Hydrogen sulfide perturbs mitochondrial bioenergetics and triggers metabolic reprogramming in colon cells

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Running Title: SQR is a respiratory shield

Abstract

Unlike most other tissues, the colon epithelium is exposed to high levels of H₂S derived from gut microbial metabolism. H₂S is a signaling molecule that modulates various physiological effects. It is also a respiratory toxin that inhibits complex IV in the electron transfer chain (ETC). Colon epithelial cells are adapted to high environmental H₂S exposure as they harbor an efficient mitochondrial H₂S oxidation pathway, which is dedicated to its disposal. Herein, we report that the sulfide oxidation pathway enzymes are apically localized in human colonic crypts at the host-microbiome interface, but that the normal apical-to-crypt gradient is lost in colorectal cancer epithelium. We found that sulfide quinone oxidoreductase (SQR), which catalyzes the committing step in the mitochondrial sulfide oxidation pathway and couples to complex III, is a critical respiratory shield against H₂S poisoning. H₂S at concentrations ≤20 µM stimulated the oxygen consumption rate in colon epithelial cells but, when SQR expression was ablated, H₂S concentrations as low as 5 µM, poisoned cells. Mitochondrial H₂S oxidation altered cellular bioenergetics, inducing a reductive shift in the NAD⁺/NADH redox couple. The consequent electron acceptor insufficiency caused uridine and aspartate deficiency and enhanced glutamine-dependent reductive carboxylation. The metabolomic signature of this H₂S-induced stress response mapped in part, to redox-sensitive nodes in central carbon metabolism. Colorectal cancer tissues and cell lines appeared to counter the growth restricting effects of H₂S by overexpressing sulfide oxidation pathway enzymes. Our findings reveal an alternative mechanism for H₂S signaling, arising from alterations in mitochondrial bioenergetics that drives metabolic reprogramming.

Microbes that liberate substantial quantities of H₂S via sulfate reduction or desulfuration of sulfur-containing amino acids (1) are among the ~100 trillion gut resident organisms (2). The adaptive mechanisms used by the colonic epithelium to counter exposure to potentially high luminal H₂S, estimated to range from ~0.2-2.4 mM (3,4) in different parts of the colon, are largely unknown. Mitochondrial sulfide quinone oxidoreductase (SQR)(1) catalyzes the first step in the canonical sulfide oxidation pathway and utilizes coenzyme Q (CoQ) as an electron acceptor (Fig. 1a,b) (5,6). High sulfide oxidation flux can potentially perturb mitochondrial bioenergetics by limiting the pool of oxidized CoQ, which accepts electrons from complexes I and II, and also supports other metabolic pathways. Furthermore,

¹Abbreviations used. CoQ: coenzyme Q; CRC: colorectal cancer; ETC: electron transfer chain, OCR: oxygen consumption rate; SQR: sulfide quinone oxidoreductase;
high H\textsubscript{2}S exposure can inhibit respiration by poisoning complex IV in the electron transfer chain (ETC) (7). There is limited understanding of the metabolic impacts of high mitochondrial H\textsubscript{2}S oxidation flux in colonocytes, resulting from their exposure to this respiratory toxin. Furthermore, antithetical findings on H\textsubscript{2}S (e.g. that it is proliferative versus antiproliferative or pro- versus anti-inflammatory), confound our understanding of its biological effects (8).

A critical function of mitochondrial respiration in proliferating cells is to recycle reduced cofactors (e.g. NADH, FADH\textsubscript{2}) and maintain a supply of electron acceptors. Rapidly proliferating cells faced with an insufficiency of electron acceptors due to mitochondrial dysfunction, become auxotrophic for uridine and pyruvate (9). While uridine deficiency is explained by the CoQ dependence of dihydroorotate dehydrogenase in the de novo pyrimidine synthesis pathway, electron acceptor deficiency, which creates a growth-restricting aspartate shortage, can be alleviated by exogenous pyruvate (10,11). Aspartate is needed for nucleotide and protein synthesis (Fig. 1a). Since aspartate is more oxidized than the nutrients from which it is derived (i.e. glucose and glutamine), its synthesis requires a steady supply of NAD\textsuperscript{+}.

Epithelial cells generally retain the capacity to proliferate through adulthood, and epithelial cancers are common (12). While most cancer cells exhibit the Warburg effect i.e., increased glycolysis in the presence of oxygen (13), mitochondrial respiration is needed to support rapid growth; respiration inhibitors block cell proliferation (14,15). It is not known how colorectal cancer (CRC) cells surmount the potentially growth-inhibiting effect of luminal H\textsubscript{2}S.

We hypothesized that exposure of colonocytes to high levels of H\textsubscript{2}S simultaneously stimulates sulfide oxidation flux and inhibits the ETC, leading to a functional insufficiency of electron acceptors. We demonstrate herein that the growth restriction imposed by H\textsubscript{2}S can be alleviated by exogenous uridine and aspartate and is partially circumvented by elevated expression of the sulfide oxidation pathway enzymes in CRC. We report that several H\textsubscript{2}S-induced metabolite changes in central carbon metabolism map to redox reactions, which are sensitive to perturbations in the NAD/NADH ratio. Our study provides novel insights into an alternative mechanism of H\textsubscript{2}S signaling, i.e. via redox-linked metabolic reprogramming that emanates from the changes in the mitochondrial ETC.

Results

Localization of the sulfide oxidation pathway enzymes in normal and CRC tissue-The localization and expression levels of the sulfide oxidation pathway enzymes in normal and malignant human colonic tissue was compared. In normal colon epithelium, SQR, TST (rhodanese) and ET\textsc{h}E1 exhibited strong apical localization in colonic crypts, i.e. at the host-microbiota interface (Fig. 2a). A similar localization pattern has been reported for the human sulfurtransferase, TSTD1 (16). In contrast, CRC tissue showed diffuse localization of all three enzymes, while the intensity of staining suggested higher expression levels (Fig. 2b).

Next, the expression levels of the sulfide oxidation pathway enzymes were examined in seven resected CRC tissue specimens and patient-matched normal tissues. Western blot analysis revealed significant differences in enzyme levels in 5 of 7 CRC samples, while the remaining two CRC samples showed expression levels that were similar to normal tissue (Fig. 3). Elevated expression of SQR and ET\textsc{h}E1 was observed in all six CRC cell lines that were studied (HT29, LoVo, Caco-2, RKO, DLD-1 and HCT116) compared to the nonmalignant colon cell line, HCEC (Fig. 4a,b). The increase in SQR and ET\textsc{h}E1 protein levels was not due to increased mitochondrial density, as the cardiolipin content in CRC and HCEC cells was comparable (Fig. 4c).

