Published online 27 August 2016

Nucleic Acids Research, 2017, Vol. 45, No. 1 ***435–445***
doi: 10.1093/nar/gkw745

Reactive sulfur species regulate tRNA methylthiolation and contribute to insulin secretion

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Received June 16, 2016; Accepted August 16, 2016

ABSTRACT

The 2-methylthio (ms^2) modification at A37 of tRNAs is critical for accurate decoding, and contributes to metabolic homeostasis in mammals. However, the regulatory mechanism of ms^2 modification remains largely unknown. Here, we report that cysteine hydropersulfide (CysSSH), a newly identified reactive sulfur species, is involved in ms^2 modification in cells. The suppression of intracellular CysSSH production rapidly reduced ms^2 modification, which was rescued by the application of an exogenous CysSSH donor. Using a unique and stable isotope-labeled CysSSH donor, we show that CysSSH was capable of specifically transferring its reactive sulfur atom to the cysteine residues of ms^2-modifying enzymes as well as ms^2 modification. Furthermore, the suppression of CysSSH production impaired insulin secretion and caused glucose intolerance in both a pancreatic β-cell line and mouse model. These results demonstrate that intracellular CysSSH is a novel sulfur source for ms^2 modification, and that it contributes to insulin secretion.

INTRODUCTION

tRNAs undergo a wide variety of post-transcriptional modifications that are essential for accurate and efficient decoding in all living organisms (1,2). The 2-methylthio (ms^2) modification is an evolutionarily conserved modification found at position 37 of tRNAs (2). In mammalian cells, two forms of ms^2-modified nucleotide have been identified: 2-methylthio-N^6-threonylcarbamoyadenosine (ms^2t^6A) and 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A) (3,4). Cdk5 regulatory subunit associated protein 1-like-1 (CDKAL1) catalyzes the methylthiolation of t^6A to form ms^2t^6A at A37 of cytosolic $tRNA^{Lys(UUU)}$ (5). On the other hand, Cdk5 regulatory subunit-associated protein 1 (CDK5RAP1) catalyzes the methythiolation of i^6A to form ms^2i^6A at A37 of four mitochondrial tRNAs: mt-$tRNA^{Ser(UCN)}$, mt-$tRNA^{Trp}$, mt-$tRNA^{Phe}$ and mt-$tRNA^{Tyr}$ (4). The ms^2 modification stabilizes codon–anticodon binding through a cross-strand interaction that contributes to accurate decoding (6). Indeed, ms^2 deficiency in cytosolic $tRNA^{Lys(UUU)}$ impairs proinsulin biosynthesis and causes the development of type 2 diabetes (5). Similarly, ms^2 deficiency in ms^2i^6A impairs mitochondrial protein synthesis and leads to the development of myopathy in mice and mitochondrial diseases in humans (4).

Given the essential role of ms^2 modification in regulating protein synthesis, the regulatory mechanism of ms^2 modification has attracted wide attention. All ms^2-modifying enzymes share similar functional domains: UPF0004, radical S-adenosylmethionine (SAM) and tRNA-binding domains (3). The UPF0004 and radical SAM domains form [4Fe-4S] clusters through conserved Cys residues in each domain (7). The methyl group in ms^2 is apparently derived from SAM (7), whereas the origin of the sulfur atom in ms^2 has remained unknown for decades. Recently, Forouhar *et al.* showed that the UPF0004 domain contained exogenous sulfide species that might provide the sulfur atom for ms^2 modification using a defined reconstitution system (8). These findings have shed light on the molecular origin

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of sulfur in ms^2. However, whether the exogenous sulfide species also exist in living cells and how these sulfides are involved in ms^2 modification remain largely unexplored.

The ms^2 modification requires the conversion of a C–H bond to C–S bond, which is a challenging reaction (3). It is thus predicted that reactive sulfur species might be required to initiate the conversion. Cells contain various sulfur species including cysteine hydropersulfide (CysSSH) and hydrogen sulfide (H_2S)(9–12). These sulfur species are mainly produced by cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CTH) (12). CysSSH is highly reactive due to its marked nucleophilicity (12,13). More importantly, it can readily initiate thiol redox exchange, leading to protein Cys-polythiolation (12). Given its ability to transfer sulfur, CysSSH could potentially participate in ms^2 modification. In the present study, we investigated the potential role of the CysSSH in the regulation of ms^2 modification in tRNAs and its physiological relevance *in vivo*.

MATERIALS AND METHODS

Reagents

The oxidized CysSSH donor was chemically synthesized as described previously (12). Briefly, 10 mM cysteine hydrochloride was reacted with 10 mM Na_2S or $Na^{34}SH$ in 50 ml of 10 mM NaOH solution in the presence of 5% I_2 at room temperature. CysS-S_2-SCys and CysS-$^{34}S_2$-SCys were purified by high pressure (or high performance) liquid chromatography. Monobromobimane, β-Cyano-L-alanine and Deferoxamine were purchased from Sigma. Predesigned siRNAs against *CBS* and *CTH* (siRNA for *CBS*: s63475, siRNA for *CTH*: CTHHSS102445) were purchased from Invitrogen and Ambion, respectively. Control siRNA was purchased from Invitrogen. Methylsulfonyl benzothiazole (MSBT) and biotinylated α-cyano ester (CN-biotin) were synthesized as described previously (12). DAPI solution and 3′,6′-dil(*O*-thiosalicyl) fluorescein (SSP4) were purchased from Dojindo, Japan. Hexadecyltrimethylammonium Bromide (CTAB) was purchased from Tokyo Chemical Industry, Japan.

Mass spectrometric analysis of ms^2 modification

For mass spectrometric analysis of ms^2 in HeLa cells, total RNA was purified by TRIzol reagent. For bacterial RNA, TOP10 competent cells were transformed with pET21a-CDKAL1 plasmids. A single colony was cultured in LB medium with constant shaking until the OD_{600} reached 0.7. Next, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, WAKO) was added to the culture, which was then incubated overnight. Total RNA from the bacteria was purified using a TRIzol Max Bacterial RNA Isolation Kit (Invitrogen) following the manufacturer's instructions. Twenty micrograms of RNA were digested with 2.5 U of Nuclease P1 (WAKO) and 0.2 U of bacterial alkaline phosphatase (Takara) in 5 mM ammonium acetate and 20 mM HEPES-KOH, pH 7.0, for 3 h at 37°C. The samples were subjected to mass spectrometry (Agilent 6460). The ms^2 modification was detected by a multiple reaction monitoring (MRM) method using the positive mode. The MRM parameters for ms^2t^6A were as follows: precursor ion: m/z 459.4, product ion: m/z 326.8, collision energy: 14, Fragmentor 125 V.

Mass spectrometry for examination of polysulfide in cells

Cysteine polysulfide (CysSSH) was detected using monobromobimane (Invitrogen), as described previously (12). Monobromobimane efficiently and specifically reacts with CysSSH to form a CysS-S-bimane adduct. HeLa cells were grown on a 10-cm dish, washed with phosphate-buffered saline and directly treated with 200 μl of 5 mM monobromobimane dissolved in methanol. Cells were collected, sonicated and incubated at 37°C for 15 min. Insoluble materials were removed by brief centrifugation at 10 000 × *g* for 10 min. Supernatants were diluted with distilled water and subjected to mass spectrometry using the MRM method in the positive ion mode. The MRM parameters for bimane adducts were as described previously (12).

