Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway

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Haem oxygenase (HO)-1/carbon monoxide (CO) protects cancer cells from oxidative stress, but the gas-responsive signalling mechanisms remain unknown. Here we show using metabolomics that CO-sensitive methylation of PFKFB3, an enzyme producing fructose 2,6-bisphosphate (F-2,6-BP), serves as a switch to activate phosphofructokinase-1, a rate-limiting glycolytic enzyme. In human leukaemia U937 cells, PFKFB3 is asymmetrically di-methylated at R131 and R134 through modification by protein arginine methyltransferase 1. HO-1 induction or CO results in reduced methylation of PFKFB3 in varied cancer cells to suppress F-2,6-BP, shifting glucose utilization from glycolysis toward the pentose phosphate pathway. Loss of PFKFB3 methylation depends on the inhibitory effects of CO on haem-containing cystathionine β-synthase (CBS). CBS modulates remethylation metabolism, and increases NADPH to supply reduced glutathione, protecting cells from oxidative stress and anti-cancer reagents. Once the methylation of PFKFB3 is reduced, the protein undergoes polyubiquitination and is degraded in the proteasome. These results suggest that the CO/CBS-dependent regulation of PFKFB3 methylation determines directional glucose utilization to ensure resistance against oxidative stress for cancer cell survival.
Carbon monoxide (CO), a gas mediator generated by haem oxygenase (HO), has been shown to serve as multiple biological actions on neurovascular transmission, modulation of inflammatory responses and apoptosis via cGMP-dependent and -independent manners. There is a body of evidence that HO-1 expression contributes to cancer survival, chemoresistance and tumour invasiveness in different types of cancer cells. Among these studies, much attention has been paid to roles of anti-oxidative biliverdin/bilirubin and CO for protecting cancer cells, although the gas-sensitive discernible molecular mechanisms remained largely unknown.

We have recently shown that physiological levels of CO exert its inhibitory action on haem-containing cystathionine β-synthase (CBS), which is the rate-limiting enzyme determining the ratio between remethylation and trans-sulphuration metabolism. Since CBS can generate hydrogen sulphide (H₂S) through the reaction utilizing cysteine and homocysteine as substrates, CO-dependent inhibition of the enzyme causes H₂S suppression and thereby plays regulatory roles for organ homeostasis. Besides its role for H₂S generation, CBS plays a role in regulating substrates for remethylation cycle including homocysteine, methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). CO-dependent inhibition of CBS can regulate up- and/or downregulation of protein arginine methylation through regulating cellular levels of SAM, the methyl donor, and SAH, an inhibitor of protein methyltransferases, while target proteins modified by protein arginine methylation/demethylation by CO remain unknown.

In clinical oncology, much attention has long been paid to CBS as a key regulator of cancer development and chemoresistance. Children with Down syndrome with acute myeloid leukaemia have significantly higher event-free survival rates compared with those with non-DS acute myeloid leukaemia. This effect is linked to greater sensitivity to anti-cancer reagents and higher transcript levels of CBS as the chromosome 21-localized gene. On the other hand, CBS dysfunction resulting from polymorphism mediates (Fig. 1). The cells exhibiting HO-1 induction by the haemin treatment significantly decreased total amounts of the 13C-labelled glycolytic metabolites (Σglycolysis), while increasing the flux towards PPP significantly, as assessed by total amounts of the 13C-labelled PPP metabolites (ΣPPP). Such a shift from glycolysis to PPP was seen in the cells treated with CO-releasing molecule (CORM-2) for 60 min at 100 μM, showing significant increases in Xu-5P/Ru-5P and Sed-7P, and decreases in BPG and PEP in glycolytic pathway, while glucose uptake rates were unchanged (Supplementary Fig. 2).

