Cysteiny1-tRNA synthetase governs cysteine polysulfdation and mitochondrial bioenergetics

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Cysteine hydropersulfide (CysSSH) occurs in abundant quantities in various organisms, yet little is known about its biosynthesis and physiological functions. Extensive persulfide formation is apparent in cysteine-containing proteins in Escherichia coli and mammalian cells and is believed to result from post-translational processes involving hydrogen sulfide-related chemistry. Here we demonstrate effective CysSSH synthesis from the substrate l-cysteine, a reaction catalyzed by prokaryotic and mammalian cysteinyl-tRNA synthetases (CARSs). Targeted disruption of the genes encoding mitochondrial CARSs in mice and human cells shows that CARSs have a crucial role in endogenous CysSSH production and suggests that these enzymes serve as the principal cysteine persulfide synthases in vivo. CARSs also catalyze co-translational cysteine polysulfidation and are involved in the regulation of mitochondrial biogenesis and bioenergetics. Investigating CARS-dependent persulfide production may thus clarify aberrant redox signaling in physiological and pathophysiological conditions, and suggest therapeutic targets based on oxidative stress and mitochondrial dysfunction.

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Cysteine hydrosulfide (CysSSH) is found physiologically in prokaryotes, eukaryotic cells, and mammalian tissues. Previously, we unequivocally verified the presence of remarkable amounts of CysSSH, glutathione persulfide (GSSH), and longer chain sulfur compounds (polysulfides, including CysS/GS-\((\text{S}_i)\) to \(-\text{H}\)) in cultured cells and tissues in vivo in mice and humans. The chemical properties and abundance of these species suggest a pivotal role for reactive persulfides (i.e., compounds containing an—S\(\text{H}\) group) in cell-regulatory processes. Researchers proposed that CysSSH and related species can behave as potent antioxidants and cellular protectants, and may function as redox signaling intermediates. Persulfides are also essential structural components of several proteins and enzymes, e.g. serving as metal ligands in iron-sulfur clusters (or sulfide donors) and in iron-cysteine and zinc-cysteine complexes. In fact, the existence of a cell reservoir for sulfane sulfur (sulfur-bonded \(\text{H}\)) is increasingly being recognized, the chemical biology and physiological functions of these species are not known with any certainty. Current dogma holds that persulfide/polysulfide formation arises as a result of hydrogen sulfide (\(\text{H}_2\text{S}\)) oxidation or chemical reaction with nitric oxide. Two \(\text{H}_2\text{S}\)-generating enzymes involved in sulfur-containing amino acid metabolism—cystathionine \(\gamma\)-lyase (cystathionase, CSE) and cystathionine \(\beta\)-synthase (CBS)—can catalyze CysSSH biosynthesis using cystine (CysSSCys) as a substrate. However, the observed \(K_m\) is high, and both cells and mice lacking CSE and/or CBS still display appreciable levels of CysSSH. This suggests the possibility that alternative processes may be responsible for endogenous persulfide production. Thus, it appears that other biosynthetic routes of CysSSH formation exist that have yet to be identified.

This study reveals that cysteinyl-tRNA synthetases (CARSs), in addition to their canonical role in protein translation, act as the principal cysteine persulfide synthases (CPERSs) in vivo. CARSs play a novel and prominent role in endogenous production of both LMW polysulfides and polysulfidated proteins that are abundantly detected in cells and in mice. Notably, CARS2, a mitochondrial isoform of CARS, is involved in mitochondrial biogenesis and bioenergetics via CysSSH production.

**Results**

**Redox property of cysteine and protein polysulfides.** CysSSH has unique redox-active properties that distinguishes it from the cysteine (CysSH) thiol. In evaluating the physiological rationale for biological CysSSH production, our present study confirmed that cysteine persulfide/polysulfides (CysSSH/CysS\(-\text{SSCys})\) possess mixed sulfur reactivity—both nucleophilic and electrophilic (Supplementary Figs. 1 and 2)—a property that is unique and distinct from that of other simple biologically relevant thiols. The dual electrophilic-nucleophilic character of hydrosulfurides is well documented (the anionic RSS\(^{-}\) species being nucleophilic and the protonated RSSH species possessing electrophilic properties akin to disulfides, RSSR)\(^{-}\). Moreover, dialkyldisulfides can also be nucleophilic and electrophilic—cleavage of S-S bonds is established. The unique properties and reactivity of polysulfides allowed us to develop several analytical techniques aimed at determining endogenous production of LMW and protein-bound polysulfides (Supplementary Fig. 3). We first developed a convenient method for selective detection of polysulfidated proteins: the biotin-polyethylene glycol (PEG)-conjugated maleimide (biotin-PEG-MAL) labeling gel shift assay (PMSA; Supplementary Fig. 3a). PMSA demonstrated extensive protein-bound cysteine polysulfidation (Supplementary Fig. 4), not only for recombinant proteins, prepared in an *Escherichia coli* cell expression system (Supplementary Table 1) but also for endogenous proteins expressed in mammalian cells.

We then used liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with \(\beta\)-(4-hydroxyphenyl)ethyl iodoacetamide (HEP-IAM) as a trapping agent to identify and precisely quantify various hydropolythiols, and also to verify the site specificity of polysulfidation as well as the number of sulfur atoms involved in proteins (Supplementary Fig. 5, and Supplementary Table 2). We chose HEP-IAM for the LC-ESI-MS/MS analyses, as described recently because of its mild electrophilicity that ensures specific labeling of hydropolythiols to form stable adducts without appreciable artifactual decay related to their dual nucleophilic and electrophilic character (Supplementary Fig. 2). In fact, we quantified CysS\(-\text{SSCys})\ as a substrate in alcohol dehydrogenase 5 (ADH5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by LC-MS/MS analysis, after premonophosphorylation of the HEP-IAM-labeled proteins, which revealed that more than 70% of cysteine residues were polysulfidated (Fig. 1a and Supplementary Fig. 6), a result consistent with the PMSA profile alluded to above (Supplementary Fig. 4). The treatment of ADH5 with N-ethylmaleimide (NEN) indeed completely abrogated the HEP-IAM labeling of CysSSH and CysSSH/SSSSH as evidenced by LC-ESI-MS/MS analysis shown in Supplementary Fig. 6b. This data indirectly supports the electrophilic decomposition of protein-bound cysteine polysulfides induced by a strong electrophile NEM. Additional LC-quadrupole (Q)-time-of-flight (TOF)-MS analyses identified sites of polysulfide formation and the sulfur chain length in each protein (Supplementary Fig. 7).