Imaging of intracellular polysulfides by SSP4

Endogenous reactive sulfur species including CysSSH were observed using the sulfane sulfur-specific fluorescent probe SSP4, as described previously (12) SSP4 is a modified version of SSP2 that was developed for bioimaging sulfane sulfurs in living cells (14). HeLa cells were cultured on 35-mm glass-bottomed dishes (IWAKI) for polysulfide imaging. The cells were washed with serum-free Dulbecco's modified Eagle's medium (DMEM), followed by the addition of 20 μM SSP4 in serum-free DMEM containing 500 μM CTAB at 37°C for 15 min. The cells were then washed twice with phosphate buffered saline (PBS) and counterstained with DAPI in PBS at room temperature for 10 min. Fluorescence was observed using an FV1000 confocal microscope (OLYMPUS). The average fluorescence was quantified using the software FLUOVIEW Ver. 4.2 (OLYMPUS).

Polysulfide-specific biotin-labeling assay

HeLa cells transfected with the pCMV-Myc-CDKAL1 plasmid vector were homogenized in lysis buffer (10 mM Tris-HCl, 1% NP-40 and 150 mM NaCl, pH 7.4) containing a protease inhibitor cocktail (Roche) and immediately incubated with 2 mM MSBT at 37°C for 30 min. Lysates were subsequently reacted with 4 mM CN-biotin at 37°C for 30 min. After the insoluble materials were removed by a brief centrifugation at 10 000 × *g* for 10 min, the biotin-labeled proteins were purified using streptavidin Sepharose beads (GE Healthcare) at 4°C for 3 h. After an extensive wash with lysis buffer, the biotin-labeled proteins were eluted by the addition of SDS sample buffer (50 mM Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol and 0.005% BPB). Cy-polythiolation of CDKAL1 was detected by Western blotting using anti-Myc antibody (Wako).

Mass spectrometric analysis of cysteine polysulfides in the CDKAL1 protein

HeLa cells were transfected with the pCMV-Myc-CDKAL1 vector for 48 h, followed by treatment with 100 μM CysS-S_2-SCys or CysS-$^{34}S_2$-SCys for 6 h. The cells

were then homogenized in lysis buffer (10 mM Tris-HCl, 1% NP-40 and 150 mM NaCl, pH 6.8) and immediately reacted with 1 mM monobromobimane at 37°C for 15 min. The lysates were incubated with anti-Myc antibody at 4°C for 1 h. Myc-CDKAL1 protein was then precipitated by Dynabeads Protein G (Life Technologies). After an extensive wash with lysis buffer, the Myc-CDKAL1 proteins captured on the Dynabeads were digested with 0.5 mg/ml pronase (Calbiochem) in sodium phosphate buffer (pH 6.0) at 37°C for 2 h. The supernatants were directly subjected to mass spectrometric analysis for the detection of CysS-S-bimane adducts, as described above.

Measurement of insulin secretion

The mouse pancreatic beta cell line was seeded in 24-well plates and treated with 500 μM BCA for 24 h. After incubation, the cells were washed with Krebs–Ringer bicarbonate buffer (KRB buffer: 115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 20 mM HEPES, pH 7.4, 0.1% bovine serum albumin) containing 2.8 mM glucose, followed by incubation in the same buffer for 1 h at 37°C. The cells were stimulated with KRB buffer containing 2.8 mM glucose (low glucose) for 30 min, followed by stimulation with KRB buffer containing 16.7 mM glucose (high glucose). The insulin levels in the incubation buffers were measured using an ELISA kit (Shibayagi) following the manufacturer's instructions.

Animals

Cth-deficient mice on C57BL6/6J background were established by Dr. Ishii (15). Animals were housed at 25°C with 12-h light/12-h dark cycles. High-fat chow (D12451, 45% kcal% fat) was purchased from Research Diets. All animal procedures were approved by the Animal Ethics Committee of Kumamoto University (approval ID: A27-037R1).

Cell culture

HeLa cells established from a human cervical cancer cell line were cultured in DMEM high-glucose medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, HyClone) at 37°C and 5% CO_2. Two mouse pancreatic β-cell lines was established from insulinoma that developed in transgenic mice expressing the SV40 antigen under the control of the insulin promoter (16). Male mice at the age of 8 weeks were sacrificed and insulinoma tissues were hand-picked under a microscope. The insulinoma was minced and cultured in DMEM high-glucose medium (GIBCO) supplemented with 10% FBS (HyClone) at 37°C and 5% CO_2.

Quantitative polymerase chain reaction (PCR) analysis of tRNA modification

Total RNA was extracted from the lysates of cultured cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The ms^2 modification levels were analyzed using a quantitative PCR-based method as described previously (17). The levels of ms^2-modified $tRNA^{Lys(UUU)}$ were normalized to the total $tRNA^{Lys(UUU)}$ (17). In all experiments, the ms^2 modification levels in control cells are expressed as 100%. The modification levels in compound-treated cells are expressed as levels relative to those in control cells. The sequences of primers were as follows:

Primers for detecting ms^2t^6A in human and mouse cells
$tRNA^{Lys}$_forward primer: GTCGGTAGAGCATCAGACTT
$tRNA^{Lys}$_reverse primer r1: CCTGGACCCTCAGATTAAAA
$tRNA^{Lys}$_reverse primer r1: GAACAGGGACTTGAACCCTG

Gene expression analysis

RNA was extracted from the cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. A PrimerScript RT Reagent Kit (TAKARA) was used to generate cDNA. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (TAKARA). The results were normalized to beta-2 microglobulin.

Plasmids

For overexpression of the CDKAL1 protein in HeLa cells, cDNA encoding *CDKAL1* was subcloned into the pCMV-Myc vector (Clontech). For the expression of CDKAL1 in *E. coli*, CDKAL1 was subcloned into the pET21a vector (Novagen). For the construction of CDKAL1 carrying Cys-to-Ala mutations, Cys 83, Cys 109 and Cys 138 in the UPF0004 domain and Cys 214, Cys 218 and Cys 221 in the radical SAM domain were mutated to Ala using QuikChange II Site-Directed Mutagenesis Kits (Agilent Technologies). Plasmids were used to transfect HeLa cells with Lipofectamine 2000 (Invitrogen).

Measurement of blood glucose and plasma insulin levels

The glucose tolerance test was performed in either male mice fed normal chow at the age of 6 weeks old or in male mice fed a high-fat diet for 20 weeks. Briefly, mice were fasted for 20 h (6:00 pm to 14:00 am), followed by the intraperitoneal injection of glucose (1 g/kg). The plasma glucose level was determined by a glucometer (ACCU-CHEK, Aviva; Roche). Plasma insulin levels were determined using an ELISA kit (Shibayagi, Tokyo, Japan) following the manufacturer's instructions.