\textit{SQR} is a respiratory shield against H\textsubscript{2}S-Sulfide concentrations below \textasciitilde20 \textmu M stimulated the O\textsubscript{2} consumption rate (OCR) in human colon cell lines, but inhibited OCR at concentrations \textgtr30 \textmu M (Fig. 5a, S1), presumably as complex IV inhibition became more dominant. The recovery time for return to the basal OCR increased as the H\textsubscript{2}S concentration increased.

While SQR overexpression in Chinese Hanster ovary (CHO) cells was reported to increase H\textsubscript{2}S-dependent OCR (17), the effect of attenuating SQR expression on the sensitivity of the ETC to
H_{2}S has not been assessed. Compared to HT29 CRC cells transfected with a scrambled shRNA sequence (HT29SQRscr), SQ expression was markedly diminished in two HT29SQRkd knockdown cell lines as assessed by Western blot analysis (Fig. 5b). SQ activity was measured in these cell lines as described (5). Activity was significantly diminished in the two HT29SQRkd cell lines (4-5 nmol product min^{-1} mg^{-1} protein) compared to the control HT29SQRscr line (134 nmol product min^{-1} mg^{-1} protein), confirming successful knockdown of SQ protein levels.

Both HT29SQRkd cell lines were highly sensitive to H_{2}S poisoning, and OCR activation was not observed at any sulfide concentration (Fig. 5c). Inhibition of oxygen consumption was observed even at 5 \mu M H_{2}S, revealing a key role for SQ in shielding cells from respiratory poisoning by H_{2}S.

The sulfide oxidation pathway generates reactive sulfur species that could be important in signaling (18,19). We therefore used the fluorescent SSP4 probe (20) to examine whether SQ activity modulates sulfane sulfur levels. Persulfide labeling was reduced in HT29SQRkd compared to control HT29SQRscr cells (Fig. 5d), consistent with the postulated role of the sulfide oxidation pathway in potentiating small molecule and protein persulfidation (18).

SQ knockdown-sensitive changes in reactive cysteine modifications in the mitochondrial proteome-We identified changes in oxidative cysteine modifications in the mitochondrial proteome, which are sensitive to the presence or absence of SQ. For this, HT29SQRkd cells were labeled with standard amino acids, while HT29SQRscr cells were labeled with heavy isotopic amino acids ([6-^{13}C,4-^{15}N]-arginine and [6-^{13}C]-lysine) (Fig. S2). Following mitochondrial enrichment and iodoacetamide-alkyne treatment, mitochondria from both cell lines were mixed, and a cleavable Azo-tag was introduced via click chemistry to enrich for reactive cysteines that were subsequently identified by MS/MS analysis as described previously (21). Most modified cysteines in HT29SQRscr (heavy, H) cells displayed a decrease in cysteine thiol reactivity relative to HT29SQRkd (light, L) cells (i.e. higher L/H ratios). To account for differences in protein levels between HT29SQRkd and HT29SQRscr, cysteine-reactivity changes were corrected for protein abundance to focus on cysteine modifications that were sensitive to the presence or absence of SQ.

Peptides identified with higher L/H isotope ratios, indicated cysteines with higher reactivity (i.e. due to decreased posttranslational modification) in the absence of SQ, potentially implicating them as targets of GSSH (a product of the SQ reaction) or as targets of an enzymatic reaction downstream of SQ, e.g. a sulfurtransferase. From >250 mitochondrial proteins, >500 cysteine residues could be quantified, of which >200 had L/H ratios >2, indicating a widespread SQ-sensitive decrease in mitochondrial cysteine reactivity (Table S1).

Proteins with the highest cysteine L/H ratios included: TOMM70A (translocase of outer mitochondrial membrane 70A) with an L/H of ~13, ISCU (iron-sulfur cluster assembly enzyme) with an L/H of ~3.25, and the mitochondrial antioxidant enzymes Txn2/Txnr2 (thioredoxin 2/thioredoxin reductase 2) with an L/H of ~3.5. TST, which forms a persulfide intermediate with GSSH as a substrate, showed a L/H ratio of 2.1 for the active site CyS_{258} containing peptide (Fig. 6a). Only 3 proteins had cysteines with a >2-fold decrease in their L/H ratio, including glyceraldehyde 3-phosphate dehydrogenase, previously identified as a persulfidation target (22) and fatty acid synthase. Since both these proteins are cytosolic, their identification indicated that some carryover of abundant cytoplasmic proteins had occurred in our mitochondrial preparation.

Pathway enrichment analysis of proteins with L/H ratios >2, identified a number of processes involved in energy metabolism, as expected for the mitochondrial proteome (Fig. 6b).

H_{2}S-induced metabolite changes map onto energy metabolism-We used LC-MS based metabolomics analysis to identify changes induced in HT29 CRC cells upon exposure to 0.1 mM H_{2}S for 1 h. Both increases and decreases were observed with a subset of metabolites mapping either directly or indirectly onto central carbon and energy metabolism (Fig. 7). The majority of the glycolytic and TCA cycle metabolites that exhibited significant (p <0.05) H_{2}S-sensitive changes, were substrates or products of redox cofactor-dependent enzymes, suggesting that H_{2}S
induces a perturbation in redox homeostasis (Fig. 8).

H₂S Restricts Proliferation-OCR experiments revealed that H₂S concentrations >30 µM inhibited respiration in intact cells (Fig. 5a, S1), and that the basal rate was recovered at longer times (60 min, not shown). Because cell proliferation is monitored over longer time periods and because H₂S is volatile and lost from culture dishes, monitoring the effects of H₂S on proliferation necessitated the use of a different study design. Thus, repeated bolus administration of H₂S (0.1 or 0.3 mM) over 72 h was used as described under Experimental Procedures. At a concentration of 0.3 mM, HT29 CRC cells were more resistant to the antiproliferative effects of H₂S compared to the non-cancer HCEC cells (Fig. 9a). At a lower concentration (i.e. 0.1 mM) only HCEC cells exhibited growth restriction compared to the four CRC cell lines that were tested (Fig. 9b).

H₂S causes electron acceptor insufficiency- The dual effects of H₂S on the ETC, i.e. stimulation of O₂ consumption by the sulfide oxidation pathway and inhibition of complex IV, predicts that H₂S exposure would lead to an over-reduced CoQ pool. Thus, the antiproliferative effect of H₂S could result in part from a general insufficiency of electron acceptors that are regenerated by the ETC, which in turn, restricts aspartate and uridine pools needed for biomass production (Fig. 1a). Consistent with this prediction, a decrease in the NAD⁺/NADH ratio was observed in cells exposed to H₂S (Fig. 10a). A further reduction in the NAD⁺/NADH ratio was seen in H₂S-treated HT29SQRkd1 cells in comparison to HT29SQRscr cells (Fig. 10b), consistent with their greater sensitivity to H₂S, resulting in ETC inhibition (Fig. 5c).