**Protein polysulfidation induced by cysteinyl-tRNA synthetase.** Because such extensive protein polysulfidation is unlikely to occur effectively by simple chemical means, we hypothesized that CysSSH and CysS\(-\text{SSCys})\ may be incorporated during protein translation. To evaluate this hypothesis, we analyzed the incorporation of CysSSH/CysS\(-\text{SSCys})\ into tRNA via cysteinyl-tRNA synthetase (CARS) from *E. coli* (EcCARS) by using synthetic Cys\(-\text{SSCys})\ and LMW-MS/MS analyses (Supplementary Fig. 8). We observed effective production of CysSSH-bound tRNA (Cys\(_{\text{tRNA}}\text{CysSSH})\), which indeed suggests translational incorporation of CysSSH/CysS\(-\text{SSCys})\ into proteins. Unexpectedly, we identified extremely high levels (>80% of total cysteine residues) of tRNA-bound cysteine persulfide, trisulfide, and even tetrasulfide, when using simple (native) cysteine with EcCARS (Fig. 1b and Supplementary Fig. 9). As an important result, these cysteine polysulfides bound to tRNA were effectively incorporated into nascent polypeptides, which is synthesized de novo in the ribosomes (Fig. 1c), as verified by a modification of the puromycin-associated nascent chain proteinics (PUNCH-P) method here termed PUNCH-PsP, PUNCH for Polysulfide Proteomics (Supplementary Fig. 10). This PUNCH-PsP analysis allowed us to obtain specific and selective identification of the intact forms of Cys\(-\text{SSCys})\ residues in the nascent peptides of GAPDH present only within the ribosomes of *E. coli*, as Supplementary Fig. 10a shows. We clearly identified high degrees of polysulfidation occurring at the \(247\text{Cys}\) residue of the mature GAPDH protein expressed and synthesized in *E. coli*. All native forms of CysSSH, CysSSH, and CysSSH residues were efficiently recovered from the native whole GAPDH protein and the extension of polysulfidation reached more than 60% of the \(247\text{Cys}\) residue of mature protein (Supplementary Fig. 10c). All these rigorous LC-Q-TOF analyses unambiguously revealed that extensive and
prevalent cysteine polysulfidation is introduced co-translationally and sustained in the mature protein physiologically present even in the post-translational processes of the cells.

Consistent with these findings, EcCARS itself appeared to have strong catalytic activity for generating CysS-(S)n-H (CysSSH and CysSSSH) from the natural substrate cysteine (Fig. 1d). The persulfide synthase activity of EcCARS depended partly on added pyridoxal phosphate (PLP) (Fig. 2a) but not on ATP and tRNA: the latter two being required for Cys-tRNA\textsuperscript{Cys}(S)n-H\textsuperscript{(S)} synthesis by EcCARS. Persulfide generation by EcCARS was enantioselective, because only l-cysteine but not d-cysteine demonstrated activity, which ruled out nonspecific post-translational polysulfidation. Furthermore, we performed a stable isotope (\textsuperscript{34}S) tracer experiment combined with LC-MS/MS-based HPE-IAM assay to clarify the catalytic mechanism of cysteine polysulfidation by EcCARS (Supplementary Fig. 11). Specifically, by means of LC-MS/MS analysis for the enzymatic reaction with stable isotope \textsuperscript{34}S-labeled cysteine as a substrate, we found that EcCARS catalyzed the cleavage of a sulfur atom from one cysteine and its transfer to another cysteine to form CysSSH, as Supplementary Fig. 11a illustrates.

Identification of CARSs as CPERSs. Kinetic analyses confirmed that, because of a very low Michaelis constant \(K_m\) and high catalytic rate constant \(k_{cat}\), EcCARS is very efficient in producing CysSSH, i.e., functioning as a CPERS, with a high affinity for cysteine (Supplementary Fig. 12 and Supplementary Table 3), in particular when compared with the kinetic parameters of other enzymes such as CSE (Supplementary Table 3).\textsuperscript{21} Although the \(k_{cat}/K_m\) value is almost equal to values of EcCARS, CSE, and CBS utilize only cysteine (but not cystine) as a substrate, which is quite distinct from CARSs that use cysteine (but not cystine) for CysSSH production.\textsuperscript{2} In addition, because the intracellular cysteine content range is physiologically at low micromolar or submicromolar concentrations, which are far lower than the \(K_m\) value of CSE (more than 200 \(\mu\)M), CSE cannot directly utilize cysteine for persulfide production. Also, the cystine/CSE reaction may not compete successfully with the reactions with other enzymes metabolizing cysteine and substance such as glutathione, which exists abundantly in cells and thus readily interacts with cysteine under physiological conditions. The intracellular cysteine concentration is reportedly 100–1000 \(\mu\)M in cells and major organs,\textsuperscript{3} which is much higher than the \(K_m\) of CARS. These
biochemical reports, therefore, strongly suggest that CARS can function as a major source of Cys–(S)n–H generation under physiological conditions.

Investigation of EcCARS PLP-binding sites with LC-Q-TOF-MS analysis and Mascot data searches indeed revealed that lysine (K) residues, including KIIK and KMSK motifs, bound to PLP (Supplementary Fig. 13). The sequence data showed that several Lys residues, especially at the KIIK and KMSK motifs, are conserved in EcCARS and other homologues from different organisms, including mammals (Fig. 2b and Supplementary Fig. 14). Also, conserved two cysteine residues bound to the active center Zn²⁺ (Fig. 2b and Supplementary Fig. 14). To clarify the function of PLP bound to EcCARS, we constructed a series of Lys mutants of this enzyme (Supplementary Table 4) and measured enzyme activities in terms of persulphide production, similar to that of the WT cells (Fig. 2e), albeit their protein synthesis and translational activity were strongly attenuated (Fig. 2f).