Statistical analysis

All data are presented as the mean ± SEM unless otherwise indicated. Statistical analyses were performed using Prism 6 Software (GraphPad Software). An unpaired Student's *t*-test was used to test the differences between two groups. Analysis of variance (one-way ANOVA) was used to test the differences among multiple groups, followed by the Bonferroni procedure to calculate the *P*-value between two groups. A two-tailed *P*-value of 0.05 was considered significant.

RESULTS

Regulation of endogenous CysSSH by CBS and CTH

CBS and CTH are key enzymes in sulfur biology (Figure 1A). In addition to the classic cysteine biogenesis pathway,

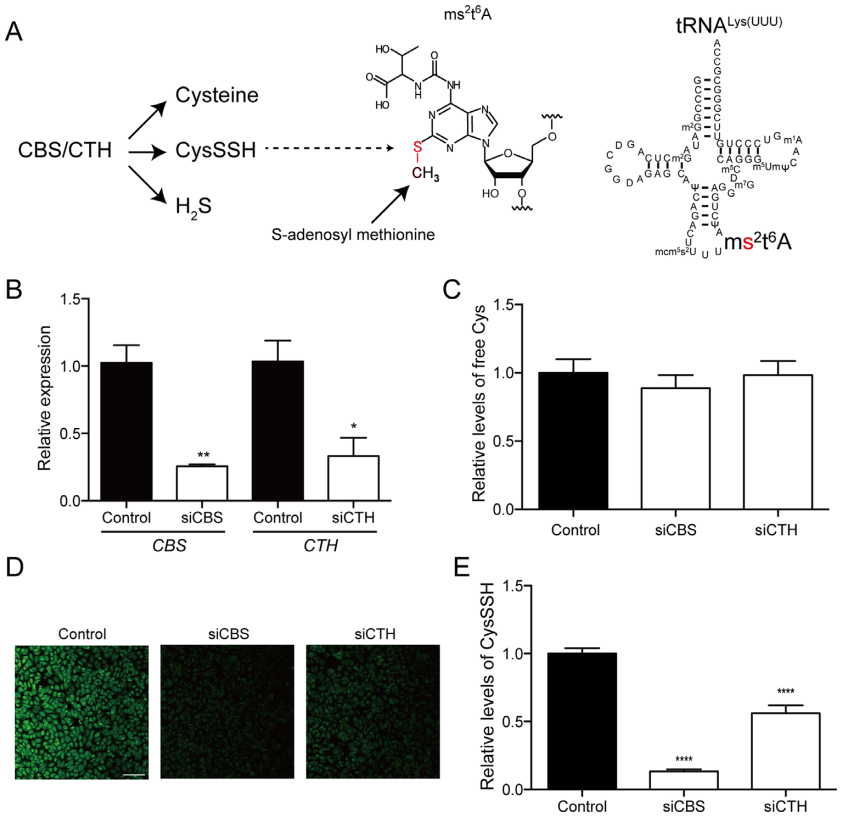

Figure 1. Regulation of endogenous cysteine hydropersulfide (CysSSH) by cystathionine beta synthase (CBS) and cystathionine gamma lyase (CTH). (**A**) Potential molecular origins of ms^2 modification of $tRNA^{Lys(UUU)}$. (**B**) The efficiency of siRNAs against *CBS* (siCBS) and *CTH* (siCTH) in HeLa cells. n = 4, $*P < 0.05$, $**P < 0.01$. (**C**) The endogenous free cysteine levels in cells treated with control siRNA and siRNAs against *CBS* (siCBS) or *CTH* (siCTH) were determined by mass spectrometry and normalized to endogenous phenylalanine levels. n = 3 each. (**D**) The endogenous CysSSH levels in cells treated with control siRNA and siRNAs against *CBS* (siCBS) or *CTH* (siCTH) were visualized by the SSP4 probe. Bar = 0.1 mm. (**E**) Relative levels of CysSSH were estimated by quantification of the intensity of SSP4 in each sample. Results are expressed as the intensity relative to that in control cells. n = 13–20, $****P < 0.0001$ versus control sample.

CBS/CTH have recently been implicated in the production of various sulfur species including CysSSH and H_2S (12, Figure 1A). To investigate the biological source of the sulfur atom in ms^2 modification, we silenced *CBS* or *CTH* in Hela cells using specific siRNAs and examined the levels of free cysteine and CysSSH (Figure 1B). The siRNAs successfully downregulated *CBS* or *CTH*, but the free cysteine level was unchanged in the siRNA-treated cells, when compared with control siRNA-treated cells (Figure 1C). To examine the endogenous CysSSH level, we applied a sulfane sulfur-specific fluorescent probe, SSP4, in HeLa cells (12). The reactive sulfur atom in CysSSH, but not cysteine or glutathione, is capable of reacting with the SSP4 probe to release its fluorophore (14). There was a significant decrease of SSP4 fluorescence in *CBS*- and *CTH*-silenced cells, when compared with control cells (Figure 1D and E). These results demonstrate that the downregulation of *CBS* or *CTH* selectively reduced the CysSSH level in HeLa cells.

Regulation of ms^2 modification by CysSSH in living cells

Next, we investigated whether CysSSH is involved in the regulation of ms^2 modification of tRNAs. *CBS* and *CTH* were silenced by siRNAs, and the levels of ms^2 modification in cytosolic $tRNA^{Lys(UUU)}$ were examined using quantitative PCR (17). There was a significant decrease in the ms^2 levels in *CBS*- and *CTH*-silenced cells, when compared with control cells (Figure 2A). In addition to the treatment with the siRNAs, we also chemically downregulated CysSSH using β-cyano-L-alanine (BCA), a classic inhibitor of CTH (18). BCA markedly reduced the intracellular CysSSH level, as indicated by the reduction of SSP4 fluorescence (Figure 2B). Accordingly, the ms^2 modification was significantly decreased in BCA-treated cells (Figure 2C). Furthermore, co-application of BCA and *CBS*-targeting siRNA synergistically reduced the ms^2 modification level (Figure 2D).

To further demonstrate that CysSSH is involved in the regulation of ms^2 modification, we aimed to modulate ms^2 modification by supplementing exogenous CysSSH. We chemically synthesized a CysSSH donor, CysS-S_2-SCys. Upon uptake by cells, CysS-S_2-SCys is rapidly broken down to CysSSH due to the reductive cellular environment. Indeed, the application of 100 μM of the CysSSH donor to HeLa cells for 1 h resulted in a marked increase of the intracellular CysSSH level (Supplementary Figure S1, Vehicle: 3.8 nM, CysSSH: 71 nM). HeLa cells were treated with specific siRNAs against *CBS* and *CTH*, followed by application of the CysSSH donor (Figure 2E). As expected, CysSSH donor supplementation successfully reversed the