The results shown in Fig. 1 led us to hypothesize that HO-1 induction can inhibit the activity of PFK-1/FBPase; the hypothesis was in good agreement with increases in PPP metabolites. As expected, the PFK-1 activities in the U937 cell lysates were significantly lower in 5μM CO-treated or haemin-treated cells than the control cells (Fig. 2a). PFK-1 activity is reported to be influenced by several allosteric effectors including ATP, citrate and F-2,6-BP downregulated by CO treatment significantly coincided with a decrease in F-2,6-BP, a potent allosteric activator of PFK-1 (Fig. 2b). The CO-treated U937 cells exhibited a time-dependent inhibition of F-2,6-BP that was insensitive to actinomycin D treatment, suggesting the inhibition process does not require de novo transcription of PFKFBs, the F-2,6-BP-generating enzymes (Fig. 2c,d). We also analysed mRNA expression levels of several glycolytic enzymes in U937 cells treated with or without CO treatment. No obvious differences in their expression levels were observed (Supplementary Fig. 3). Since PFKFB3 and PFKFB4 constitute major isoforms in U937 leukaemia cells, effects of the isoyme knockdown were examined (Fig. 2e), indicating that PFKFB3 knockdown, but not PFKFB4, significantly suppressed cellular F-2,6-BP contents.

As mentioned in the Introduction section, CBS is a CO-sensitive key enzyme that regulates protein arginine methylation, leading us to examine whether the CO/CBS system is involved in mechanisms for CO-sensitive downregulation of F-2,6-BP in the cell. As seen in Fig. 2f, CBS knockdown by siRNA significantly suppressed basal F-2,6-BP contents in the same cells. Such cells rendered insensitive to the same concentration of CO. We next examined whether CO suppresses trans-sulphuration via CBS inhibition. As expected, the CO treatment significantly inhibited intracellular conversion of 15N-labelled methionine to N-cystathionine by CBS. This result is in good agreement with our previous studies in vivo. Furthermore, the CBS knocked down cells suppressed the basal flux for the cystathionine generation, and became insensitive to the CO application (Fig. 2g). Collectively, these results raised a possibility that CO modulates glucose biotransformation through post-translational mechanisms involving arginine methylation of PFKFB3 in the cells.

Results

CO-dependent suppression of F-2,6-BP is mediated by CBS. Differences in glucose biotransformation between human monoblastic leukaemia U937 cells pretreated with 25 μM haemin for 6 h and untreated controls were examined by metabolome analysis based on capillary electrophoresis mass spectrometry (CE-MS), showing that the sum of metabolites belonging to glycolysis was decreased, while those belonging to PPP was increased significantly by HO-1 induction by haemin (Supplementary Fig. 1). Under these circumstances, ATP and energy charge values were not changed by the haemin treatment.

In order to determine the actual conversion of glucose to the downstream pathways, we added 13C₆-glucose in culture for 5 min to determine amounts of 13C-labelled metabolic intermediates (Fig. 1). The cells exhibiting HO-1 induction by the haemin treatment significantly decreased total amounts of the 13C-labelled glycolytic metabolites (Σglycolysis), while increasing the flux towards PPP significantly, as assessed by total amounts of the 13C-labelled PPP metabolites (ΣPPP). Such a shift from glycolysis to PPP was seen in the cells treated with CO-releasing molecule (CORM-2) for 60 min at 100 μM, showing significant increases in Xu-5P/Ru-5P and Sed-7P, and decreases in BPG and PEP in glycolytic pathway, while glucose uptake rates were unchanged (Supplementary Fig. 2).