Our computational modeling of the three-dimensional structure of EcCARS supported PLP binding to the particular Lys residues at the KIIK and KMSK motifs. Also, this modeling revealed that PLP-bound motifs have a vicinal location within 10–20 Å distance but apparently distinct from both the ATP-binding HIGH motif and the Zn²⁺-binding active site of the EcCARS for Cys-tRNA<sub>(S)</sub>ynthesis. A commensurate change in the binding capacity and/or stability of

**Fig. 2** Cys–(S)<sub>n</sub>–H biosynthesis catalyzed by EcCARS and its various mutant EcCARSs. a Cys–(S)<sub>n</sub>–H (CysSSH and CysSSSSH) biosynthesis from cysteine catalyzed by EcCARS as a function of reaction time and the presence or absence of PLP. Cys–(S)<sub>n</sub>–H production was analyzed by using the HPE-IAM labeling with LC-MS/MS analysis for the reaction of recombinant EcCARS (200 μg/ml) with 100 μM cysteine in the presence or absence of 50 μM PLP. The data are means ± s.d. (n = 3). *P < 0.05. b General structure (upper panel) and conserved amino acid alignments (lower panel) of bacterial, human, and rodent CARSs. c, e Enzyme activities of EcCARS lysine (K) mutants and cysteine (C) mutants to form CysSSH. WT and EcCARS K and C mutants, 200 μg/ml each, reacted with 25 μM cysteine at 37 °C for 30 min. Data represent means ± s.d. (n = 3). ***P < 0.001. The enzyme activity of EcCARS Lys mutants (d) and Cys mutants (f) was assessed by the PUREfrex assay with the cell-free translational reactions for ALDH1A1 (55 kDa), ADH5 (40 kDa), GAPDH (36 kDa), and ETHE1 (28 kDa), with protein syntheses being identified by western blotting.
PLP seems to exist, caused by the mutation of any one of four Lys residue among four Lys residues because each single Lys mutation at the KIJK and KMSK motifs greatly affected all CysS–(S)_n–H synthesis activity of EcCARS (Fig. 2c). One possible explanation for the commensurate effect is that PLP may need multiple Lys residues, rather than a single Lys binding, to exhibit stable binding and full catalytic activity of CARS to function as CPERS during CysS–(S)_n–H formation. That is, for their stable binding and catalytic activity, PLP-dependent catalytic activity may need stabilization by a multiple Lys binding, because CysSSH produced by CARS, due to its highly nucleophilic nature, may readily interfere with the electrophilic aldehyde group of PLP to form an imine (Schiff base) linkage on the Lys residues, which would cause instability of the catalytic activity of PLP bound to these particular Lys residues of CARS. This interpretation receives support from by the aforementioned computational structural analysis showing the close localization (in 20 Å) of these Lys residues at KIJK and KMSK motifs (Fig. 3a). Together these data suggest that EcCARS is indeed an efficient CPERS enzyme with independent catalytic functions in aminocetyl-tRNA biosynthesis.

CARS2 functions as a CPERS conserved in mammals. Two different CARSS exist in mammals: CARS1 (cytosolic) and CARS2 (mitochondrial)30–32. Both CARSS (mouse CARS1 and human CARS2, which we tested herein) had strong CysS–(S)_n–H producing activities, which depended on the presence of PLP (Fig. 3b–d). Also, a very nice correlation was found between the CPERS activity and PLP content of CARS2 containing varied amounts of PLP incorporated after treatment with different concentrations of PLP (Fig. 3e). To clarify how much cellular CysS–(S)_n–H originated from CARS1 and CARS2 in human cells, we attempted to disrupt CARS1 and CARS2 genes in HEK293T cells via the CRISPR/Cas9 system in HEK293T cells. We could not obtain CARS1-knockout (KO) cells, but we successfully established CARS2 KO cells. We selected one of the clones, carrying a 30-bp deletion plus an 8-bp insertion just downstream of the translation-initiating codon in the CARS2 first exon, was selected for LC-MS/MS analysis (Supplementary Fig. 15). CysS–(S)_n–H and GSSH levels decreased significantly in CARS2 KO cells (Fig. 4a, b), which suggests that CARS2 is a major producer of persulfide. Because we still detected a low level of CARS2 in CARS2 KO cells (Fig. 4c), we also treated the cells with siRNA against CARS2, which resulted in the 67 and 42% decreases in CysSSH and GSSH levels, respectively (Fig. 4a, b). When we knocked down CARS1 in CARS2 KO cells, CysSSH decreased only marginally, which suggests a predominant role of CARS2 in the production of CysSSH. Immunoblot analysis and immunostaining verified the reduced CARS2 and CARS1 protein levels in CARS2 KO cells and in cells with CARS1 or CARS2 siRNA (Fig. 4c and Supplementary Figs 16 and 17).

Markedly reduced persulfide formation in CARS2 KO cells was recovered by adding back WT CARS2. CARS2 C78/257D mutant rescued the persulfide production of CARS2 KO cells, but K124/127A and K317/320A mutants (mutants of KIIK and KMSK motifs, respectively), did not (Fig. 4d, e). The CARS2 KO cells had a markedly decreased Cys-tRNA synthetase activity, and again adding back the C78/257D mutant resulted in lost Cys-tRNA synthetase activity, as assessed by the expression of mitochondrial cytochrome c oxidase subunit 1 (MTCO1 encoded by mitochondrial DNA), but still retained full CPERS activity; conversely, K124/127A and K317/320A mutants had impaired CPERS functions but retained Cys-tRNA synthetase activity (Fig. 4f, g). These results clearly verify that CARS2 truly functions as a CPERS in mammals and that this function is separate from cysteinyl-tRNA synthetase activity.