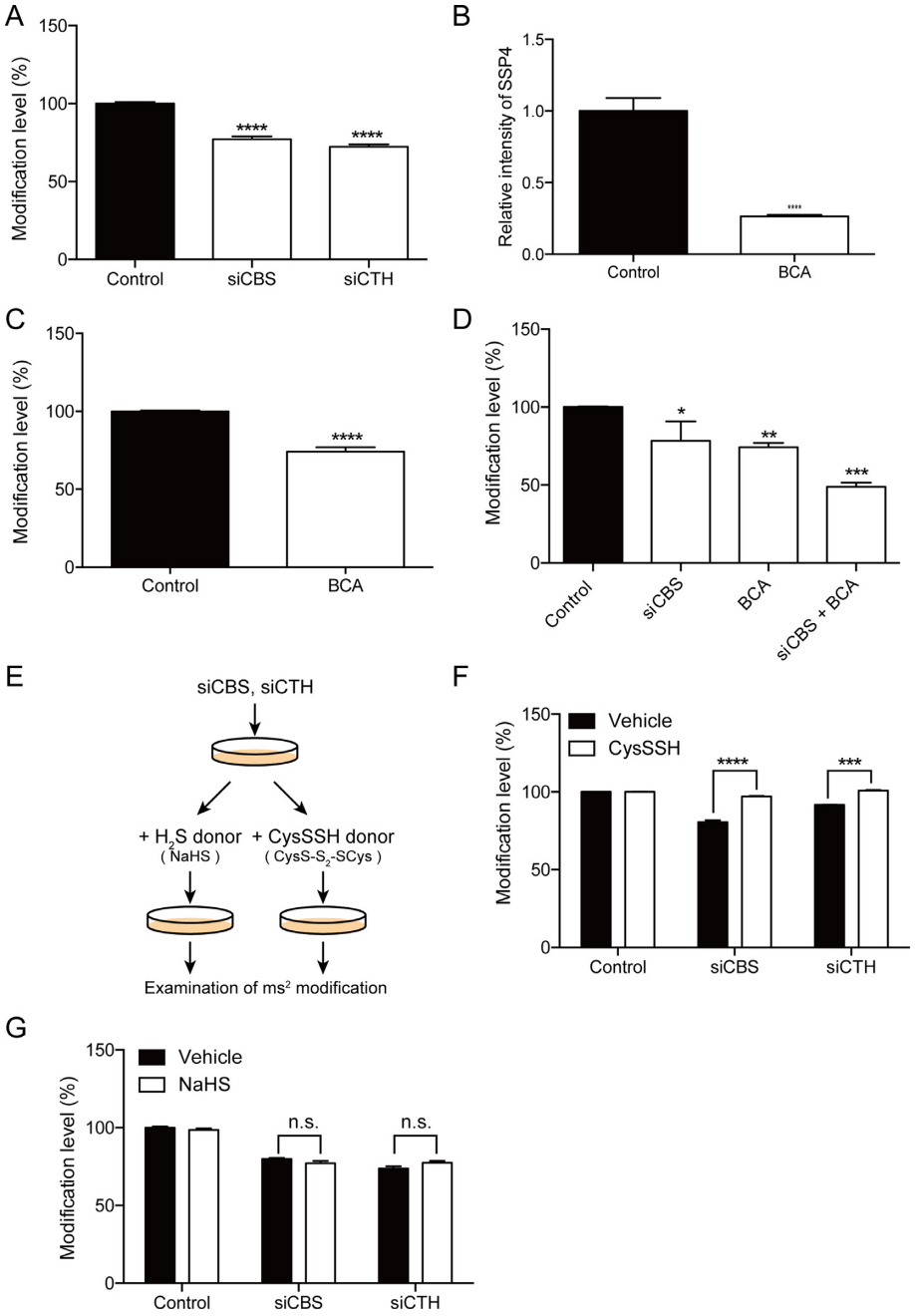

Figure 2. (**A**) Regulation of ms^2 modification by CysSSH in intact cells. Silencing of *CBS* and *CTH* significantly reduced the ms^2 level of $tRNA^{Lys(UUU)}$. n = 4, $^{****}P < 0.0001$. (**B**) Cells treated with 500 μM BCA exhibited a significant decrease in endogenous CySSSH levels. n = 8–9, $^{****}P < 0.0001$. (**C**) Treatment with 500 μM BCA significantly decreased the ms^2 modification levels of $tRNA^{Lys(UUU)}$. n = 4, $^{****}P < 0.0001$. (**D**) Inhibition of CBS and CTH with siCBS and BCA synergistically suppressed endogenous CysSSH levels. n = 4, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**E**) Experimental design for rescuing ms^2 modification by the exogenous CysSSH donor or H_2S donor. (**F**) The CysSSH donor, but not (**G**) H_2S donor, effectively reversed the suppression of ms^2 modification levels of $tRNA^{Lys(UUU)}$ by silencing *CBS* or *CTH*. n = 4, $^{****}P < 0.0001$. n.s.: not significant.

ms^2 modification, which was downregulated by silencing *CBS* or *CTH* (Figure 2F).

Both CBS and CTH are capable of producing H_2S, which is also actively involved in diverse biological activities (19). Therefore, to investigate the potential contribution of H_2S to ms^2 modification, we applied NaHS, a donor of H_2S, to HeLa cells with silencing of *CBS or CTH* (Figure 2E). However, NaHS was not able to reverse the ms^2 modification level of $tRNA^{Lys(UUU)}$ in *CBS*-silenced or *CTH*-silenced cells (Figure 2G). Taken together, these results suggest that CBS/CTH-mediated CysSSH production is selectively involved in the regulation of ms^2 modification.

Selective chasing of reactive sulfur atom by stable isotope labeling

To clarify which sulfur atom in CysSSH is utilized for ms^2 modification, we synthesized a 'heavy' CysSSH donor (CysS-$^{34}S_2$-SCys), in which reactive sulfur atoms were selectively and stably labeled with heavy isotope ^{34}S instead of natural ^{32}S (Figure 3A). The heavy CysSSH donor markedly and selectively induced the intracellular Cys$S^{34}SH$ level as quickly as 1 h after application (Supplementary Figure S2A and B). Notably, application of the Cys$S^{34}SH$ donor did not give rise of S^{34}-labeled free cysteine (Supplementary Figure S2C and D). These results clearly demonstrate that the Cys$S^{34}SH$ donor enables us to selectively track the mobilization of reactive sulfur atoms by the differential molecular mass.

We applied the Cys$S^{34}SH$ donor to HeLa cells and examined whether Cys$S^{34}SH$ is capable of providing a 'heavy' sulfur atom for ms^2 modification (Figure 3A). All chemical species contain a trace of the isotope, and exhibit a fixed isotopic distribution pattern. Theoretically, the abundance of naturally occurring $m^{34}s^2t^6A$ (m/z 461) only accounts for 8.19% of the abundance of ms^2t^6A (m/z 459). If the heavy ^{34}S of Cys$S^{34}SH$ is actively incorporated in the sulfur atom of ms^2t^6A, there will be an unnatural increase of the abundance of $m^{34}s^2t^6A$ (Figure 3A). However, if ms^2 modification utilizes unlabeled sulfur atoms of cysteine, the abundance of $m^{34}s^2t^6A$ would remain unchanged (Figure 3A). This isotopic distribution can be monitored by the ratio of the abundance of $m^{34}s^2t^6A$ to the abundance of ms^2t^6A ($m^{34}s^2t^6A$/ ms^2t^6A), which thus reflects the incorporation of the reactive ^{34}S atom. When Hela cells were treated with 100 μM of the CysSSH donor, the abundance of $m^{34}s^2t^6A$ accounted for 6.49% of the abundance of ms^2t^6A, which is close to the theoretical ratio (Figure 3B and C). Upon treatment with 100 μM of the Cys$S^{34}SH$ donor, there was a marked increase of $m^{34}s^2t^6A$; the abundance of $m^{34}s^2t^6A$ in Cys$S^{34}SH$ donor-treated cells accounted for 20.5% of the abundance of ms^2t^6A, which is far beyond the natural distribution (Figure 3B and C). This evidence strongly suggests that a substantial portion of ms^2 modification is likely to be derived from the reactive sulfur atom of CysSSH.

