Hu A, Azad RF, Anderson KP, Coen DM. Human cytomegalovirus mutant with sequence-dependent resistance to the phosphorothioate oligonucleotide fomivirsen (ISIS 2922). *Antimicrob Agents Chemother* 1998; 42:971-3; PMID:9559825
- Roush W. Antisense aims for a renaissance. *Science* 1997; 276:1192-3; PMID:9182327; <https://doi.org/10.1126/science.276.5316.1192>
- Stein CA, Hansen JB, Lai J, Wu S, Voskresenskiy A, Hog A, Worm J, Hedtjarn M, Souleimanian N, Miller P, et al. Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. *Nucleic Acid Res* 2010; 38:e3; PMID:19854938; <https://doi.org/10.1093/nar/gkp841>
- Nashimoto M. Conversion of mammalian tRNA 3' processing endoribonuclease to four-base-recognizing RNA cutters. *Nucleic Acid Res* 1995; 23:3642-7; PMID:7478991; <https://doi.org/10.1093/nar/23.18.3642>
- Nashimoto M. Specific cleavage of target RNAs from HIV-1 with 5' half tRNA by mammalian tRNA 3' processing endoribonuclease. *RNA* 1996; 2:523-4; PMID:8718682
- Nashimoto M, Geary S, Tamura M, Kaspar R. RNA heptamers that direct RNA cleavage by mammalian tRNA 3' processing endoribonuclease. *Nucleic Acid Res* 1998; 26:2565-72; PMID:9592138; <https://doi.org/10.1093/nar/26.11.2565>
- Nashimoto M. Anomalous RNA substrates for mammalian tRNA 3' processing endoribonuclease. *FEBS Lett* 2000; 472:179-86; PMID:10788607; [https://doi.org/10.1016/S0014-5793\(00\)01462-9](https://doi.org/10.1016/S0014-5793(00)01462-9)
- 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; 32:4429-38; PMID:15317868; <https://doi.org/10.1093/nar/gkh774>
- Takaku H, Minagawa A, Takagi M, Nashimoto M. A novel 4-base-recognizing RNA cutter that can remove the single 3' terminal

- nucleotides from RNA molecules. *Nucleic Acid Res* 2004; 32:e91; PMID:15247324; <https://doi.org/10.1093/nar/gnh092>
24. Shibata HS, Takaku H, Takagi M, Nashimoto M. The T loop structure is dispensable for substrate recognition by tRNase ZL. *J Biol Chem* 2005; 280:2326-34; PMID:15824113; <https://doi.org/10.1074/jbc.M502048200>
 25. Takahashi M, Elbarbary RA, Abe M, Sato M, Yoshida T, Yamada Y, Tamura M, Nashimoto M. Elimination of specific miRNAs by naked 14-nt sgRNAs. *PLoS One* 2012; 7:e38496; PMID:22675569; <https://doi.org/10.1371/journal.pone.0038496>
 26. Takahashi M, Elbarbary RA, Nakashima A, Abe M, Watanabe N, Narita M, Takahashi M, Tamura M, Yoshida T, Nashimoto M. A naked RNA heptamer targeting the human Bcl-2 mRNA induces apoptosis of HL60 leukemia cells. *Cancer Lett* 2013; 328:362-8; PMID:23092557; <https://doi.org/10.1016/j.canlet.2012.10.016>
 27. Watanabe N, Narita M, Yamahira A, Taniguchi T, Furukawa T, Yoshida T, Miyazawa T, Nashimoto M, Takahashi M. Induction of apoptosis of leukemic cells by TRUE gene silencing using small guide RNAs targeting the WT1 mRNA. *Leuk Res* 2013; 37:580-5; PMID:23403166; <https://doi.org/10.1016/j.leukres.2013.01.015>
 28. Iizuka S, Oridate N, Nashimoto M, Fukuda S, Tamura M. Growth inhibition of head and neck squamous cell carcinoma cells by sgRNA targeting the cyclin D1 mRNA based on TRUE gene silencing. *PLoS One* 2014; 9:e114121; PMID:25437003; <https://doi.org/10.1371/journal.pone.0114121>