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PRMT1 contributes to protein arginine methylation of PFKFB3. We found that human PFKFB3 possessed two RRX(R) motifs, presumably recognized by protein arginine methyltransferase 1 (PRMT1) in the primary structure (Supplementary Fig. 4). The fact led us to examine whether the enzyme modification by arginine methylation is involved in mechanisms for attenuating F-2,6-BP levels through the CO/CBS system.
Figure 1 | HO-1 shifts glucose metabolism towards PPP. U937 cells were treated with 25 μM haemin for 6 h. Cells were then incubated with 13C6-labelled glucose for 5 min. Data show amounts of 13C-labelled metabolites in glycolysis and PPP (open bar, control; closed bar, haemin-treated cells). \( \sum \) glycolysis; total sum values of 13C6-G-6-P, 13C6-F-1,6-BP, 13C3-GA3P, 13C3-DHAP and 13C3-BPG. \( \sum \) PPP indicates total sum values of 13C6-G-6-P, 13C5-R-5-P, 13C5-Ru-5-P and 13C3-GA3P. BPG is expressed as total sum values of 2,3-BPG and 1,3-BPG. Inset, HO-1 expression in haemin-treated cells. Data are expressed as mean \( \pm \) s.d. (nmol 10^{-8} cells) of five separate experiments. * \( P < 0.05 \) versus controls (unpaired Student’s t-test). ND; not detected; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-BP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; BPG, bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; 6-PG, 6-phosphogluconate; Ru-5-P, ribulose-5-phosphate; R-5-P, ribose-5-phosphate; Xy-5-P, xylulose-5-phosphate; Sed-7P, sedoheptulose-7-phosphate; Ery-4P, erythrose-4-phosphate.
pathway. To test this hypothesis, arginine methylation of PFKFB3 was detected in vitro by the incorporation of [3H]-methyl group derived from [3H]-SAM with autoradiography (Fig. 3a). Among different PRMT isozymes, PRMT1 and PRMT4 can transfer methyl group towards GST-tagged PFKFB3. In order to identify the residues of arginine methylation, three human PFKFB3 fragments including full-length (1–520), N-terminal (1–246) and C-terminal (247–520) were constructed, since the latter two fragments contain a single RXRR (131-RERR-134) or RXR (443-RER-445) domain, respectively (Fig. 3b). In vitro methylation assay in the presence of PRMT1 or 4 and the radioactive SAM showed that RXRR (131-RERR-134) or RXR (443-RER-445) domain respectively (Fig. 3b). To note was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f). To be noted was the fact that dimethylated PFKFB3 occurs in the untreated control cells, while unmethylated PFKFB3 (Fig. 3f).
disappearance of methylated PFKFB3 that coincided with notable downregulation of total PFKFB3 amounts in the cells (Fig. 4b). On the other hand, the transfectant of mutant HO-1 (H25A), which is unable to catalyse haem because it lacks the histidine residue for the axial haem ligand and thereby serves as a dominant negative for the cellular catalytic activity of HO-1 (ref. 25), exhibited robust elevation of the methylated enzyme. Under these circumstances, total amounts of PFKFB3 were also maintained.
**Figure 4 | HO-1/CO triggers K142 polyubiquitination of PFKFB3.** (a) Demethylation of PFKFB3 in U937 cells treated with haemin for 6 h. (b) Alterations in arginine methylation status of PFKFB3 in U937 cells stably transfected with WT and mutant (H25A) HO-1 that lacks in catalytic activity. Note that transfection with WT but not with H25A mutant HO-1 causes PFKFB3 demethylation. Lysates were analysed by western blotting with anti-dimethyl-PFKFB3, anti-PFKFB3 and anti-HO-1 antibodies. (c) Effects of CrMP, an HO inhibitor of methylation of PFKFB3, indicating that the catalytic activity of HO is necessary for PFKFB3 demethylation. (d)Temporal alterations in methylation of PFKFB3 in U937 cells transfected with siRNA (control or siCBS) exposed to 5 μM CO. Lysates were analysed by western blotting using anti-dimethyl-PFKFB3, anti-PFKFB3 and anti-iCBS antibodies. In the right panels, note suppression of basal me-PFKFB3 signals and disappearance of the CO-induced signal inhibition in siCBS-transfected cells. (e) Effects of Cdh1-silencing in HeLa cells on R131/134 methylation of PFKFB3. Note that knockdown of Cdh1 increases methylated PFKFB3. (f) Role of HO-1 catalytic activities in the regulation of demethylation and polyubiquitination of PFKFB3. PFKFB3 endogenous polyubiquitination (Ub–PFKFB3) was observed by WT, but not by H25A mutant HO-1 transfection. (g) Effects of PRMT1 knockdown on PFKFB3 polyubiquitination. U937 cells were transfected with siRNA against control (scramble) or against PRMT1. Lysates were immunoprecipitated with anti-polyubiquitin antibody. The eluates are analysed with anti-PFKFB3 antibody. Lysates for input were immunoblotted with anti-dimethyl-PFKFB3 (R131/134), anti-PFKFB3 and anti-iCBS antibodies. In the right panels, note suppression of basal me-PFKFB3 signals and disappearance of the CO-induced signal inhibition in siCBS-transfected cells. (h) Effects of Cdh1-silencing in HeLa cells on R131/134 methylation of PFKFB3. Note that knockdown of Cdh1 increases methylated PFKFB3. (i) Role of HO-1 catalytic activities in the regulation of demethylation and polyubiquitination of PFKFB3. PFKFB3 endogenous polyubiquitination (Ub–PFKFB3) was observed by WT, but not by H25A mutant HO-1 transfection. (j) Effects of PRMT1 knockdown on PFKFB3 polyubiquitination. U937 cells were transfected with siRNA against control (scramble) or against PRMT1. Lysates were immunoprecipitated with anti-polyubiquitin antibody. The eluates are analysed with anti-PFKFB3 antibody. Lysates for input were immunoblotted with anti-dimethyl-PFKFB3 (R131/134), anti-PFKFB3 and anti-iCBS antibodies. In the right panels, note suppression of basal me-PFKFB3 signals and disappearance of the CO-induced signal inhibition in siCBS-transfected cells.
exhibited downregulation of polyubiquitinated PFKFB3 (Fig. 4i). In both experiments, K142A mutant PFKFB3 did not undergo polyubiquitination in response to the HO-1 induction or to PRMT1 overexpression in U937 cells. These results collectively suggested that the methylation switch determines PFKFB3 ubiquitination at K142. Finally, MGI132, a proteosomal inhibitor, increased basal amounts of methylated PFKFB3 with total PFKFB3 being unchanged (Fig. 4j). Although the relationship between proteosomal degradation and fates of PFKFB3 methylation should be further examined, these results suggest that R131/134-unmethylated form of PFKFB3 serves as a target of polyubiquitination at K142, and that HO-1/CO accelerates this process.