We also evaluated the potential contribution of CSE and CBS to the endogenous persulfide production in HEK293T cells. Silencing of CSE and CBS suppressed the persulfide production, but notably, intracellular cysteine (CARS substrate) levels were significantly decreased (Supplementary Fig. 18). In CARS2 KO cells, knockdown of CSE and CBS also reduced cysteine levels but not persulfide production (Supplementary Fig. 18). Therefore, cysteine production is dependent on both CSE and CBS, and thus cysteine is provided via the metabolic pathways mediated by CSE/ CBS in each cell line irrespective of CARS2 expression.
addition, almost two thirds of CysSSH seems to be supplied by CARS2 in HEK293T cells based on the decrease by almost two thirds in the CYSSSH levels. The rest of CYSSSH in the CARS2 KO cells were not derived from CSE/CBS expressed in HEK293T cells, since no further reduction of CYSSSH was obtained even by CSE/CBS knockdown in CARS2 KO cells. These results suggest that CSE and CBS do not contribute directly to persulfide production but rather may promote the biosynthesis of cysteine and its supply to CARS, at least in this cultured cell model under physiological conditions.

To further clarify CPERS functions of CARS2 in vivo, we generated the CARS2-deficient mice by using CRISPR/Cas9 technology. As Fig. 5 illustrates, a guide RNA (gRNA) was designed against exon 1 of CARS2. We established a mutant mouse line with a mutant CARS2 allele (line 1) that had a 200-bp deletion containing a translation-initiating codon in exon 1 (Fig. 5a, b). Mating of F1 CARS2 heterozygous KO (CARS2+/−) mice produced WT and CARS2−/− mice, but no homozygous mice (viable offsprings included 20 WT mice and 19 CARS2+/- mice), which suggests that CARS2−/− mice are embryonic lethal. CARS2+/- mice were normally born without any apparent abnormalities in macroscopic appearance or growth profiles during the observation period of at least 6 months after birth, but they demonstrated reduced mitochondrial expression of CARS2 protein by half and marked attenuation of CYSSSH production; in contrast, we observed no appreciable change in mitochondrial DNA-encoded MTCO1, which indicated intact Cys-tRNA synthetase activity in the liver and other tissues of CARS2+/- mice (Fig. 5c–e and Supplementary Fig. 19a). Therefore, we quantified the sulfide metabolites in the liver of CARS2+/- mice and their WT littermates via LC-MS/MS analysis with HPE-1AM as described earlier. As we expected, CARS2+/- mice showed a striking difference in persulfide production compared with the WT littermates (Fig. 6a, b). Endogenous levels of CYSSSH and all other derivatives (e.g., GSSH, HS−, thiosulfate, and hydroxysulfides) decreased by 50% or more in the liver and lung of CARS2+/- mouse compared with WT mice.

To exclude the possibility of off-target effects by the gRNA used to produce line 1 CARS2+/- mice, we developed another strain of CARS2+/- mice (line 2) with an alternative gRNA targeting CARS2 exon 3. Line 2 CARS2+/- mice had phenotypes almost identical to those of line 1 (Supplementary Figs. 20 and 21). That heterozygous CARS2 mutant mice manifested a CYSSSH reduction by ~50% should be noted; it suggests that CARS2 contributes almost entirely to the CYSSSH production in mouse tissues under physiological conditions. As an important finding, CARS2 disruption did not alter expression levels of other sulfide-metabolizing enzymes, including CSE, CBS, and 3-mercaptopropionate sulfur transferase (3-MST) (Fig. 5e, Supplementary Figs. 19b, c and 21), which emphasized the sole
contribution of CARS2 to endogenous persulfide biosynthesis in vivo.

To explore the possibility that CARS2, a mitochondrial protein, can produce CysSSH and provide it to the whole cell, we isolated mitochondria from mouse liver and measured the release of de novo-synthesized CysSSH from the mitochondria (Supplementary Fig. 22). A large fraction of CysSSH was indeed released from mitochondria isolated from the liver. The lower panel shows the densitometric analysis of the western blot. Data are means ± s.d. (n = 3). ***P < 0.001. CysSSH production in mitochondria isolated from the liver of WT and Cars2−/−/ mice. Various concentrations of isolated mitochondria were reacted with HPE-IAM for 1 h, followed by LC-MS/MS analysis (see Supplementary Methods for details). Mitochondria were obtained from line 2 Cars2−/− mice (Supplementary Figs. 20 and 21). *P < 0.05, WT vs. Cars2−/−/ mice (two-way ANOVA).

CARS-mediated polysulfidation and mitochondrial morphology. Unexpectedly, Cars2 KO cells showed markedly altered mitochondrial morphology (i.e., shrunken or fragmented appearance), which greatly improved when Cars2 was added back, as seen with the MitoTracker Red fluorescent mitochondrial stain (Fig. 8a and Supplementary Fig. 17c), transmission electron microscopy (Fig. 8b), and immunofluorescence staining for translocase of outer mitochondrial membrane 20 (TOMM20) and CARS2 (Supplementary Fig. 17a, b). Not only WT Cars2 but also the C78/257D mutant induced a strikingly improved mitochondrial morphology, but other Lys mutants tested did not (Fig. 8a, b and Supplementary Fig. 17c). In line with these findings, deletion of Cars2 activated dynamin-related protein (Drp1), a major mediator of mitochondrial fission33, and Drp1 GTPase activity was significantly attenuated by adding back the WT Cars2 and C78/257D mutant, thereby producing CysSSH without Cars2 activity, but not by adding back the K317/320A mutant (Fig. 8c). Usually, Drp1 in HEK293T cells was extensively polysulfidated (Fig. 8d), as evidenced by our new biotin-PEG-MAL capture method (Supplementary Fig. 3b). However, Drp1 polysulfidation was markedly suppressed by both Cars2 KO and additional Cars2/1 double-knockdown, respectively (Fig. 8d and Supplementary Fig. 23). Because Drp1 is likely activated via chemical depoly sulfidation or a post-transitional process operated physiologically by the Trx−/−TrxR system, for example, we identified Drp1 as a major signal effector molecule reversibly regulated through a unique polysulfidation and depolsulfidation process (Fig. 8e).