As a further test of our model, we assessed the ability of pyruvate and/or uridine to alleviate H₂S-imposed growth restriction. Proliferation of HT29 and HCEC cells was fully rescued by uridine and pyruvate and partially rescued when either nutrient was supplied individually (Fig. 10c). Furthermore, aspartate, combined with uridine, also rescued growth, demonstrating that aspartate could effectively substitute for pyruvate to relieve the growth-limiting effect of H₂S.

H₂S stimulates reductive carboxylation- A signature metabolic response in cells experiencing ETC restriction, is stimulation of the reductive carboxylation of glutamine-derived α-ketoglutarate to drive citrate synthesis (23,24). Reductive carboxylation relies on NADPH-dependent isocitrate dehydrogenase 1 and 2 to generate citrate, which can be distinguished from the TCA cycle-dependent oxidative decarboxylation by the pattern of label incorporation from [U-¹³C]-glutamine into citrate (Fig. 11a). Mass isotopomer analysis revealed an increase in the fractional enrichment of m+5 citrate and a decrease in m+4 citrate in H₂S-treated versus untreated controls, supporting an increased flux of glutamine-derived α-ketoglutarate into citrate via reductive metabolism (Fig. 11b).

Discussion

While most mammalian cells synthesize H₂S and maintain low steady-state levels (10-80 nM (25,26)) of this respiratory toxin (27,28), epithelial cells in tissues like colon are also routinely exposed to high levels of exogenous H₂S (3). It is not known whether H₂S is a mediator of productive mutualism at the host-microbiota interface or a byproduct of such an interaction. Strikingly, the sulfide oxidation pathway enzymes are heavily localized at the surface of colonic crypts, i.e. at the host-microbiota interface (Fig. 2).

In contrast, these enzymes are diffusely distributed, and as discussed later, overexpressed in CRC tissue.

The metabolic response of colon epithelial cells to potentially high chronic H₂S exposure is not known. In this study, acute responses to H₂S were monitored in OCR experiments at relatively low concentrations of H₂S (5 to 40 µM). Our longer term studies e.g. proliferation and metabolic labeling assays, used higher concentrations of H₂S (0.1-0.3 mM), due to its volatility, and rapid loss from the medium under cell culture conditions. We note, however, that these concentrations are likely to be at the low end of endogenous luminal H₂S concentrations, which are reported to range from ~0.2-2.4 mM (3,4).

Short-term exposure of colon cell lines to H₂S (>30 µM) inhibited respiration (Fig. S1), which was reversible ((17) and not shown). The presence of SQR was critical for shielding the ETC from H₂S. When SQR was knocked down, cells became
very sensitive to respiratory inhibition, which was observed even at 5 µM H₂S concentration (Fig. 5c). These results indicate that SQR levels are likely to be an important determinant of tissue sensitivity to H₂S.

Repeated and longer-term exposure to H₂S restricted growth (Fig. 9) and limited regeneration of oxidized cofactors that are needed for nutrient oxidation. This was reflected in a reductive shift in the NAD⁺/NADH ratio (Fig. 10a). In SQR knockdown cells, the magnitude of the reductive shift was greater as expected from their greater sensitivity to ETC inhibition by acute H₂S exposure (Fig. 10b). Notably, the SQR knockdown cells did not exhibit a lower NAD⁺/NADH ratio in the absence of exogenous H₂S. Thus, the residual SQR levels were sufficient to clear H₂S derived via endogenous synthesis, and/or endogenous H₂S synthesis was negligible under our growth conditions.

H₂S-induced redox perturbation (i.e. as indicated by the altered NAD⁺/NADH ratio), is expected to have pleiotropic metabolic effects. Thus, the cytosolic aspartate transaminase GOT1, which typically consumes aspartate and α-ketoglutarate, forming oxaloacetate and glutamate, reverses direction when the ETC is inhibited (11). GOT1 is a component of the malate-aspartate shuttle that moves reducing equivalents into the mitochondrion. In response to a drop in aspartate levels, due to ETC inhibition, GOT1 reversal opens a route for cytoplasmic aspartate synthesis (Fig. 8, green arrows). Under these conditions, glutamine is the major carbon source for aspartate synthesis driven via the reductive carboxylation pathway (10,11). Thus, glutamine-derived citrate is cleaved to oxaloacetate, which in turn, is transaminated by GOT1 to form aspartate. Our finding that H₂S-induced growth restriction is alleviated by exogenous uridine and aspartate (Fig. 10c), is consistent with the model that repeated H₂S exposure inhibits the ETC. Increased m+5 labeling of citrate from [U-¹³C]-glutamine further supported increased reductive carboxylation activity under these conditions (Fig. 11b).

The antiproliferative effect of H₂S raised the obvious question about how CRC cells overcome this restriction. We found a correlation between malignancy and overexpression of sulfide oxidation pathway enzymes for clearing H₂S in human CRC tissue and cell lines (Fig. 3,4). Increased levels of SQR and ETHE1 relative to the levels in normal colon epithelium were observed in 5 of 7 CRC patient samples compared to normal tissue margins and in all six malignant cell lines compared to a nonmalignant colon-derived cell line. While TST was also increased in 5/7 CRC samples, a change was not consistently seen in the CRC cell lines compared to the non-malignant cell line. Increased levels of these enzymes should increase H₂S oxidation capacity and confer protection against its inhibitory effects. Consistent with the lower expression levels of SQR and ETHE1, the nonmalignant HCEC cells were more sensitive to growth restriction by H₂S than the malignant cell lines (Fig. 9).

What is the mechanism by which H₂S induces metabolic reprogramming? H₂S is widely assumed to signal via cysteine persulfidation (22), a posttranslational modification of cysteine residues. As discussed below, this mechanism fails to explain how specific cysteines are targeted for persulfidation, and how this highly reactive modification is stabilized, to have a lifetime that is sufficient for transducing the signal (18). We posit that H₂S signals via changes in the ambient or local redox state. In this mechanism, H₂S induces alterations in mitochondrial bioenergetics that can trigger retrograde metabolite signaling, beginning with metabolic reprogramming in the mitochondrion.