**CO modulates PFKFB3 methylation to protect from oxidants.**

As a result of the decrease in F-2,6-BP generation by decreased PFKFB3 methylation, U937 cells exposed to CO or to HO-1 induction by haemin exhibited significant elevation of cellular amounts of NADPH that coincided with increasing NADPH/NADP⁺ ratio (Fig. 5a). U937 cells exposed to CO or those pretreated with haemin became significantly resistant to H₂O₂ (Fig. 5b). Since CO exposure as well as the HO-1 induction decreased amounts of trans-sulphuration metabolites via CO-dependent CBS inhibition, total amounts of reduced and oxidized glutathione (GSH + GSSG) were modestly suppressed in U937 cells. Even under such circumstances, the ratio between GSH and GSSG was significantly increased in the CO-treated cells, suggesting that anti-oxidative capacity of the cell was potentiated (Fig. 5c). Moreover, the cells transfected with siCBS or siPRMT1 exhibited lesser sensitivity to H₂O₂ that coincided with increased NADPH contents (Supplementary Fig. 7). In the same cells, we examined effects of the CBS knockdown by siRNA on cisplatin sensitivity. CBS knockdown caused a decrease in methylated PFKFB3 in the cells (Fig. 5d), and acquired chemoresistance against cisplatin (Fig. 5e).

Using HEK293 cells, which enabled us to prepare stable transfectants easily, we examined effects of exogenously introduced WT–PFKFB3 and the unmethylable R131/134K mutant. As seen, the WT-transfectant increased, and the R131/134K mutant transfectant decreased basal contents of F-2,6-BP versus the mock transfectant (Fig. 5f). Moreover, transfection of PRMT1, but not that of PRMT4, increased F-2,6-BP contents in WT–PFKFB3-expressing cells. By contrast, the R131/134K mutant exhibited significantly lower contents of F-2,6-BP, and did not increase in response to PRMT1 overexpression (Supplementary Fig. 8). The cells overexpressing the R131/134K mutant significantly increased cellular amounts of NADPH and the NADPH/NADP⁺ ratio more than mock and WT transfectants (Fig. 5g,h). Under these circumstances, the cells overexpressing R131/134K mutant PFKFB3 exhibited lesser susceptibility against H₂O₂ than those of mock or WT transfectants (Fig. 5i), showing dominant negative effects of the mutant. These results collectively suggested that the R131/134-unmethylated form of PFKFB3 is crucial for supporting cellular NADPH against oxidative stress.