We examined the efficacy of the Cys$S^{34}SH$ donor by subjecting cells to various conditions. The incorporation of ^{34}S was dose-dependent, with an optimal concentration of 100 μM (Figure 3D). The incorporation of ^{34}S peaked 6 h after treatment (Figure 3E). The utilization of Cys$S^{34}SH$ donor allowed us to examine the turnover rate of ms^2 modification *in vivo*. HeLa cells were labeled with the Cys$S^{34}SH$ donor for 6 h, followed by various chasing periods. The ^{34}S in $m^{34}s^2t^6A$ decreased slowly and had mostly disappeared at 48 h after treatment (Figure 3F). The slow turnover rate of ms^2-containing tRNA is in agreement with the half-life of cytosolic tRNAs *in vivo* (20), and reflects the partial labeling efficiency by the Cys$S^{34}SH$ donor. Taken together, these results clearly demonstrate that the reactive sulfur atom of CysSSH is transferred for the ms^2 modification of tRNA under physiological conditions.

Polysulfidation of CDKAL1 in intact cells

A previous study of an *in vitro* reconstituted bacterial ms^2-modifying enzyme, MiaB, revealed the existence of exogenous sulfur atoms in the enzyme (8). It has been proposed that the enzyme-conjugating polysulfide might be utilized for ms^2 modification. We speculated that CDKAL1 may undergo CysSSH-mediated protein polysulfidation, and this is subsequently utilized for ms^2 modification. To investigate the potential polysulfidation of CDKAL1, we performed a polysulfide-specific biotin-labeling assay in HeLa cells (12). This unique method enables the selective conjugation of biotin to the reactive sulfur residue present in polysulfide-containing proteins (Figure 4A). The biotin-labeled protein can be specifically enriched by streptavidin-beads and subjected to the downstream applications (Figure 4A). HeLa cells expressing Myc-CDKAL1 were subjected to the biotin-labeling assay. Subsequently, the enriched polysulfide-containing proteins were examined by Western blotting using anti-Myc antibody. As expected, Myc-CDKAL1 was clearly detected in the polysulfide-containing protein fraction (Figure 4B).

The UPF0004 domain of bacterial MiaB protein forms a cluster [4Fe–4S] and has also been proposed to form a polysulfide moiety (3,8). Consistent with the bacterial model, mutation of the Cys residues in the UPF0004 domain of CDKAL1 completely eliminated its activity (Supplementary Figure S3). To examine whether the polysulfidation of CDKAL1 occurred in the UPF0004 domain of intact cells, CDKAL1 in which the Cys residues were mutated to Ala was subjected to the polysulfide-specific biotin labeling assay (Figure 4C). Compared with wild-type CDKAL1, CDKAL1 carrying Cys-to-Ala mutations in the radical SAM domain exhibited a slight decrease in polysulfidation. Intriguingly, the polysulfidation level was markedly decreased in CDKAL1 carrying Cys-to-Ala mutations in the UPF0004 domain. Mutations in both the UPF0004 and radical SAM domains almost eliminated the polysulfidation from the CDKAL1 protein. These results suggest that the UPF0004 domain is the major polysulfidation site in CDKAL1.

CysSSH-mediated polysulfidation of CDKAL1

To investigate whether the polysulfidation of CDKAL1 is also mediated by the reactive sulfur atom of CysSSH, we treated HeLa cells with the Cys$S^{34}SH$ donor, and examined ^{34}S-containing polysulfidation in CDKAL1 (Figure 5A). In analogy with the experiment that aimed to detect ^{34}S incorporation in ms^2 modification shown in Figure 3A, the formation of ^{34}S-containing polysulfide in Cys residues of CDKAL1 would result in an increased abundance of Cys$S^{34}SH$, which would lead to a shift in the isotopic distribution of CysSSH (Figure 5A). HeLa cells expressing Myc-CDKAL1 were treated with the Cys$S^{34}SH$ donor for 1 h. Subsequently, Myc-CDKAL1 was immunoprecipitated with anti-Myc antibody. The immunoprecipitaed proteins were digested by pronase and subjected to mass spectrometry. In control cells treated with the CysSSH donor, abundance of natural occurring Cys$S^{34}SH$ accounted for 1% of the abundance of CysSSH in Myc-CDKAL1 (Figure 5B). After application of the Cys$S^{34}SH$ donor, the abundance of