Oxidative cysteine modifications have emerged as second messengers for transducing oxidant signals into cellular responses (29). While the types of regulatory cysteine modifications continue to grow, insight into how target specificity is achieved in all such redox signaling pathways is limited. Furthermore, it is challenging to separate modifications that are functionally consequential from those that are likely to be solely collateral effects of an oxidant signaling response. Similarly, the mechanism for achieving target specificity during formation of a cysteine persulfide, presumably via the reaction of an oxidized cysteine (e.g. cysteine sulfenic acid) with H₂S (30), is not known. In addition, the potential involvement of low-molecular-weight persulfide donors e.g. GSSH, a product of the SQR reaction (5,31), in protein persulfidation, is not known.
Our analysis of changes in oxidative cysteine modifications in the mitochondrial proteome that are sensitive to SQR, revealed a large number of targets of which some (e.g. TST) are known to be persulfidated (32) (Table S1). A very large number of persulfidation targets have also been reported in other studies that specifically identified persulfidated proteins (33,34). However, characterization of H\(_2\)S signaling via persulfidation, which is a labile and reactive modification, remains to be rigorously demonstrated. Our study establishes an alternative mechanism by which H\(_2\)S signals, i.e. via reprogramming energy metabolism, which is initiated by a shift in mitochondrial bioenergetics with consequent effects in the cytoplasm. A fundamental open question is why eukaryotic cells produce H\(_2\)S at all. A provocative implication of our study is that metabolic modulation by H\(_2\)S might be a key regulatory strategy that is used by cells under certain conditions.

**Experimental Procedures**

**Immunohistochemistry**—Formalin-fixed paraffin-embedded colon tissues cross-sections from three patients were performed as described previously using the ABC technique (16,35). Briefly, human colon tissue was mounted on glass slides in pairs (normal and cancer tissue) and the slides were completely de-paraffinized by immersion in xylene twice for 10 min each and rehydrated with water following incubation in graded ethanol (100, 90 and 70%). The antigen retrieval step was performed before immunostaining by microwaving the slides for 10 min in citrate buffer (pH 6.0; Biogenex, San Ramon, CA) followed by incubation in 3 % H\(_2\)O\(_2\) in methanol to block endogenous tissue peroxidase activity. The sections were blocked with 1.5% rabbit (for SQR and TST staining) or mouse (for ETHE1 staining) serum for 1 h and incubated with the specific antibody overnight at 4 °C. Rabbit anti-ETHE1 (Abcam, [EPR11697] ab174302), rabbit anti-SQR (Proteintech, 17256-1-AP) and mouse anti-TST (Proteintech, 66018-1-Ig) antibodies were used. Slides were washed with PBS and incubated with a biotinylated secondary antibody for 30 min at room temperature. The antigen signal was amplified using the Avidin-Biotin Complex method (Vectastain ABC kit, catalogue no. PK-6105; Vector Laboratories, Burlingame, CA). The antigen-antibody-Avidin complex was detected using the chromogenic substrate 3,3’-diaminobenzidine, which produced a dark brown color. Immunostained sections were counterstained with hematoxylin and examined by light microscopy. pTNM pathological classification was used to stage tumor tissues (36).

**Western Blot Analysis**—Pieces of frozen human colon tissue (obtained from the Tissue and Molecular Pathology Core, University of Michigan) were disrupted in Ripa lysis and extraction buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM deoxycholic acid, 0.5% NONIDET P-40, 2 tablets/100ml of cOmplete™ of mini, EDTA-free protease inhibitor cocktail (Roche), and 25 µg/ml phenylmethylsulfonylfluoride) using a glass homogenizer. Cell cultures were washed twice with cold PBS and then the plates (with cells) were frozen on dry ice. Then, cells were scraped off the plates in Ripa buffer followed by three cycles of freezing and thawing. The lysates were incubated on ice for 30 min followed by centrifugation at 14,000 x g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined using the Bradford reagent (BioRad) and bovine serum albumin as a standard. Cell lysates were separated on SDS-polyacrylamide gels along with known amounts of purified recombinant proteins used as standards and the proteins were transferred to PVDF membranes. The antibodies used for the immunohistochemistry experiments described above, were also used for Western blot analysis. Tomm20 was detected in colon cell lines using a rabbit polyclonal antibody from Proteintech (11802-1-AP). Rabbit or mouse secondary antibodies conjugated to horseradish peroxidase were used to visualize signals using the chemiluminescent peroxidase substrate kit SuperSignal West Dura (Thermo Scientific). Equal loading of the samples was verified by staining the PVDF membranes with Ponceau S or Coomassie Blue.

**Gene Silencing**—Gene silencing was performed using shRNA vectors containing SQR-targeting shRNA sequences were purchased from Sigma (clone ID: NM_021199.1-1306s1c1, and...
NM_021199.1-838s1c1, Mission shRNA Library). For the negative control, an shRNA vector with scrambled sequence was used (Sigma). Lentiviral particles containing the shRNA targeting or scrambled sequences were obtained by transfecting Hek293T cell lines with packaging plasmids and the shRNA vector. Viral particles were added to HT29 cells and the transected cells were selected with 1 µg/ml puromycin.

**OCR Measurements**

-O2 consumption by cell suspensions was measured using a respirometer (Oroboros Instruments Corp., Innsbruck, Austria) equipped with two polarographic oxygen-sensing electrodes in two 2 ml chambers. For this, confluent cells (70-80%) in 10 cm plates were trypsinized and suspended in cell culture medium. Cells were pelleted by centrifuging at 2,000 x g for 5 min at 4 °C, the medium was aspirated, and the cells were suspended in 2.2 ml DPBS (PBS plus CaCl2 and MgCl2 (Gibco)) supplemented with 20 mM HEPES, pH 7.4, and 5 mM glucose, and stored on ice. The cell suspension was diluted 2-fold inside the chambers of the respirometer and stirred at 25 °C. The rate of O2 consumption by cells was stabilized within 5-10 min, and then known concentrations of Na2S were added using a Hamilton gas tight syringe. OCRs were normalized to protein concentration in the cell suspension. For protein analysis, cell suspensions were diluted 1:1 with lysis buffer (20 mM HEPES, pH 7.4, 25 mM KCl, 0.5% NP 40 (v/v), protease inhibitor cocktail for mammalian tissue (Sigma), 1% (v/v)). The cells were disrupted by repeated freeze/thaw cycles, centrifuged, the supernatant was collected, and protein concentration in the supernatant was measured using the Bradford reagent (Bio-Rad).

**Persulfide Analysis in Live Cells**

HT29 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM glutamine, in a 37 °C incubator with an atmosphere of 5% CO2:95% air. To assess persulfide levels in live cells, the fluorescent probe SSP4 (Sulfane Sulfur Probe 4, Dojindo Molecular Technologies, MD), was used. Cells were plated on 35 mm glass-bottom dishes (MatTek corporation, USA) and cultured overnight. Then, cells were either untreated or treated with 0.1 mM Na2S for 1 h followed by 15 min incubation in serum-free medium containing the surfactant CTAB (0.5 mM, hexadecyltrimethylammonium bromide), and 10 µM SSP4. Analysis of cell fluorescence was carried out using an IX70 inverted fluorescence microscope (Center for Live Cell Imaging, University of Michigan), equipped with an ×100 magnification oil immersion objective (Ex: 482 nm, Em: 515 nm). Images were post-processed and the fluorescence intensity was quantified using ImageJ software.