As shown in structure of PFKFB3 monomer (Supplementary Fig. 6), positively charged R131/134 residues might strengthen fructose-6-phosphate (F-6-P) binding to R133 as their proximal residue. To examine whether an unmethylable and uncharged mutant of PFKFB3, which weakens F-6-P binding to R133, might facilitate a shift of glucose metabolism from glycolysis to PPP to upregulate NADPH, we established HEK293 cells overexpressing R131/134K mutant PFKFB3 (Mock, WT, and Mutant). Western blotting revealed that R131/134K mutant PFKFB3 diminished basal F-6-P contents versus WT PFKFB3 in HEK293 cells (Supplementary Fig. 9). The cells overexpressing the R131/134K mutant revealed increased NADPH contents and NADPH/NADP⁺ ratio more than mock and WT transfectants (Supplementary Fig. 10). Moreover, transfection of PRMT1, but not PRMT4, increased F-2,6-BP contents in WT–PFKFB3-expressing cells. By contrast, the R131/134K mutant exhibited significantly lower contents of F-2,6-BP, and did not increase in response to PRMT1 overexpression (Supplementary Fig. 11).

**Figure 5** | Protective effects of CO on oxidative cell injury through enhancing NADPH–glutathione system in U937 cells. (a) Intracellular NADPH levels and NADPH/NADP⁺ ratio in U937 cells treated with CO or with haemin. Data indicate mean ± s.d. (n = 4–6). *P < 0.05 versus controls. (b) Inhibitory effect of CO or haemin on H₂O₂-induced cell death. Cells were exposed to 0.5 mM H₂O₂ for 1 h. Data indicate mean ± s.d. of four separate experiments. *P < 0.05 versus controls. (c) Alterations in reduced and oxidized glutathione levels in U937 cells treated with CO and haemin. Data indicate mean ± s.d. (n = 5–8). *P < 0.05 versus controls. Procedures for treatment with CO at 5 μM for 30 min or with haemin at 25 μM for 6 h were identical throughout experiments. (d) The plot shows the methylation status of PFKFB3 in U937 cells transfected with siCBS. The expression level of GAPDH is used as a loading control. (e) Effect of CBS knockdown on cisplatin-induced apoptosis in U937 cells. U937 cells were treated with increasing concentrations of cisplatin for 6 h. Cell viability was determined as fluorescence signal derived from annexin V-FITC in the apoptotic cells. Data indicate the values of relative cell death as means ± s.d. of four separate experiments. *P < 0.05 versus control without cisplatin treatment. Panels (f–i): Effects of overexpression of WT–PFKFB3 or mutant R131/134K–PFKFB3 on redox metabolites in HEK293 cells. (f) F-2,6-BP contents, (g) NADPH contents, (h) NADPH/NADP⁺ ratio and (i) sensitivity to H₂O₂. In these experiments, HEK293 cells were transfected with mock, WT or nonmethylable mutant (R131/134K) PFKFB3. Data indicate mean ± s.d. of five separate experiments. *P < 0.05 (mock versus R131/134K), †P < 0.05 (WT versus R131/134K). ANOVA with Fischer’s multiple comparison test was used for determining statistical significance throughout this figure.
Figure 6 | Overexpression of unmethylable PFKFB3 mutants shifts glucose metabolism towards PPP. HEK293 cells were transfected with WT-PFKFB3, R131/134L- or R131/134K-PFKFB3 mutant. These cells were incubated with the medium containing 1 g l\(^{-1}\) \(\text{^{13}C}_6\)-labelled glucose for 5 min. Measurements of \(\text{^{13}C}\)-labelled metabolites were performed by CE-MS. (a) Differences in \(\text{^{13}C}\)-labelled metabolites in glycolysis and PPP between the cells transfected with WT-PFKFB3 (open bars), those transfected with R131/134K-PFKFB3 (grey bars) and those transfected with R131/134L-PFKFB3 (closed bars). Data are expressed as mean ± s.d. (nmol mg\(^{-1}\) protein) of six separate experiments *P < 0.05 versus controls. ND, not detected. (b) The two nonmethylable mutants significantly increase PPP flux and suppress glycolysis. Differences in biotransformation of glucose in the cells carrying WT-PFKFB3 or two nonmethylable mutant: R131/134K and R131/134L. \(\Sigma\text{^{13}C}\)-glycolysis and \(\Sigma\text{^{13}C}\)-PPP are described in Fig. 1 and Supplementary Fig. 1. BPG is expressed as total sum values of 2,3-BPG and 1,3-BPG. Note that the R131/134L mutant displays greater suppression of the glycolytic flux than the R131/134K mutant (*P < 0.05). Data are expressed as mean ± s.d. (nmol mg\(^{-1}\) protein) of six to seven separate experiments. *P < 0.05 versus the cells carrying WT-PFKFB3. ANOVA with Fischer’s multiple comparison test was used throughout this figure.
the R131/134K or R131/134L mutant PFKFB3, and compared the $^{13}$C$_6$-glucose fluxes versus the cells overexpressing WT PFKFB3 (Fig. 6). The cells expressing the R131/134L mutant and those overexpressing R131/134K exhibited a significant metabolic shift from glycolysis to PPP. To be noted was the fact that the R131/134L mutant-expressing cells exhibited greater decrease in $\Sigma$glycolysis values than the R131/134K mutant-expressing cells, suggesting a potent inhibitory effect of the latter mutant on the PFKFB3 activity. These results showed that the two different unmethylable mutants of PFKFB3 facilitate directional glucose utilization towards PPP.