We next examined Cars2 contribution to mitochondrial biogenesis and function. Mitochondrial DNA normalized against

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**Fig. 5** Generation of Cars2-deficient mice via the CRISPR/CAS9 system. a Schematic illustration of the mouse Cars2 gene structure and sequences of WT and mutant alleles around the target locus. Green and black letters indicate the first exon and intron of Cars2, respectively. The targeted locus of gRNA and protospacer-adjacent motif (PAM) sequence were indicated in the WT sequence are indicated by underlined and bold letters, respectively. A modified allele sequence obtained from the Cars2-edited mouse (line 1) is shown below. b Detection of mutations introduced by gRNA-Cas9 targeting Cars2 via PCR with genomic DNA from WT and Cars2+/− mice. Cars2+/−, Cars2 heterozygous KO mice, M: DNA molecular weight marker. c Western blotting of CARS2 and mitochondrial proteins, e.g., MTCO1 and SDHA, from mitochondria isolated from the liver. The lower panel shows the densitometric analysis of the western blot. Data are means ± s.d. (n = 3). ***P < 0.001. d CysSSH production in mitochondria isolated from the liver of WT and Cars2+/− littermate mice. Various concentrations of isolated mitochondria were reacted with HPE-IAM for 1 h, followed by LC-MS/MS analysis (see Supplementary Methods for details). Mitochondria were obtained from line 2 Cars2+/− mice (Supplementary Figs. 20 and 21). *P < 0.05, WT vs. Cars2−/−/ mice (two-way ANOVA). e Western blotting of CARS1, CSE, CBS, and 3-MST with liver tissue obtained from WT and Cars2−/−/ mice. Supplementary Fig. 19 provides full blot images. The right panels show the densitometric analysis of the CARS1 and CARS2 immunoblots. Data are means ± s.d. (n = 3). ***P < 0.001.
nuclear DNA was reduced in CAR2 KO cells, which was similarly restored by WT CAR2 and C78/257D but not by Lys mutants (Supplementary Fig. 24a), which suggests that CAR2-derived persulphide enhances mitochondrial biogenesis. Mitochondrial membrane potential was decreased in CAR2 KO cells, but it recovered or even increased when the WT and C78/257D mutant were added back or overexpressed but not when Lys mutants were used (Fig. 8f and Supplementary Fig. 25a). We also used an extracellular flux analyzer to measure the oxygen consumption rate (OCR) in HEK293T CAR2 KO cells. The OCR in CAR2 KO cells was ~50% of that in WT cells (Fig. 8g), consistent with the incomplete elimination of CAR2 protein and thereby attenuated expression of MTCO1 in CAR2 KO cells (Fig. 4g). The decrease of OCR in CAR2 KO cells was recovered by introduction of WT CAR2 and C78/257D mutant but not by Lys mutants (Fig. 8g and Supplementary Fig. 25b). A novel concept emerging from these observations is that CAR2-derived cysteine persulphides play an important role in the electron transport chain (ETC) in mitochondria, which sheds light on a completely new and fundamental role of persulphides in supporting mitochondrial bioenergetic function.

**CAR2 linked up to mitochondrial ETC.** In our efforts to elucidate the mechanism of how CAR2-derived CysSSH contributes to the mitochondrial bioenergetics function, we noticed a quite different profile of the products of human CAR2 in the cell-free enzyme reaction compared with cellular CAR2 metabolism in HEK293T cells in culture (Fig. 9a, b). Although CAR2 synthesized mostly CysSSH/SSSH in a cell-free solution (Fig. 3c, d), preferential formation of HS⁻ (H₂S) together with thiosulfate (S₂O₃²⁻) over CysSSH was evident with HEK293T cells. We thus hypothesized that the mitochondrial compartment is a unique metabolic environment in which de novo CysSSH synthesized by CAR2 may be further metabolized, possibly being coupled with the mitochondrial ETC.

To understand how the ETC function and CysSSH derived from CAR2 are associated (Fig. 8g and Supplementary Fig. 25b), we examined the effect of ETC suppression on the metabolic profile of CysSSH and its derivatives in HEK293T cells (Fig. 9c–h). We then used two approaches to inhibit the ETC in the cells: one method was to use a specific inhibitor of complex III, antimycin A (Fig. 9c–e), and the other ETC disrupter used was ethidium bromide to induce mitochondrial DNA deprivation (Fig. 9f–h and Supplementary Fig. 24b; see Supplementary Methods for details). Both ETC suppressive treatments caused a significant increase in CysSSH and simultaneous reduction of HS⁻ production, as assessed by the HPE-IAM labeling LC-MS/MS analysis (Fig. 9c–h). These inverse and stoichiometric relationships between CysSSH and hydrosulphide anion (HS⁻) formation strongly suggested an ETC activity-dependent conversion of CysSSH to HS⁻ mediated via the ETC occurring in the cells (Fig. 9e, h). We interpret these results to mean that CysSSH derived from CAR2 in mitochondria is effectively reduced by accepting an electron from the ETC to release HS⁻ (H₂S), as Fig. 9i illustrates.

These data thus provide robust support for the idea that the CAR2-CysSSH pathway is involved in the mitochondrial function because CAR2-dependent CysSSH production is functionally integrated into and tightly linked to the mitochondrial ETC, which is in turn involved in the energy metabolism, as Fig. 10 illustrates. In fact, low (nM) concentrations of H₂S reportedly sustained the ETC function possibly mediated by sulfide:quinone reductase and other potential enzymes that oxidize sulfides to thiosulfate (S₂O₃²⁻)⁴⁴⁻³⁸. How H₂S is supplied endogenously in mitochondria remained unclear, however. Our earlier and current studies suggest that CSE, CBS, and 3-MST are not major sources of H₂S in mitochondria in
various mammalian cell lines and in mice in vivo (Fig. 5e and Supplementary Fig. 18)7,20–24. In this context, our study is the first to verify that HS\(^-\) (or H\(_2\)S) is indirectly formed from CARS2 via CysSSH generation in the mitochondrial environment (Figs. 9i and 10). Moreover, our recent study determined that CysSSH contributed to the endogenous formation of iron-sulfur clusters14. Because iron-sulfur clusters are known to be synthesized and utilized in complexes I-III of the ETC in mitochondria39, and are actively transported extramitochondrially, the CysSSH-dependent HS\(^-\) metabolism may be coupled with the generation of iron-sulfur centers of the mitochondrial ETC and cytosolic formation and maintenance of various iron-sulfur complex machineries as well. Our reasonable conclusion is, therefore, that CARS2 functions as a major CPERS, which in turn promotes mitochondrial biogenesis and bioenergetics (Fig. 10).