 29. Shih JD, Hunter CP. SID-1 is a dsRNA-selective dsRNA-gated channel. *RNA* 2011; 17:1057-65; PMID:21474576; <https://doi.org/10.1261/rna.2596511>
 30. Elhassan MO, Christie J, Duxbury MS. Homo sapiens systemic RNA interference-defective-1 transmembrane family member 1 (SIDT1) protein mediates contact-dependent small RNA transfer and microRNA-21-driven chemoresistance. *J Biol Chem* 2012; 287:5267-77; PMID:22174421; <https://doi.org/10.1074/jbc.M111.318865>
 31. Jialin G, Xuefan G, Huiwen Z. SID1 transmembrane family, member 2 (Sidt2): a novel lysosomal membrane protein. *Biochem Biophys Res Commun* 2010; 402:588-94; PMID:20965152; <https://doi.org/10.1016/j.bbrc.2010.09.133>
 32. Duxbury MS, Ashley SW, Whang EE. RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem Biophys Res Commun* 2005; 331:459-63; PMID:15850781; <https://doi.org/10.1016/j.bbrc.2005.03.199>
 33. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW, 3rd, et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 2009; 10:R130; PMID:19919682; <https://doi.org/10.1186/gb-2009-10-11-r130>
 34. Chapel A, Kieffer-Jaquinod S, Sagne C, Verdon Q, Ivaldi C, Mellal M, Thirion J, Jadot M, Bruley C, Garin J, et al. An extended proteome map of the lysosomal membrane reveals novel potential transporters. *Mol Cell Proteomics* 2013; 12:1572-88; PMID:23436907; <https://doi.org/10.1074/mcp.M112.021980>
 35. Schroder B, Wrocklage C, Pan C, Jager R, Kusters B, Schafer H, Elsassner HP, Mann M, Hasilik A. Integral and associated lysosomal membrane proteins. *Traffic* 2007; 8:1676-86; PMID:17897319; <https://doi.org/10.1111/j.1600-0854.2007.00643.x>
 36. Aizawa S, Fujiwara Y, Contu VR, Hase K, Takahashi M, Kikuchi H, Kabuta C, Wada K, Kabuta T. Lysosomal putative RNA transporter SIDT2 mediates direct uptake of RNA by lysosomes. *Autophagy* 2016; 12:565-78; PMID:27046251; <https://doi.org/10.1080/15548627.2016.1145325>
 37. Li W, Koutmou KS, Leahy DJ, Li M. Systemic RNA interference deficiency-1 (SID-1) extracellular domain selectively binds long double-stranded RNA and is required for RNA transport by SID-1. *J Biol Chem* 2015; 290:18904-13; PMID:26067272; <https://doi.org/10.1074/jbc.M115.658864>
 38. Gerasimenko JV, Tepikin AV, Petersen OH, Gerasimenko OV. Calcium uptake via endocytosis with rapid release from acidifying endosomes. *Curr Biol* 1998; 8:1335-8; PMID:9843688; [https://doi.org/10.1016/S0960-9822\(07\)00565-9](https://doi.org/10.1016/S0960-9822(07)00565-9)
 39. Zhang X, Jin Y, Plummer MR, Pooyan S, Gunaseelan S, Sinko PJ. Endocytosis and membrane potential are required for HeLa cell uptake of R.I.-CKTat9, a retro-inverso Tat cell penetrating peptide. *Mol Pharm* 2009; 6:836-48; PMID:19278221; <https://doi.org/10.1021/mp800121f>
 40. Juriano RL. The delivery of therapeutic oligonucleotides. *Nucleic Acid Res* 2016; 44:6518-48; PMID:27084936; <https://doi.org/10.1093/nar/gkw236>
 41. Aizawa S, Contu VR, Fujiwara Y, Hase K, Kikuchi H, Kabuta C, Wada K, Kabuta T. Lysosomal membrane protein SIDT2 mediates the direct uptake of DNA by lysosomes. *Autophagy* 2017; 3:218-22; PMID:27846365; <https://doi.org/10.1080/15548627.2016.1248019>