**SILAC Labeling and SQR-sensitive Reactive Cysteine Modification**

Metabolically light HT29SQRkd1 and heavy HT29SQRscr cells were generated by 6x passaging in SILAC DMEM media (minus L-lysine and L-arginine) supplemented with 10% dialyzed FBS and 1% penicillin/streptomycin and either: (Light media) L-arginine (84 µg/ml), L-lysine (146 µg/ml), or (Heavy media) [6-13C,4-15N]-L-arginine (84 µg/ml), [6-13C]-L-lysine (146 µg/ml). Complete isotopic incorporation was confirmed by mass spectrometry. HT29 cells were grown at 37°C under 5% CO2 in the appropriate SILAC media supplemented with 10% FBS (Gibco) and 25 µg/ml amphotericin B, 10,000 units/ml penicillin, and 10,000 µg/ml streptomycin (Gibco). Once cells reached 100% confluency, they were harvested by scraping followed by centrifugation at 1,000 x g. The cell pellet was washed with PBS and then with roughly one pellet volume of mitochondrial isolation buffer (10 mM Tris (diluted from 100 mM Tris stock adjusted to pH 7.4 with MOPS powder), 1 mM EDTA (diluted from 100 mM EDTA stock adjusted to pH 7.4 with Tris powder), 200 mM sucrose, pH 7.4). Mitochondria were isolated as previously described (37). Briefly, cell pellets were resuspended in 10 volumes of mitochondrial isolation buffer and homogenized with 30 strokes of a teflon pestle in a pre-chilled glass homogenizer. The homogenate was centrifuged at 600 x g for 10 min at 4°C. The supernatant was collected and centrifuged twice at 600 x g for 10 min at 4°C, discarding the pellet between spins. The supernatant was then spun at 12,500 x g for 10 min at 4°C. The pellet was collected and washed 2 times in 1–2 pellet volumes of mitochondrial isolation buffer with a 10 min, 10,500 x g centrifugation at 4°C after each wash.
For analysis of cysteine reactivity, purified mitochondria were treated with 100 µM IA-alkyne in PBS for 1 h at 25°C and then lysed by sonication and appended with a chemically cleavable diazobenzene biotin-azide tag (Click Chemistry Tools, Scottsdale, AZ) by copper-assisted azide-alkyne cycloaddition chemistry as described previously(21). Light HT29SQRkd1 and heavy HT29SQRscr labeled mitochondrial lysates were then combined pair-wise and centrifuged for 10 min at 4°C to pellet precipitated protein. The pellet was resuspended by sonication in ice-cold methanol. After centrifugation, a second ice-cold methanol wash was performed and the protein pellet was then resuspended by sonication in 1 ml 1.2% SDS in PBS. Samples were heated for 5 min at 80 °C to fully solubilize protein, then centrifuged to remove copper. The solubilized protein samples were then combined with 5 ml PBS (0.2% final SDS concentration) and 100 µl streptavidin-agarose beads. Samples were incubated at 4°C overnight and then at 25°C for 2-3 h. The samples were centrifuged at 1,400 x g for 3 min and resuspended in 500 µl of 6 M urea and 10 mM DTT and heated at 65 °C for 15 min. Iodoacetamide (20 mM) was added and the samples were incubated at 37 °C for 30 min. The urea concentration was adjusted to 2 M with PBS and the beads were centrifuged and resuspended in 200 µl 2 M urea in PBS, 1 mM CaCl2 and 2 µg trypsin. Protein digestion was allowed to proceed overnight at 37 °C. The unlabeled peptide digests were discarded and the beads were washed 3x in PBS and 3x in water. The beads were then incubated with 50 µl of 50 mM sodium dithionite in PBS, at 25°C for 1 h with gentle agitation by rotation. After centrifugation, the supernatant was collected and saved. The beads were washed twice more with 75 µl of 50 mM sodium dithionite and all the collected supernatant fractions were combined. The beads were washed twice more with 75 µl water, and the supernatant fractions were combined with the previous fractions, for a total sample volume of 350 µl. To the combined supernatant fractions, 17.5 µl of formic acid was added, and the samples were stored at -20 °C.

MS analysis was performed using a Thermo LTQ Orbitrap Discovery mass spectrometer coupled to an Agilent 1200 series HPLC. Labeled peptide samples were pressure loaded onto a 250 µm fused silica desalting column packed with 4 cm of Aqua C18 reverse phase resin (Phenomenex). Peptides were eluted onto a 100 µm fused silica biphasic column packed with 10 cm C18 resin and 4 cm Partisphere strong cation exchange resin (SCX, Whatman), using a five step multidimensional LC/LC-MS/MS protocol (MudPIT) (38,39). Each of the five steps used a salt push (0%, 50%, 80%, 100%, and 100% for labeled peptides, and 0%, 25%, 50%, 80%, and 100% for tryptic peptides), followed by a gradient of 5–100 % buffer B (20% water, 80% acetonitrile, 0.1% formic acid) in Buffer A (95% water, 5% acetonitrile, 0.1% formic acid. The flow rate through the column was ~0.25 ul/min, with a spray voltage of 2.75 kV. One full MS1 scan (400–1800 MW ) was followed by 8 data-dependent scans of the n-th most intense ion. Dynamic exclusion was enabled.

The tandem MS data, generated from the 5 MudPIT runs, was analyzed by the SEQUEST algorithm(40). Static modification of cysteine residues (+57.0215 m/z, iodoacetamide alkylation) was assumed with no enzyme specificity. The precursor-ion mass tolerance was set at 50 ppm while the fragment-ion mass tolerance was set to 0 (default setting). Data was searched against a human reverse-concatenated non-redundant FASTA database containing Uniprot identifiers.
For tryptic protein abundance data, independent searches for SILAC static modifications on lysine and arginine for either light (0.0 and 0.0) or heavy (6.02013 and 10.00826) peptides were performed. MS2 spectra matches were assembled into protein identifications and filtered using DTASelect2.0 (41) to generate a list of protein hits with a peptide false-discovery rate of <5%. For cysteine-targeted data, a differential cysteine modification was allowed for the commercial diazo biotin-azide tag (+258.1481), with peptides restricted to fully tryptic (-y 2) with a found modification (-m 0) and a delta-CN score greater than 0.06 (-d 0.06). Single peptides per locus were also allowed (-p 1) as were redundant peptides identifications from multiple proteins, but the database contained only a single consensus splice variant for each protein. L/H ratios were calculated using the cimage quantification package described previously (42).