**Discussion**

Until now, endogenous persulfides were thought to be formed as a result of H\(_2\)S/HS\(^-\) oxidation via post-translational processes, and serve as protein cysteine thiol-bound intermediates of detoxification enzymes3,7,21, and as metal ligands for iron and zinc complexes11–15. While CSE and CBS can catalyze CysSSH biosynthesis by using cystine as a substrate3,4,6–10,18–21, several cells and tissues without CSE/CBS expression and CBS/CSE KO mice reportedly synthesized appreciable amount of persulfides3,20,22–24, but the source of the persulfides (polysulfides) or the sulfane sulfur reservoir has remained elusive. We here demonstrate that CARs catalyze CysS-(S)\(_n\)-H formation from cysteine and co-translational protein polysulfidation. Also, CSE and CBS may still play a major role in the CysSSH production via the direct catalytic reaction using cystine as the substrate especially under pathophysiological conditions associated with oxidative and electrophilic stress, where intracellular cysteine concentrations are considerably approaching the high \(K_m\) value of CSE3,7,21,40–42.

The second, even more crucial, finding is that the mitochondrion is a key cellular compartment for the formation and action of CysSSH and CysS-(S)\(_n\)-H. Notably, CysSSH is mostly generated by CARS2 localized in the mitochondria and is released extramitochondrially into the cytoplasm so that it can effectively produce CysS-(S)\(_n\)-H and protein polysulfidation in whole-cell compartments. The current study established that CARS2-derived CysSSH (CysS-(S)\(_n\)-H) indeed sustains mitochondrial biogenesis and the ETC function. While the implications of these findings await further investigation, a recent clinical study by Coughlin et al. documented an intriguing result: CARS2 mutations identified in a patient were associated with ETC impairment and mitochondrial dysfunctions31. Although the patient’s clinical symptoms resulted from loss of a canonical function of CARS2, which the neurological disorders might be caused by impairment of CPERS activity of CARS2 is plausible, and thus this impaired activity may overlap with the observed impairment of Cys-tRNA aminoacylation.

The nature of sulfane sulfur or polysulfides has continued to be a puzzle for a long time, because of a complicated polysulfide chemistry with dual electrophilic and nucleophilic characteristics. Previous reports demonstrated the ability of a trisulfide species to react with numerous electrophiles. For example, Fletcher and Robson reported that thiocysteine (cystine trisulfide, CysSSSCys) readily reacted with electrophilic halogens (e.g., Br\(_2\)), which resulted in cleavage of the S–S bond26. A review by Parker and Kharasch also discussed numerous examples of the electrophilic cleavage of the S–S bond in disulfides by electrophilic reagents such as protons, sulphenium ions, and halogens26. More recently (and directly relevant to our studies), Abdolrasulnia and Wood reported that CysSSSCys reacted readily with iodoacetic acid (a well-established thiol-modifying agent) to ultimately give carboxymethylthiocysteine (CysSSS–CH\(_2\)COOH)27, which is consistent with the idea that a nucleophilic sulfur atom of the polysulfide reacted with the electrophilic iodoacetic acid species and led to S–S bond cleavage. Previous examination of the reaction of electrophiles with disulfides (the simplest of all polysulfides) is entirely consistent with this idea28. Thus, ample precedence for the nucleophilic character of polysulfides exists, by capitalizing on such a unique property, we are now able to identify the cysteine and protein polysulfidation occurring endogenously by means of a conventional PMSA or capturing.
assays and even by using HPE-IAM labeling LC-MS/MS analysis. The present discovery of a novel polysulfide biosynthesis, therefore, can now explain substantial endogenous generation of sulfane sulfur, which we clarified as composed of various polysulfide derivatives and which is biosynthesized by CPERSs and CARSs. Our findings raise a number of important questions; however, for example, why are such protein-bound cysteines abundantly bound? And, what function does this modification play in compartments other than mitochondria? Determining how CPERS activity is regulated will also be important. Given the powerful effects of persulphides on mitochondrial morphology and bioenergetics, the availability of persulphides in cells must be subject to stringent regulation. Although CPERSs play a critical role in generating CysSSH, the Trx-TrxR system may help maintain cellular persulphide concentrations within certain limits by controlling the rate of persulphide degradation.

Some aminocetyl-tRNA synthetases reportedly possess functions in physiological processes besides their role in translation. The mitochondria-promoting functions of CARS2 suggest its non-canonical roles and therefore may therefore represent “moonlighting” roles of CARS2. However, CARSs effectively synthesize cysteine polysulphides, and this process is closely related to the initial translational process of de novo synthesis of nascent polypeptides in ribosomes (cf. Fig. 1b and Supplementary Fig. 10). The CPERS function of CARSs is apparently associated not only with translation but also with the mitochondrial respiration, which indicates that CARSs, rather than having a moonlighting role, have a primary function of producing persulphides.

In conclusion, our discovery of reactive persulphide production mediated by the CARS or CPERS pathway and the potent effects on mitochondrial functions observed would seem to represent a significant evolution of molecular and cell biology, thereby inviting a paradigm shift in the current understanding of cellular translation, redox signaling, and energy metabolism (Fig. 10). Our discovery of CARS and CPERS as a major source of reactive persulphides in biology may usher in a new era of modern redox biology and life science research that will help to recognize the great potential to invigorate translational studies in a variety of disease processes known to be associated with aberrant redox regulation and mitochondrial dysfunction.
**Methods**