**CO decreases methylated PFKFB3 to cause chemoresistance.** As described above, methylation levels of PFKFB3 determines the capacity of NADPH/GSH system to ameliorate oxidative stress in human monoblastic leukaemia U937 cells. We examined whether the decrease in methylated PFKFB3 serves as a switch for directional glucose metabolism towards PPP in different types of cancer cells. Human-derived HCT116 colon cancer cells and HEK293 cells were transfected with control non-targeted shRNA, or with different forms of short hairpin RNA interference (shCBS#1 and #2). Stable CBS knockdown by shCBS decreased amounts of methylated PFKFB3 in both cell-types, while amounts of total PFKFB3 protein were unchanged (Fig. 7a). Maintenance of the total PFKFB3 amounts in shCBS-treated cells was not observed in those treated with the transient siCBS experiments using U937 cells (Figs 4d and 5d). In HCT116 cells in culture, CBS knockdown increased NADPH contents and NADPH/NADP$^+$ ratio (Fig. 7b), and GSH contents and GSH/GSSG ratio (Fig. 7c). Knocking down CBS or PRMT1 for decreasing methylated PFKFB3 significantly attenuated endogenous generation of reactive oxygen species (ROS) in the cells (Fig. 7d). The cells carrying control shRNA exhibited cell death in response to cisplatin in HCT116 cells expressing shCBS#2 stably. Data indicate the mean ± s.d. ($n$ = 4 independent experiments with unpaired Student’s t-test). *$P$ < 0.05 versus controls. (c) Alterations in GSH, GSSG and GSH/GSSG ratio in CBS knockdown HCT116 cells. Data indicate the mean ± s.d. ($n$ = 6 independent experiments; unpaired Student’s t-test). *$P$ < 0.05 versus controls. n.s., not significant. (d) Suppression of intracellular ROS level in the cells silencing of CBS or PRMT1 by shRNA. Data indicate the values of relative ROS levels (mean ± s.d., $n$ = 8); *$P$ < 0.05 versus controls.