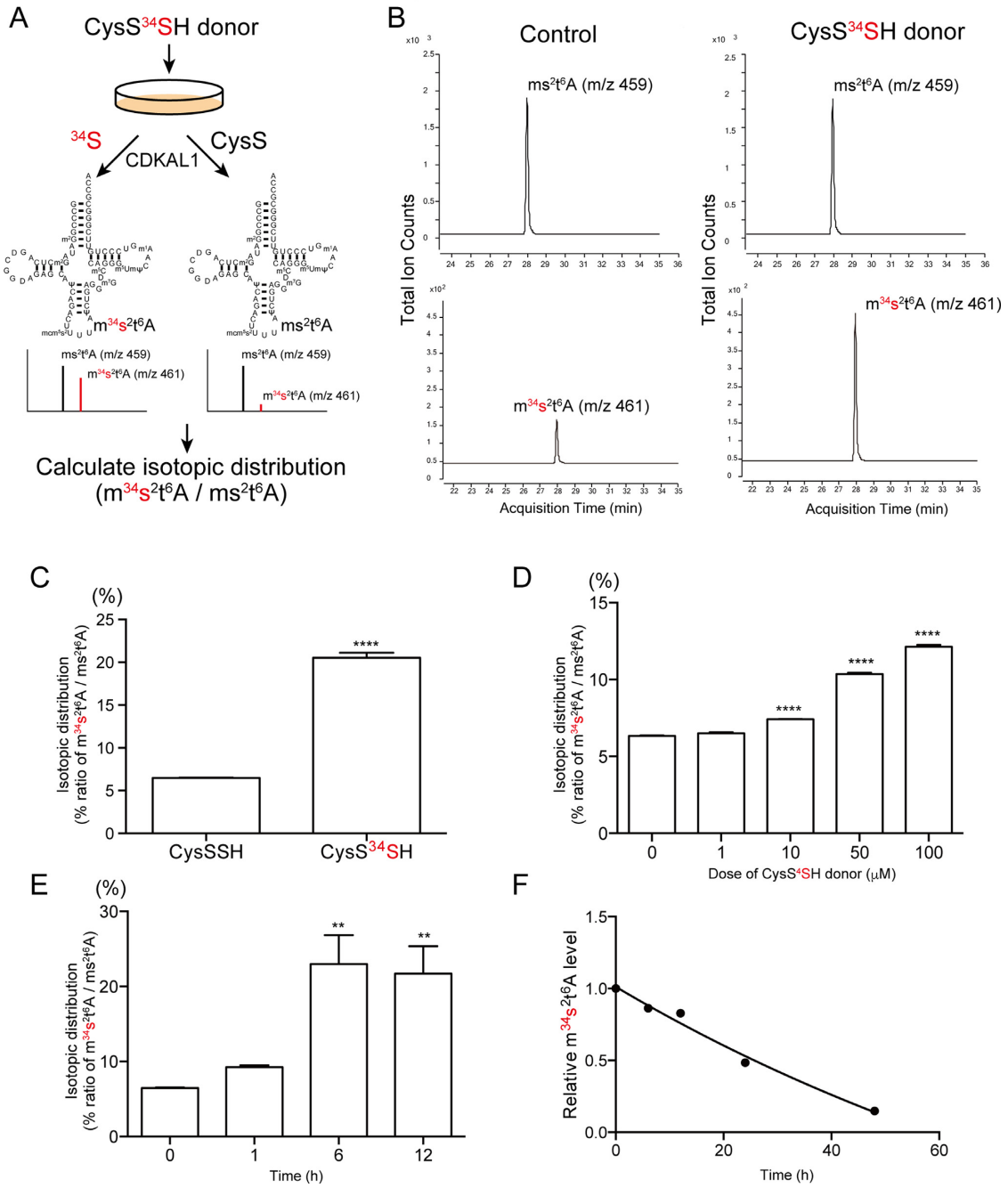

Figure 3. Incorporation of reactive sulfur atom in ms^2 modification. (**A**) Experimental design to identify the sulfur source for ms^2 in intact cells. The red letters represent the stable isotope-labeled reactive sulfur atoms of CysSSH. (**B**) HeLa cells were treated with 100 μM of CysSSH or the $CysS^{34}SH$ donor. Subsequently, total RNA was purified and subjected to mass spectrometry. Representative mass chromatograms of ms^2t^6A and $m^{34}s^2t^6A$ are shown. The $CysS^{34}SH$ donor markedly increased the $m^{34}s^2t^6A$ level. (**C**) The isotopic distribution (relative abundance of $m^{34}s^2t^6A$ to ms^2t^6A) of ms^2t^6A in cells treated with vehicle (CysSSH donor) or the $CysS^{34}SH$ donor. n = 4 each, $^{****}P < 0.0001$. The isotopic distribution of ms^2t^6A in response to different (**D**) doses or (**E**) periods of treatment with 100 μM of the $CysS^{34}SH$ donor. $^{**}P < 0.01$, $^{****}P < 0.0001$, n = 4 each. (**F**) Pulse-chasing of $m^{34}s^2t^6A$ showing the relatively slow rate of decrease in the amount of $m^{34}s^2t^6A$ in cells. Data points represent the relative change in the isotopic distribution of ms^2t^6A from four replicates.

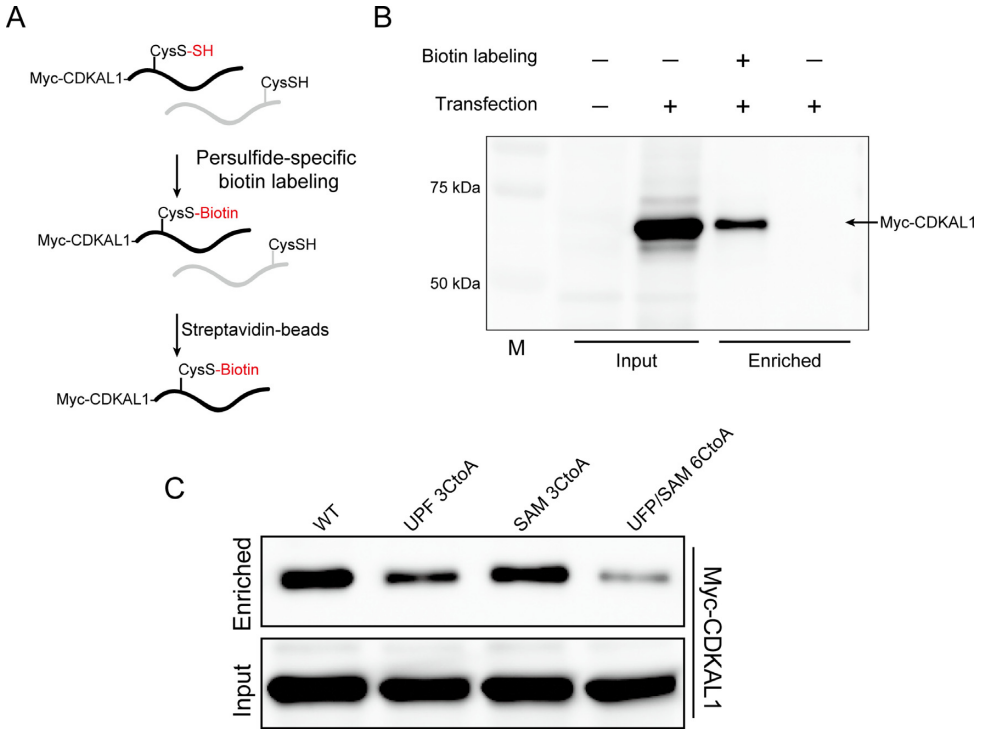

Figure 4. Detection of polysulfidation in CDKAL1. (**A**) Illustration of the polysulfide-specific biotin labeling assay used to identify the polysulfidation in proteins. (**B**) HeLa cells overexpressing Myc-CDKAL1 were subjected to a polysulfide-specific biotin labeling assay. Myc-CDKAL1 was detected in enriched proteins using streptavidin beads. (**C**) The polysulfidation levels in wild-type CDKAL1 (WT) or mutant CDKAL1 carrying Cys-to-Ala mutations in the UPF0004 domain (UPF 3CtoA), radical SAM domain (SAM 3CtoA) or UPF0004/radical SAM domains (UPF/SAM 6CtoA) were evaluated using a polysulfide-specific biotin labeling assay.