Label-free targeted QqQ metabolomics analysis—Targeted metabolomics was performed as described (43). Briefly, an Agilent 1290 UHPLC and 6490 Triple Quadrupole (QqQ) mass spectrometer (LC-MS) were used for label-free targeted metabolomics. Agilent MassHunter Optimizer and Workstation Software LC/MS Data Acquisition for 6400 Series Triple Quadrupole B.08.00 were used for standard optimization and data acquisition. Agilent MassHunter Workstation Software Quantitative Analysis Version B.0700 for QqQ was used for raw data processing and initial analysis.

For reversed-phase chromatography (RPC), a Waters Acquity UPLC BEH TSS C18 column (2.1 x 100mm, 1.7µm) was used with mobile phase (A) consisting of 0.5 mM NH₄F and 0.1% formic acid in water; mobile phase (B) consisting of 0.1% formic acid in acetonitrile. The following gradient was used: mobile phase (B) was held at 1% for 1.5 min, increased to 80% in 15 min, then to 99% in 17 min and held for 2 min before returning to the initial conditions and held for 10 min. For hydrophilic interaction chromatography (HILIC), a Waters Acquity UPLC BEH amide column (2.1 x 100mm, 1.7µm) was used with mobile phase (A) consisting of 20 mM ammonium acetate in water at pH 9.6; mobile phase (B) consisting of acetonitrile. The following gradient was used: mobile phase (B) was held at 85% for 1 min, decreased to 65% in 12 min, then to 40% in 15 min and held for 5 min before returning to the initial conditions and held for 10 min.

Both columns were at 40 °C and 3 µl of each sample was injected into the LC-MS with a flow rate of 0.2 ml/min. Calibration of TOF MS was achieved through Agilent ESI-Low Concentration Tuning Mix. Optimization was performed on the 6490 QqQ in the positive or negative mode for the RPC or HILIC respectively for each of 220 standard compounds to get the best fragment ion and other MS parameters for each compound. Retention time for each of the 220 standards was measured from a pure standard solution or a mixture of standards. The LC-MS/MS method was created with dynamic multiple reaction monitoring with retention times, retention time windows, and multiple reaction monitoring for all 220 standard compounds. Key parameters of AJS ESI in both the positive and the negative acquisition modes are: Gas temp 275°C, Gas Flow 14 l/min, Nebulizer at 20 psi, SheathGasHeater 250°C, SheathGasFlow 11 l/min, and Capillary 3000 V. For MS: Delta EMV 200V or 350V for the positive or negative acquisition mode respectively and Cycle Time 500 ms and Cell Acc 4V for both modes were used.

For data analysis, the pre-processed data with Agilent MassHunter Workstation Software Quantitative Analysis were post-processed for further quality control in the programming language R. We calculated the coefficient of variation (CV) across replicate samples for each metabolite given a cut-off value of peak areas in both the positive and the negative modes. We then compared distributions of CVs for the whole dataset for a set of peak area cut-off values of 0, 1000, 5000, 10000, 15000, 20000, 25000 and 30000 in each mode. A noise cut-off value of peak areas in each mode was chosen by manual inspection of the CV distributions. Each sample was then normalized by the total intensity of all metabolites to reflect the same protein content as a normalization factor. We then retained only those metabolites with at least 2 replicate measurements. The remaining missing value in each condition for each metabolite was filled with the median of the other replicate measurements. Finally, each metabolite abundance values in each sample was divided by the mean of all abundance values across all samples for statistical analyses and
visualizations among metabolites. The statistical significance test was done by a two-tailed t-test with a significance threshold of 0.05 for those metabolites filtered with CV <1 for all conditions. The p-values were not adjusted in favor of more flexible biological interpretation for downstream analysis.

\[ \text{NAD}^+/\text{NADH} \] measurement-The NAD\(^+\)/NADH ratio in HT29 (in control, HT29\(^{SQIkdi}\) and HT29\(^{SQRscr}\) and HCEC cells was measured using the NAD/NADH-Glo Assay (Promega) luminescence assay with slight modifications. Briefly, cells were plated in 12-well plates and grown overnight in an atmosphere containing 2\% O\(_2\), 5\% CO\(_2\), and 93\% N\(_2\) at 37 °C. Then, the medium was changed before the cells were treated with 0.1 mM Na\(_2\)S at 0 and 3 h and harvested at 4 h. The untreated and Na\(_2\)S treated cells were quickly washed twice with cold PBS. Then, 200 µl ice cold lysis buffer containing 1\% CTAB in 0.2 N NaOH diluted 1:1 with cold PBS was added and the samples were divided in two equal aliquots for NAD\(^+\) and NADH analysis.

For NAD\(^+\) measurement, samples were diluted 1:1 with 0.4 N HCl solution and incubated at 60 °C for 15 min to degrade NADH. To measure NADH, the samples were heated at 60 °C for 15 min to selectively degrade NAD\(^+\). Then, NADH and NAD\(^+\) samples were incubated at room temperature for 10 min and mixed 1:1 with 0.25 M Tris base in 0.2 N HCl for the NADH sample and 1:1 with 0.5 M Tris base for NAD\(^+\). The NAD/NADH-Glo detection reagent was added to each sample (1:1 v/v) and incubated at room temperature for 30 min. A luminometer was used to record luminescence.

Cell proliferation assay-Cell proliferation rate was assessed in an oxygen-regulated incubator containing an atmosphere of 2\% O\(_2\), 5\% CO\(_2\) and 93\% N\(_2\). Briefly, 5 X 10^4 cells were cultured in 6-well plates containing RPMI 1640 medium with 2 mM glutamine, 10% FBS, and 1\% penicillin/streptomycin. After 15 h, 0.1 or 0.3 mM Na\(_2\)S (as specified in the figure legend) was added every 12 h for 72 h. The culture medium was changed every 24 h. To rescue cells from Na\(_2\)S-dependent growth restriction, the culture medium was supplemented with 100 µM of uridine, pyruvate and/or 10 mM aspartate as specified in the figure legend. Cells proliferation was measured using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay. For this, MTT (5 mg/ml in PBS) was added at the desired time point to each well at a dilution of 1:10 and incubation was continued at 37 °C for 30 min. Then, the medium was aspirated and 1 ml DMSO was added per well to dissolve formazan, formed by MTT reduction. The optical density of formazan in DMSO, proportional to cell number, was measured at 553 nm. Absorption was normalized to cell number in each well at the time of seeding.