**Figure 7 | CBS knockdown suppresses PFKFB3 methylation to cause chemoresistance.** (a) Evaluations of non-overlapping shRNAs against human CBS (shCBS#1 and #2) on PFKFB3 R131/134 methylation in HCT116 (left) and HEK293 (right). Lysates were immunoblotted with anti-dimethyl-PFKFB3 (R131/134), anti-PFKFB3 and anti-CBS antibodies. The expression level of GAPDH was used as a loading control. (b) Intracellular NADPH contents and NADPH/NADP$^+$ ratio in HCT116 cells expressing shCBS#2 stably. Data indicate the mean ± s.d. ($n$ = 4 independent experiments with unpaired Student’s t-test). *$P$ < 0.05 versus controls. (c) Alterations in GSH, GSSG and GSH/GSSG ratio in CBS knockdown HCT116 cells. Data indicate the mean ± s.d. ($n$ = 6 independent experiments; unpaired Student’s t-test). *$P$ < 0.05 versus controls. n.s., not significant. (d) Suppression of intracellular ROS level in the cells silencing of CBS or PRMT1 by shRNA. Data indicate the values of relative ROS levels (mean ± s.d., $n$ = 8); *$P$ < 0.05 versus controls. (e) Effects of CBS knockdown on the toxicity of cisplatin in HCT116 cells. HCT116 cells were treated with increasing concentrations of cisplatin for 16 h. Values of relative cell death are expressed as mean ± s.d. ($n$ = 4). *$P$ < 0.05 versus controls without cisplatin. †$P$ < 0.05 for shCBS#2 versus control without cisplatin. (f) Alterations in endogenous ROS levels with increasing concentrations of cisplatin in the cells carrying control or shCBS#2. Note that treatment of cisplatin upregulates intracellular ROS levels in a dose-dependent manner in the control cells, while those transfected with shCBS#2 displayed the partial suppression of cellular ROS levels. Data indicate mean ± s.d. for eight separating experiments. *$P$ < 0.05 versus controls (without cisplatin). †$P$ < 0.05 for the cells transfected with shCBS#2 versus controls in the presence of 50µM cisplatin. Statistical analysis used in (d-f) was ANOVA with Fischer’s multiple comparison test.
cisplatin in a dose-dependent manner, while those carrying shCBS exhibited significant suppression of the cisplatin sensitivity (Fig. 7e) that coincided with attenuating the dose-dependent augmentation of oxidative stress by cisplatin (Fig. 7f). The CBS knockdown in HeLa cells also increased NADPH contents and the NADPH/NADP⁺ ratio, which coincided with ROS suppression and decreased sensitivity to cisplatin (Supplementary Figs 9a–c). Such a chemoresistance against cisplatin was reproduced by PRMT1 knockdown (Supplementary Fig. 9f), being in good agreement with a notion that a decrease in methylatable PFKFB3 increases anti-oxidative capacity of cancer cells.

CBS knockdown increases GSH/GSSG to stimulate tumour growth. In our studies, CBS knockdown in HCT116 cells or overexpression of unmethytable R131/134K mutant in HEK293 cells significantly accelerated cell proliferation (Supplementary Fig. 10), suggesting a critical role of CBS in modulating PFKFB3 methylation to enhance GSH/GSSG against oxidative stress. We therefore inquired whether CBS knockdown in HCT116 cells results in enhancement of GSH/GSSG in vivo, since increasing GSH/GSSG might help cancer proliferation so far as our recent observation showed that cancer cells underwent increased oxygen consumption to expose themselves to oxidative stress during G2/M phase28.

Taking advantage of quantitative imaging mass spectrometry (Q-IMS) to determine regional contents of metabolites in tumours in vivo as well as clinical relevance of transportal metabolic metastasis of colon cancer in the liver, we examined GSH and GSSG using an experimental model of xenograft transplantation of HCT116 cells in superimmunodeficient NOG mice28–30. HCT116 cells stably transfected with shCBS against two different target sequences (shCBS#1 and #2) or with non-targeted control shRNA were injected into spleens of NOG mice to induce liver metastasis. At 2 weeks after the cell transplantation in spleen, the size of tumours derived from CBS-knockdown cells was significantly greater than the non-target control shRNA-expressing tumour (Fig. 8a). The relative percentages of tumour occupancy of shCBS-carrying HCT116 cells in the host liver were significantly greater than the non-target control. The greatest effects of growth stimulation were seen in the shCBS#2-treated cells (Fig. 8b). These results are consistent with a notion that enhancement of NADPH/GSH system by CBS knockdown benefits tumour growth in vivo. So far as judged by CE-MS analyses using snap-frozen tumour-bearing liver samples, CBS knockdown significantly decreased GSSG contents, and resultanty increased the GSH/GSSG ratio in tumour-bearing liver tissues as a whole. The tissue contents of total glutathione in vivo were unchanged by shCBS#2 treatment (Fig. 8c).

To further examine local contents of these metabolites in individual tumours, Q-IMS based on matrix-assisted laser desorption/ionization (MALDI) was carried out (Fig. 9a). As judged by imaging UDP-N-acetyl-hexosamine (UDP-HexNAc) as a tumour marker30, tumours derived from CBS-knocked down HCT116 cells developed the size of metastases, being consistent with the results in Fig. 8a,b. The development of tumour growth in shCBS-transfected HCT116 xenografts coincided with a decrease in regional GSSG contents ([GSSG]app in Fig. 9b) and an increase in the ratio of [GSH]app/[GSSG]app in metastases with statistical significance. These results suggested that the increase in GSH/GSSG in HCT116-derived tumours results not only from the increasing size of metastases but also from metabolic alterations in local cancer cells forming individual tumours in vivo.