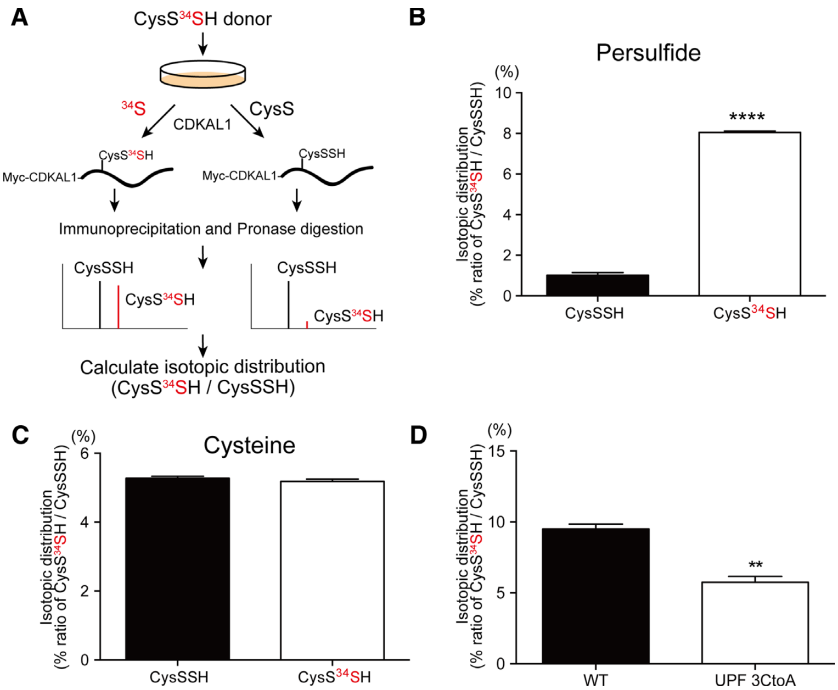

Figure 5. CysSSH-mediated polysulfidation of CDKAL1. (**A**) Experimental design to detect ^{34}S-labeled polysulfide in the CDKAL1 protein. (**B** and **C**) HeLa cells overexpressing Myc-CDKAL1 were treated with vehicle (CysSSH donor) or the CysS^{34}SH donor for 1 h. Myc-CDKAL1 was immunoprecipitated and subjected to mass spectrometry to determine the isotopic distribution of polysulfidation in Cys residues. The isotopic distribution of CysSSH (abundance of CysS^{34}SH relative to that of CysSSH) is shown in (B). The isotopic distribution of the Cys residue (abundance of CysS^{34}H relative to that of CysSH) is shown in (C). $^{****}P < 0.0001$, n = 4. (**D**) HeLa cells expressing wild-type Myc-CDKAL1 or mutant CDKAL1 carrying Cys-to Ala mutations in the UPF0004 domain were treated with the CysS^{34}SH donor, followed by mass spectrometric analysis. The mutant CDKAL1 had less ^{34}S incorporation in Cys residues in CDKAL1, when compared with the wild-type CDKAL1. n = 3 each, $^{**}P < 0.01$.

$CysS^{34}SH$ accounted for as high as 8% of the abundance of CysSSH (Figure 5B). In contrast, the $CysS^{34}SH$ donor did not change the isotopic distribution of cysteine residue ($Cys^{34}SH$/CysSH) in CDKAL1 protein (Figure 5C). Furthermore, we applied the $CysS^{34}SH$ donor to HeLa cells expressing Myc-CDKAL1 with or without mutations in the UPF0004 domain, and examined polysulfidation by mass spectrometry. There was a significant reduction of ^{34}S-containing polysulfidation in mutant CDKAL1, when compared with the wild-type (Figure 5D). These results strongly suggest that CysSSH selectively transferred the reactive sulfur atom to Cys residues of CDKAL1, and induced polysulfidation in the UPF0004 domain of CDKAL1.

In addition, we aimed to exclude the possibility that the [4Fe-4S] clusters might non-specifically transfer sulfur atoms to cysteine residues of CDKAL1 protein during sample preparation under oxidized conditions. HeLa cells were treated with an iron-chelating reagent, deferoxamine, to degenerate [4Fe-4S] clusters and then subjected to mass spectrometry. The polysulfidation in the CDKAL1 protein was not affected by deferoxamine (DFOM) (Supplementary Figure S4A–D), whereas the reagent significantly impaired CDKAL1 activity (Supplementary Figure S4A–E). Taken together, these results suggest that cysteine residues in UPF0004 of CDKAL1 undergo CysSSH-mediated polysulfidation and the sulfur of the polysulfidated Cys residues is ultimately transferred for ms^2 modification.

Regulation of ms^2 modification and insulin secretion by CysSSH in pancreatic β-cells

CDKAL1-medated ms^2 modification regulates insulin biosynthesis in pancreatic β-cells, and has been implicated in the development of type 2 diabetes (5). Given the important role of CysSSH in ms^2 modification, we aimed to investigate whether CysSSH contributes to insulin secretion through the regulation of ms^2 modification in pancreatic β-cells. We established two pancreatic β-cell-derived cell lines and applied BCA to reduce the intracellular CysSSH levels. The suppression of CysSSH production by BCA significantly decreased the ms^2 modification level in both β-cell lines (Figure 6A and Supplementary Figure S5A). We treated β-cell lines with BCA and then stimulated the cells with 2.8 mM (low) glucose and 16.7 mM (high) glucose. BCA significantly decreased glucose-stimulated insulin secretion in these cells (Figure 6B and Supplementary Figure S5B).

Next, we investigated whether or not modulation of the CysSSH level affects ms^2 modification and glucose metabolism *in vivo*. Cth-deficient mice developed normally but exhibited high-fat diet-induced metabolic defects (15,21). Indeed, when the mice were fed normal chow, the hepatic CysSSH level in Cth-deficient mice was comparable with that of wild-type mice (Supplementary Figure S6A). To accelerate the diabetic phenotype, we fed the mice a high-fat diet for 20 weeks. The hepatic CysSSH level of Cth-deficient mice was then significantly lower than that of wild-type mice (Supplementary Figure S6B). Accordingly, there was a significant reduction of ms^2 modification in pancreatic islets of Cth-deficient mice, when compared with wild-type mice (Figure 6C). These results suggest that CysSSH is associated with ms^2 modification *in vivo*.

To examine whether a decrease of ms^2 modification affects insulin secretion, mice were injected with glucose and the blood insulin level was examined. The Cth-deficient mice fed a high-fat diet exhibited impaired insulin secretion, when compared with wild-type mice (Figure 6D). To examine whether the impairment of insulin secretion was associated with a decrease of glucose metabolism, the mice were injected with glucose and the blood glucose levels were measured. The blood glucose level in Cth-deficient mice was significantly higher than that in control mice (Figure 6E). Taken together, these results suggest that CysSSH contributes to ms^2 modification and glucose metabolism *in vivo*.

DISCUSSION

The present study provides direct evidence that the intracellular CysSSH is closely involved in the regulation of ms^2 modifications in mammalian tRNAs. Using the unique CysSSH donor in combination with precision mass spectrometry-based analytic methods, our results clearly demonstrate that the reactive sulfur of CysSSH rapidly initiates protein polysulfidation in ms^2-modifying enzymes and mediates the sulfur insertion of ms^2 modification. Furthermore, the suppression of CysSSH production resulted in a decreased ms^2 level, which ultimately led to the impairment of insulin secretion *in vivo*.

The regulatory mechanism of ms^2 modification in mammalian cells has remained largely unknown. In general, all ms^2-modifying enzymes require [4Fe-4S] clusters for their catalytic activities. Based on studies of biotin synthase, a [4Fe-4S] cluster-containing thiotransferase, it was previously assumed that the sulfur atom in ms^2 might be derived from the sacrifice of the sulfur atom from its own [4Fe–4S] cluster (22). Recently, a structural study of bacterial MiaB protein questioned this self-sacrifice model, and proposed that the sulfur atom of ms^2 might be derived from an extra sulfur group conjugated to the enzyme (8). However, the extra sulfur-regulated ms^2 modification could be formed as a byproduct during the *in vitro* reconstitution of [4Fe–4S] clusters. Using chemically defined $CysS^{34}SH$ donors, we were able to provide direct evidence that the extra sulfur group of CDKAL1 was indeed formed in intact mammalian cells, and that the extra sulfur was derived from the reactive sulfur of CysSSH. In addition, the majority of the polysulfidation was found in the UPF0004 domain of CDKAL1 that was also in agreement with a previous prediction (8). Taken together, these results suggest that CysSSH-mediated protein polysulfidation is a physiological event that contributes to ms^2 modification.