Tracing citrate formation from [U-\(^{13}\)C]-glutamine-HT29 cells were grown to ~80% confluency in 6-well plates in RPMI 1640 medium (2 mM glutamine, 10% FBS, 1% penicillin/streptomycin) and then transferred to glutamine-free DMEM containing 10% dialyzed FBS, 1% penicillin/streptomycin and supplemented with 2 mM [U-\(^{13}\)C]-glutamine (Cambridge Isotope Laboratories). Cells were cultured in an incubator with an atmosphere of 2\% O\(_2\), 5\% CO\(_2\) and 93\% N\(_2\) Cells were either untreated or treated at 0, 3, 6, 9 and 12 h with 0.1 mM Na\(_2\)S. After 13 h incubation, 1 ml 80\% cold methanol (-80 °C) was added to each well and the cells were incubated at -80 °C for 10 min followed by centrifugation at 14,000 x g for 10 min at 4 °C. The supernatants were lyophilized in a Speedvac and resuspended in 20 µl LC-MS grade water for LC-MS analysis.

For LC-MS analysis, an Agilent 1260 UHPLC and 6520 Accurate-Mass Q-TOF LC/MS was used as described previously(44). Agilent MassHunter Workstation Software LC/MS Data Acquisition for 6200 series TOF/6500 series Q-TOF (B.06.01) was used for calibration and data acquisition. In the negative ion MS acquisition mode, a Waters Acquity UPLC BEH amide column (2.1 x 100 mm, 1.7 µm) was used with mobile phase (A) consisting of 20 mM NH\(_4\)OAc in water at pH 9.6, and mobile phase (B) consisting of acetonitrile. Gradient program: mobile phase (B) was held at 85\% for 1 min, decreased to 65\% in 12 min, then to 40\% in 15 min and held for 5 min before going to the initial condition and held for 10 min. The column was at maintained at 40°C and 3 µl of each sample was injected into the LC-MS with a flow rate of 0.2 ml/min. Calibration of TOF MS was achieved through Agilent ESI-Low Concentration
Tuning Mix. Key parameters were: acquisition rate 1 spectrum/sec, mass range 100-1200 Da, gas temp 350 °C, fragmentor 150 V, skimmer 65 V, drying gas 10 l/min, nebulizer at 20 psi and Vcap 3500 V. Reference ions of 119.0363 and 980.01637 Da were used for real time mass calibration with a nebulizer at 4 psi. The reference solution comprised 10 µM purine and 1 µM HP0921.

In the positive MS acquisition mode, a Waters Acquity UPLC BEH TSS C18 column (2.1 x 100 mm, 1.7 µm) was used with mobile phase (A) consisting of 0.5 mM NH₄F and 0.1% formic acid in water, and mobile phase (B) consisting of 0.1% formic acid in acetonitrile. Gradient program: mobile phase (B) was held at 1% for 1.5 min, increased to 20% in 15 min, then to 99% in 17 min and held for 2 min before going to the initial condition and held for 10 min. The column was maintained at 40 °C and 3 µl of each sample was injected into the LC-MS with a flow rate of 0.2 ml/min. Calibration of TOF MS was achieved through Agilent ESI-Low Concentration Tuning Mix. Key parameters were: mass range 100-1200 Da, gas temp 350 °C, fragmentor 150 V, skimmer 65 V, drying gas 10 l/min, nebulizer 20 psi and Vcap 3500 V. Reference ions of 121.0509 and 922.0098 Da were used for real time mass calibration with the nebulizer at 4 psi. For data analysis, Agilent MassHunter Workstation Software Profinder B.08.00 with Batch Targeted Feature Extraction and Batch Isotopologue Extraction and Qualitative Analysis B.07.00 were used. Various parameter combinations, e.g. mass and RT tolerance, were used to find best peaks and signals by manual inspection. Key parameters were: mass tolerance = 20 or 10 ppm and retention time tolerance of 1 or 0.5 min. Isotopolog ion thresholds, the anchor ion height threshold was set to 250 counts and the threshold of the sum of ion heights to 500 counts. The coelution correlation threshold was set to 0.3.

**Measurement of cardiolipin content**

Mitochondrial cardiolipin content was determined using nonyl acridine orange (NAO) (AnaSpec, San Jose, CA). Briefly, 1 X 10⁶ cells were grown in a phenol-free DMEM medium and were treated with 100 nM NAO for 30 min. Cells were washed with cold PBS and trypsinized with phenol-free trypsin, centrifuged at 500 x g for 5 min, resuspended in cold PBS on ice and analyzed by fluorescence activated cell sorting.

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**Author Disclosure Statement**