**LC-ESI-MS/MS analyses for per/poly/sulfides.** LC-ESI-MS/MS analysis with HPE-IAM (Supplementary Fig. 5 and Supplementary Table 2) was used to determine CysSSH or Cys–(S–S)–H formed from EcCARS and CARSs. To identify Cys–(S–S)–H formed and incorporated into Cys–RNA via the enzymatic reaction of EcCARS, 200 μg/ml recombinant EcCARS was reacted with 0.5 mg/ml tRNA (Sigma-Aldrich) and Cys–(S–S)–H or 10 μM cysteine as the substrate, in 50 mM HEPES buffer (pH 7.5) containing 1 mM ATP, 25 mM KCl and 15 mM MgCl2 at 37 °C, followed by alkalization with 1 mM HPE-IAM for 20 min at 37 °C. Cys–(S–S)–H were formed from 10 μM cysteine and 30 μM Na2S5 in 30 mM HEPES buffer pH 7.5 at 37 °C for 5 min. The Cys–tRNA5S–(S–S)–H synthesized by EcCARS was precipitated by adding 10% trichloroacetic acid to the reaction mixture, followed by trapping by cotton wool filters (100 μl) placed in tipette tips. The precipitated total tRNA containing Cys–tRNA5S–(S–S)–H was washed with 10% trichloroacetic acid (200 μl twice) and with 70% ethanol (200 μl twice) to completely remove the free cysteine and Cys–(S–S)–H. Cys–HPE-IAM and Cys–(S–S)–H–HPE-IAM adducts were dissociated by alkaline heat hydrolysis of the ester bond of aminoacyl moieties of the Cys–tRNA5S and Cys–tRNA5S–(S–S)–H. The hydrolysis was performed in 20 mM Tris-HCl (pH 8.0), which contained known amounts of stable isotope-labeled internal standards, at 70 °C for 15 min. The eluted solutions were acidified with formic acid and analyzed via LC-ESI-MS/MS. Also, Cys–tRNA-bound CysSSH was identified by detecting a CysSSH–adenosine adduct formed in the Cys–tRNA molecules synthesized by EcCARS from the substrate cysteine. The CysSSH–adenosine adducts in the reaction of EcCARS with cysteine and Cys–tRNA were measured by using LC-ESI-MS/MS analysis. In brief, CysSSH incorporated into tRNA as catalyzed via EcCARS with cysteine was prepared in the same manner as that described above, followed by alkylation with HPE-IAM and acetylation with acetic anhydride, as described earlier44. After precipitation and washing of samples with ethanol, the acetylated and HPE-IAM-labeled Cys–tRNA5S–HPE-IAM was digested to generate acetylated CysSSH–HPE-IAM–bound adenosine with treatment with RNase ONE (Promega, Madison, WI) at 37 °C for 1 h, after which LC-ESI-MS/MS analysis was performed. To measure Cys–(S–S)–H generated directly by EcCARS and CARSs, recombinant EcCARS, mouse CARS1, or human CARS2 was incubated with cysteine in 50 mM HEPES buffer (pH 7.5) containing 25 mM KCl and 15 mM MgCl2 with or without 1 mM ATP at 37 °C. The mixtures were then reacted with 1 mM HPE-IAM in methanol at 37 °C for 20 min to form Cys–(S–S)–H–HPE-IAM adducts. After centrifugation, aliquots of the supernatants were diluted 10–100 times with 0.1% formic acid containing known amounts of isotope-labeled internal standards and were subjected to LC-ESI-MS/MS. To clarify the molecular mechanism of CysSSH formation, 50 μM 34S-labeled l-cysteine was reacted with 200 μg/ml EcCARS as a substrate in 30 mM HEPES buffer (pH 7.5) containing 25 mM KCl and 15 mM MgCl2 with or without 1 mM ATP at 37 °C. The mixtures were then reacted with 1 mM HPE-IAM in methanol at 37 °C for 20 min to form Cys–(S–S)–H–HPE-IAM adducts. After centrifugation, aliquots of the supernatants were diluted 10–100 times with 0.1% formic acid containing known amounts of isotope-labeled internal standards and were subjected to LC-ESI-MS/MS. To determine kinetic parameters, WT EcCARS and C28S EcCARS were incubated with different concentrations of L-cysteine in 50 mM HEPES buffer (pH 7.5) containing 25 mM KCl and 15 mM MgCl2 at 37 °C for 15–60 min. The reaction products treated with HPE-IAM were diluted with 0.1% formic acid containing known amounts of isotope-labeled internal standards, which were then subjected to LC-ESI-MS/MS as described above. To determine kinetic parameters, WT EcCARS and C28S EcCARS were incubated with different concentrations of L-cysteine in 50 mM HEPES buffer (pH 7.5) containing 25 mM KCl and 15 mM MgCl2 at 37 °C for 30 s. The reaction mixtures were treated with 1 mM HPE-IAM, followed by LC-ESI-MS/MS as described above. The data were fitted by nonlinear regression to the Michaelis–Menten equation by using GraphPad Prism software ver. 6.0 (GraphPad Software, San Diego, CA).
Cysteine persulfide synthases (CPERSs) mediate the co-translational synthesis of CysS-(S)ₙ-H, which is a key step in the biosynthesis of thiolated proteins. The physiological relevance of co-translational protein polysulfidation is highlighted in the figure, showing the regulation of mitochondrial functions by CARS2/CPERS and the antioxidant effects of CysSSH. The figure also illustrates the depolysulfidation process, which is crucial for maintaining redox homeostasis.

Identification of CysS-(S)ₙ-SH formed in nascent peptides. CysS-(S)ₙ-SH species synthesized endogenously and formed in nascent polypeptides by EcCARS in E. coli cells were analyzed by means of puromycin-associated nascent chain proteomics (PUNCH-P) [34], which was specifically modified here for polysulfated proteins (PUNCH for Polysulfate Proteomics, henceforth called PUNCH-Pp). The E. coli JM109 cells transfected with an hGAPDH expression vector (pGE-30) were cultured and hGAPDH expression was induced with IPTG as described earlier. Active, followed by collecting and sonication of the cells in cell lysis buffer containing 0.3 mg/ml lysozyme and 2 mM iAM without any reducing agents. The supernatant obtained by centrifugation was applied to the Ni-NTA column for purification. The resultant pellet of the E. coli cell lysate, the ribosomal fraction was isolated via sucrose density gradient ultracentrifugation, as reported previously [29]. The CysS-(S)ₙ-H-dependent HS⁻ metabolism might be coupled with formation of the iron-sulfur clusters, as being controlled by the mitochondrial ETC. I, II, III, and IV: complexes I, II, III, and IV; TCA tricarboxylic acid (Krebs) cycle.