 42. Fujiwara Y, Furuta A, Kikuchi H, Aizawa S, Hatanaka Y, Konya C, Uchida K, Yoshimura A, Tamai Y, Wada K, et al. Discovery of a novel type of autophagy targeting RNA. *Autophagy* 2013; 9:403-9; PMID:23291500; <https://doi.org/10.4161/auto.23002>
 43. Hase K, Fujiwara Y, Kikuchi H, Aizawa S, Hakuno F, Takahashi S, Wada K, Kabuta T. RNautophagy/DNautophagy possesses selectivity for RNA/DNA substrates. *Nucleic acids research* 2015; 43:6439-49; PMID:26038313; <https://doi.org/10.1093/nar/gkv579>
 44. Fujiwara Y, Hase K, Wada K, Kabuta T. An RNautophagy/DNautophagy receptor, LAMP2C, possesses an arginine-rich motif that mediates RNA/DNA-binding. *Biochem Biophys Res Commun* 2015; 460:281-6; PMID:25772617; <https://doi.org/10.1016/j.bbrc.2015.03.025>
 45. Fujiwara Y, Kikuchi H, Aizawa S, Furuta A, Hatanaka Y, Konya C, Uchida K, Wada K, Kabuta T. Direct uptake and degradation of DNA by lysosomes. *Autophagy* 2013; 9:1167-71; PMID:23839276; <https://doi.org/10.4161/auto.24880>
 46. Gough NR, Fambrough DM. Different steady state subcellular distributions of the three splice variants of lysosome-associated membrane protein LAMP-2 are determined largely by the COOH-terminal amino acid residue. *J Cell Biol* 1997; 137:1161-9; PMID:9166415; <https://doi.org/10.1083/jcb.137.5.1161>
 47. Feinberg EH, Hunter CP. Transport of dsRNA into cells by the transmembrane protein SID-1. *Science* 2003; 301:1545-7; PMID:12970568; <https://doi.org/10.1126/science.1087117>
 48. Pei J, Millay DP, Olson EN, Grishin NV. CREST—a large and diverse superfamily of putative transmembrane hydrolases. *Biol Direct* 2011; 6:37; PMID:21733186; <https://doi.org/10.1186/1745-6150-6-37>
 49. Sun H, Zhu X, Lu PY, Rosato RR, Tan W, Zu Y. Oligonucleotide aptamers: new tools for targeted cancer therapy. *Mol Ther Nucleic Acids* 2014; 3:e182; PMID:25093706; <https://doi.org/10.1038/mtna.2014.32>
 50. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003; 5:834-9; PMID:12942087; <https://doi.org/10.1038/ncb1038>
 51. Iguchi H, Kosaka N, Ochiya T. Secretory microRNAs as a versatile communication tool. *Commun Integr Biol* 2010; 3:478-81; PMID:21057646; <https://doi.org/10.4161/cib.3.5.12693>
 52. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci* 2010; 101:2087-92; PMID:20624164; <https://doi.org/10.1111/j.1349-7006.2010.01650.x>
 53. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 2001; 7:297-303; PMID:11231627; <https://doi.org/10.1038/85438>
 54. Kabuta T, Mitsui T, Takahashi M, Fujiwara Y, Kabuta C, Konya C, Tsuchiya Y, Hatanaka Y, Uchida K, Hohjoh H, et al. Ubiquitin C-terminal hydrolase L1 (UCH-L1) acts as a novel potentiator of cyclin-dependent kinases to enhance cell proliferation independently of its hydrolase activity. *J Biol Chem* 2013; 288:12615-26; PMID:23543736; <https://doi.org/10.1074/jbc.M112.435701>
 55. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. The role of autophagy during the early neonatal starvation period. *Nature* 2004; 432:1032-6; PMID:15525940; <https://doi.org/10.1038/nature03029>