No competing financial interest exists

**Author contributions**

M.L. performed IHC, proliferation, SQR knockdown and metabolic labeling experiments, M.L. and V.V. analyzed NAD⁺/NADH, T.B. and V.V. performed the OCR experiments, D.B. and E.W. performed and analyzed the mitochondrial oxidative cysteine labeling experiments, H.J.L. and C.L. analyzed the metabolomics data, N.S. and E.F. assisted with the human colonic IHC and SQR knockdown
experiments, M.L. and R.B. designed the study and co-wrote the manuscript, which was edited by the coauthors.
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Figure 1. A schematic diagram showing the intersection between sulfide oxidation and energy metabolism. (a). In proliferating cells, oxidation of nutrients for biomass synthesis leads to electron capture by NAD$^+$ and other cofactors, which are recycled by the ETC. An H$_2$S-induced ETC backup leads to a shortage of uridine and aspartate. DHO and DHOD are dihydroorotate and dihydroorotate dehydrogenase, respectively. (b). Reactions catalyzed by the sulfide oxidation pathway enzymes.
Figure 2. Localization of sulfide oxidation pathway enzymes in human colon tissue.
Immunohistochemical staining of human SQR, TST (rhodanese) and ETHE1 reveals a strongly apical (surface) localization in normal colon (a) but diffuse localization in CRC epithelium (b). The panels are shown at ×20 magnification and are representative of 2 independent experiments each performed in triplicate.
Figure 3. Sulfide oxidation pathway enzymes are up-regulated in human CRC samples. (a) Representative western blot analysis of the sulfide oxidation pathway enzymes (SQR, ETHE1 and TST) in paired normal and cancer tissues from 7 patients with CRC. STD denotes purified protein standards and the numbered N/C pairs denote normal margin versus cancer tissue for each matched sample. (b) Protein loading control for colon tissue samples as visualized by Coomassie Blue staining. The data are representative of 3 independent experiments.
Figure 4. Sulfide oxidation pathway enzymes are up-regulated in human CRC cell lines. (a) Representative western blot analysis of SQR, ETHE1 and TST in six human CRC cell lines and the non-malignant human colon epithelial cell line HCEC. Note that the HCEC lane was loaded with 120 µg protein whereas the others had 30 µg protein each. (b) Protein loading control for colon cell lysate samples in (a) as visualized by Ponceau S staining. The HCEC lane was loaded with 120 µg protein while the others had 30 µg protein each. The data are representative of 3 independent experiments. The dotted line indicates where the lane with the MW markers was spliced in. (c) Mitochondrial content was assessed by nonylacridine orange (NAO) fluorescence of cells sorted by FACS in the nonmalignant colon epithelial cell line, HCEC and the indicated CRC cell lines. The data represent the mean fluorescence ± SD of 2 independent experiments each performed in quadruplicate. The mean fluorescence of HCEC was arbitrarily set to 1.
Figure 5. SQR dependence of H$_2$S-induced OCR and sulfane sulfur formation. (a) Changes in OCR in a suspension of HT29 cells (HT29$_{SQRscr}$) following addition (arrow) of varying Na$_2$S concentrations. (b) SQR expression in HT29$_{SQRscr}$ and two independent SQR knockdown lines, HT29$_{SQRkd1}$ and HT29$_{SQRkd2}$ (left). Equal loading of the same membrane as visualized by Ponceau S staining (right). The dotted line indicates where the lane with the MW markers was spliced in. (c) Representative data from one of two SQR knockdown cell lines (HT29$_{SQRkd1}$) showing that they failed to activate OCR at any Na$_2$S concentration. Similar results were obtained with HT29$_{SQRkd2}$. (d) Representative images of HT29$_{SQRscr}$ cells that were untreated (upper) or treated with 0.1 mM Na$_2$S for 1 h (middle) compared to HT29$_{SQRkd1}$...
cells (lower) treated with 0.1 mM Na₂S. Cells were labeled with SSP4, which emits a green fluorescence in the presence of sulfane sulfur species (Ex: 482 nm, Em: 515 nm). Semi-quantitative analysis of the microscopic data (bottom panel) represents the mean ± SD of 3 independent experiments each performed in duplicate (*p <0.001).

Figure 6. Effect of SQR on reactive cysteine modifications in the mitochondrial proteome. (a) Differential effects on oxidative cysteine modifications in mitochondrial proteins modulated by SQR activity. L/H ratios for all identified mitochondrial cysteine-containing peptides corrected for changes in protein expression are shown; a subset is highlighted. Peptides with high levels of modified cysteine residues in the heavy sample (HT29SQRscr) display high L/H. The data are the average of 2 independent
experiments. (b) Pathway enrichment analysis of cysteine-containing peptides with L/H >2. Pathways exhibiting a >10-fold enrichment included several involved in energy metabolism.
**Figure 7. Metabolomic changes elicited by H\textsubscript{2}S.** The heat map shows differential changes (p<0.05, CV<1) in metabolites in HT29 cell lysates exposed for 1 h to 0.1 mM H\textsubscript{2}S. Metabolites are ordered by fold-change. The samples were run in quadruplicate. The suffixes, “rp” and “hn”, in the metabolite names represent measurements using reversed-phase liquid chromatography in the positive mode and hydrophilic interaction liquid chromatography in the negative mode, respectively as described under Experimental Procedures.
Figure 8. Several H$_2$S-sensitive metabolite changes map to redox active nodes in central carbon metabolism. Schematic of central carbon metabolism including the glutamine-dependent reductive carboxylation pathway (green arrows) that is stimulated by H$_2$S. Changes in metabolites are denoted by the same color scheme used in Fig. 7, i.e. blue and red arrows represent increases and decreases, respectively.
Figure 9. CRC cells are more resistant than a nonmalignant colon cell line to the antiproliferative effect of H$_2$S. (a) Effect of H$_2$S (0.3 mM) on the proliferation of HCEC and HT29 cells. Values for untreated HCEC and HT29 cells at t = 0 h were set at 1. The data (mean±SD) are representative of 3 independent experiments each performed in triplicate. (b) The proliferation rate of HCEC cells was compared to 4 other colon cancer cell lines as indicated in the absence (white bars) or presence of Na$_2$S (grey bars). Cells were treated with 0.1 mM Na$_2$S at time = 0 and every 12 h, and the medium was changed every 24 h for a total treatment time of 72 h, as described under Experimental Procedures. Proliferation was measured using the MTT assay. The data are representative of 3 independent experiments each performed in triplicate and represent the mean±SD.
Figure 10. Growth restriction by H$_2$S is due to electron acceptor insufficiency. (a) A change in the NAD$^+$/NADH ratio was observed in the presence of 0.1 mM Na$_2$S (grey) compared to its absence (white). The NAD$^+$/NADH ratios for untreated HCEC and HT29 cells were set at 1. The data are representative of 5 independent experiments each performed in triplicate, (*p < 0.001). (b) A difference in the NAD$^+$/NADH ratio was observed in HT29$^{QRscr}$ (Ctrl) and HT29$^{QRkd1}$ (KD) cells in the absence (white) and presence of 0.1 mM Na$_2$S (grey). The NAD$^+$/NADH ratio for untreated HT29$^{QRscr}$ cells was set at 1. The data are representative of mean ± SD of 3 independent experiments each performed in triplicate. The *p*-values are shown for the difference between HT29$^{QRscr}$ cells ± H$_2$S and between Na$_2$S-treated HT29$^{QRscr}$ and HT29$^{QRkd1}$ cells. The difference between untreated HT29$^{QRscr}$ and HT29$^{QRkd1}$ cells was not significant (p>0.3). (c) Proliferation of HCEC (white) and HT29 (grey) cells at 48 h following culture in the absence or presence of 0.3 mM Na$_2$S, uridine (U), pyruvate (Pyr) and aspartate (Asp) added at concentrations specified under Experimental Procedures. The data are representative of 4 independent experiments each performed in triplicate and represent the mean±SD (*p < 0.001, **p < 0.01).
Figure 11. H₂S induces a metabolic shift towards the reductive TCA cycle (a) Schematic representation of the fate of [U-¹³C]-L-glutamine (blue) metabolized in the oxidative (black arrows) versus reductive (red arrows) directions. Unlabeled atoms are shown in white. ACL is ATP citrate lyase. (b) Fractional labeling isotopologue distribution of citrate in HT29 cells labeled with [U-¹³C]-L-glutamine in the absence or presence of 0.1 mM Na₂S. Samples were analyzed by MS and the values were normalized to total protein as described under Experimental Procedures. The analysis was performed in quadruplicate and the data represent mean±SD.
Hydrogen sulfide perturbs mitochondrial bioenergetics and triggers metabolic reprogramming in colon cells

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