Supplementary Table 2 summarizes the MRM parameters for each derivative. The figure provides a comprehensive overview of the metabolic pathways involved in mitochondrial biogenesis and bioenergetics.
unique physicochemical properties of the interior structure of the polyethylene exit tunnel in the ribosome, which allowed us to obtain specific and selective identify. We generated two sets of Cas2-deficient mRNAs in Xbal, gRNAs were introduced into fertilized eggs by injecting a Leica Micromanipulator system, according to the protocol previously as previously described, we transferred the products of pseudo-pregnant females on the day of the vaginal plug. A founder mouse harboring the Cas2 mutant alleles was crossed with WT mice to obtain Cas2 heterozygous mice. After segregating the Cas2 mutant alleles, heterozygous mice with a 200-bp deletion in exon 1 line) and with a 1-bp insertion in exon 3 were used for analysis (Fig. 6; Supplementary Figs. 20 and 21).

**Mitotracker Red staining for mitochondrial morphology.** To analyze mitochondrial morphogenesis under several experimental conditions in cells, mitochondria were imaged by using the fluorescent probe Mitotracker Red CM-H$_2$Xros (Invitrogen). In brief, culture slides were coated with 0.5% polyethylene imine for more than 1 h and washed twice with PBS. CAS2 KO cells were transfected with expression plasmids for WT and individual mutants of human CARS2 via Lipofectamine 2000. At 3 days after transfection, cultured cells were washed with Hank’s buffer, incubated with 1 μM Mitotracker Red CM-H$_2$Xros at 37 °C for 30 min, rinsed twice with Hank’s buffer, and examined with a Nikon EZ-C1 confocal laser microscope (Tokyo, Japan). We used ImageJ and Prism software for image processing and quantification of mitochondrial dimensions including their length.

**Mitochondrial bioenergetic functions.** Mitochondrial function was investigated, according to a previous report with a slight modification, by measuring the basal OCR of the mitochondria under various experimental conditions in WT and CAS2 KO cells, using the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent). At the end of the experiment, rotenone and antimycin A (2.4 μM each) were added to inhibit complexes I and III of the mitochondrial electron transport chain, respectively, to determine the remaining mitochondria-independent OCR. NADH oxidase was normalized to the cell number determined at the end of the experiments by means of sulforhodamine B staining (Sigma-Aldrich, St. Louis, MO). To obtain the mitochondria-specific OCR, the rotenone/antimycin-sensitive part of cell respiration was used.

**Effect of suppression of ETC on metabolic profiles of CysSSH.** The mitochondrial ETC in HEK293T cells was inhibited either by a complex III inhibitor, antimycin A, or by elimination of mitochondrial DNA (mtDNA) induced by ethidium bromide. For the direct but partial ETC (complex III) inhibition, WT and CAS2 KO cells were treated with various concentrations of antimycin A for 1 h, followed by methanol extraction for measurement of CysSSH and its related sulfdide derivatives by HPE-IAM labeling LC-ESI-MS/MS analysis as described earlier. To investigate whether all ETC components (comprising complexes I, II, and III) or CAS2 KO HEK293T cells was eliminated specifically by treatment with ethidium bromide (30 ng/ml, 127 nM) for 12 days under standard cell culture conditions (37 °C, humidified, 5% CO$_2$/95% air) with DMEM containing 10% FBS, 1% penicillin-streptomycin, sodium pyruvate (1 mM), nonessential amino acids (1%), a prethermic black latexized medium, according to the manufacturer’s instructions. The sRNA was introduced into WT and CAS2 KO cells, as described above for CAS2 gene transfection.

**Generation of CAS2-deficient mice.** All experimental procedures conformed to “Regulations for Animal Experiments And Related Activities at Tohoku University,” and were reviewed by the Institutional Laboratory Animal Care and Use Committee of Tohoku University, and finally approved by the President of University. Only two lines of CAS2-deficient mice as follows. CAS2 gRNAs vectors were constructed with use of a pT7-sgRNA and pT7-hCas9 plasmid (a gift from Dr. M. Ikawa, Osaka University) 30. After digestion of pT7-hCas9 plasmid with EcoR1, hCas9 mRNA was synthesized by using an in vitro transcription kit (mMESSAGE mmACHINE T7 Ultra kit; Ambion, Austin, TX), according to the manufacturer’s instructions. A pair of oligonucleotides targeting CAS2 was annealed and inserted into the Bsal site of the pT7-sgRNA vector. The sequences of the gRNAs were designed as follows: 5′-GGACAGATCCGAGAAGCAG-3′ and 5′-AAATATACAAGAGCTAAGC-3′, located at exons 1 and 3 of CAS2 gene, to generate CAS2-deficient lines 1 and 2 mice, respectively. After digestion of pT7-CARSHSS101368 (Invitrogen), and CAS2, CAS2HSS128464 (Invitrogen). siRNA transfection was performed by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The siRNA was introduced into WT and CAS2 KO cells, as described above for CAS2 gene transfection.
MS analysis. Changes in the amounts of CysSSH (ΔCysSSH) and HS- (ΔHS-) induced by complex III inhibition by antimycin A or by mtDNA elimination in WT and CARS2 KO HER293T cells were then calculated.

Statistical analysis. Results are presented as means ± s.d. of at least three independent experiments unless otherwise specified. For statistical comparisons, we utilized two-tailed Student’s t test or two-way analysis of variance followed by the Student–Newman–Keuls test, with significance set at P < 0.05.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
T.A. and H.M., experiment design, biochemistry and cell biology, data analysis, animal studies, and writing the paper; T.I., M.M.A., H.I., T.S., M.J. and T.M., MS analysis, biochemistry and molecular biology, cell biology, and cell imaging; S.W., K.I., protein structural analysis; F.-Y.W., Akir.N., H.S., N.T. and K.T., cell biology and mitochondria study; M.M. and M.O., CRISPR/Cas9 technology, cell biology, and animal studies; M.N., Akir.N., S.F., K.Y. and Y.W., cell signaling, cell biology, and data analysis; P.N., M.F., J.M.F., chemical analysis and editing the paper.

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