Sulfur atoms are widely incorporated into tRNAs during a number of essential modifications, including 2-thiouridine, 4-thiouridine and 2-thiocythidine (2). To date, cysteine has been considered as the only sulfur source for these modifications (23). In the general model, cysteine desulfurase captures a sulfur atom from cysteine, and forms enzyme-bound polysulfide as the first step of the reactions (24). The activated sulfur atom is ultimately relayed to tRNAs by various enzymes. In contrast to the general model, our study showed that ms^2 modification and CDKAL1 con-

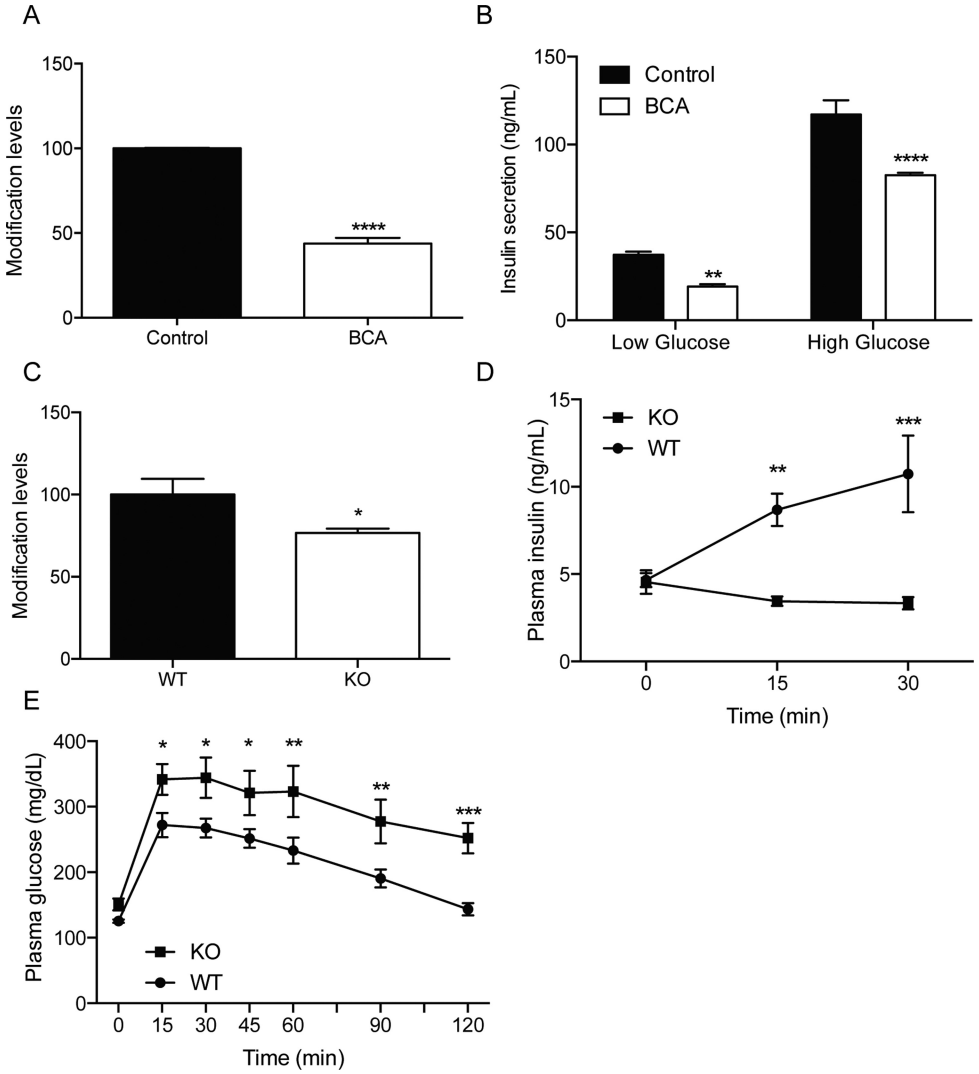

Figure 6. Regulation of insulin secretion by CySSH. (**A**) Suppression of CysSSH by 500 μM BCA significantly decreased ms^2 levels in the pancreatic β-cell line #1. n = 4, $^{****}P < 0.0001$. (**B**) The inhibition of Cth by BCA significantly impaired glucose-stimulated insulin secretion in the pancreatic β-cell line. n = 8, $^{**}P < 0.01$, $^{****}P < 0.0001$. (**C**) Wild-type (WT) mice and Cth-deficient mice (KO) were fed a high-fat diet for 20 weeks. The ms^2 levels of $tRNA^{Lys(UUU)}$ in pancreatic islets were examined. n = 4–5, $^{*}P < 0.05$. (**D**) WT and KO mice were fed a high-fat diet for 20 weeks and challenged with glucose (1 g/Kg). (**D**) Plasma insulin levels and (**E**) blood glucose levels at indicated time-points are shown. n = 4–5, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

tained a substantial portion of sulfur atoms, which were derived from the reactive sulfur atoms of CysSSH, but not cysteine. These results thus challenge the classical model, and suggest that there are multiple sulfur sources for tRNA thiolation, including the reactive CysSSH. The CysSSH probes and analytic methodologies utilized in this study would provide unique biochemical tools for studying the molecular mechanisms of these sulfur transfers in the future.

Oxidative stress impairs insulin secretion and subsequently induces glucose intolerance. Because CysSSH is highly susceptible to intracellular reactive oxygen species (12), excess oxidative stress might downregulate the CysSSH level, which in turn impairs ms^2 modification as well as insulin secretion. Consistent with this view, the CysSSH level was markedly impaired in Cth-deficient mice in a stress-dependent manner. Furthermore, the decrease of the CysSSH level was associated with a decrease of ms^2 modification as well as impaired insulin secretion in Cth-deficient mice. Nevertheless, it is conceivable that the high-fat-induced oxidative stress might also affect the redox state of [4Fe–4S] clusters of CDKAL1.

In summary, our results show that the reactive CysSSH is a novel sulfur source for ms^2 modification of tRNA and CDKAL1 in intact cells and *in vivo*. Suppression of the CysSSH level leads to the reduction of ms^2 modification and the impairment of insulin signaling.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Nobuko Maeda for technical support during the animal experiments.

Author contributions: N.T. performed all experiments and wrote the manuscript; Y.O. and T.S. synthesized the persulfide donors; S.W. performed the animal experiments and analyzed the data; T.A. provided reagents for polysulfide-specific biotin-labeling assay; I.I. established and provided Cth-deficient mice; A.F., T.K. and H.N. contributed to the discussion; and F.Y.W. and K.T. designed the experiments and wrote the manuscript.

FUNDING

Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Japan Society for the Promotion of Science (JSPS) through its 'Funding Program for Next Generation World-Leading Researchers'; Grant-in-aid for Scientific Research from the Ministry of Health, Labour and Welfare. Funding for open access charge: Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest statement. None declared.

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