Recent observations have shown that CBS might participate in the regulation of cancer proliferation and chemoresistance
through mechanisms involving H₂S/HS⁻: some studies suggest stimulatory effects while others suggest inhibitory effects on proliferation³¹–³³, indicating conflicting results of roles of H₂S/HS⁻. Although direct visualization of H₂S/HS⁻ was technically difficult in vivo, we examined whether CBS knockdown in HCT116 cells alters glutathione S-sulphonate (GSSO³⁻), a terminal oxidized product yielded by H₂S/HS⁻ from GSH and GSSG under atmospheric MALDI condition, respectively (Supplementary Fig. 11). This is because of the fact that thiol-containing compounds are automatically oxidized by ionization under atmospheric conditions in vitro³⁴; GSH and glutathione persulphide (GSS⁻), which is generated through the reaction of

Figure 9 | Increased GSH/GSSG and decreased glutathione S-sulphonate in HCT116-derived tumours knocking down CBS. (a) Representative pictures of quantitative imaging mass spectrometry (Q-IMS) to determine regional contents of reduced (GSH) and oxidized glutathione (GSSG) in tumour-bearing liver. Data showed the apparent contents of GSH at m/z 306.08, GSSG at m/z 611.14 and [GSH]ₐp∥[GSSG]ₐp ratio. UDP-HexNAc at m/z 606.07 was also visualized in the same tissue section to identify localization of HCT116-derived tumours. UDP-HexNAc was shown as a marker metabolite enriched in hepatic micrometastasis of HCT116 (ref. 30). Pseudocolor bars indicate apparent contents of metabolites (µmol per gram of tissue). The ratio image of GSH/GSSG was also generated. Scale bar, 500 µm. (b) Quantitative determination of apparent contents of GSH [GSHₐp], GSSG [GSSGₐp], GSH/GSSG [GSHₐp]/[GSSGₐp] and total glutathione contents in HCT116-derived tumours. Data indicate mean ± s.d. of five separate experiments. *P < 0.05 versus controls (unpaired Student’s t-test). (c) Representative pictures of GSO³⁻ at m/z 354.06, glutathione S-sulphonate (GSSO³⁻) at m/z 386.03, and the ratio image of GSSO³⁻ /GSO³⁻ in metastatic tumours derived from HCT116 cells treated with non-targeting control shRNA and those treated with shCBS#2. The microscopic field is identical to that shown in a. Right color bar and associated numbers indicate a.u. or ratio with red and blue representing high and low amounts, respectively. Scale bar, 500 µm.
H$_2$S/HS$^-$ with GSH, are oxidized and detected as glutathione sulphone (GSO$_3^-$) at m/z 354.06, and GSSO$_3^-$ at m/z 386.03 as stable endproducts, respectively. Namely, atmospheric MALDI detection of GSSO$_3^-$ might serve as an indirect index of endogenous H$_2$S/HS$^-$ generation.

According to the previous method showing that the mixture of GSSG with NaHS generates GSSH$^{55}$, we prepared the standard persulfide solution and exposed it to MALDI procedure to analyse the profile by tandem MS. Experiments in vitro showed generation of GSS$^-$ and GSSO$_3^-$ from GSH in the presence of NaHS (Supplementary Fig. 11a,b). These results indicate that neither GSS$^-$ nor GSSO$_3^-$ is generated from GSH without NaHS. GSSO$_3^-$ was also generated from GSG in the presence of NaHS (Supplementary Fig. 11c). Tandem MS profiles of the peak at m/z 386.03 (GSSO$_3^-$) and 354.06 (GSO$_3^-$) detected in the HCT116 xenograft-derived tumour in vivo (Supplementary Fig. 11e,g) were consistent with those detected in the NaH$_2$-derived persulfide solution containing GSSO$_3^-$ or GSO$_3^-$, respectively (Supplementary Fig. 11d–g). These results suggested that GSSO$_3^-$ as the GSS$^-$-derived oxidized product is detectable in the tumour-bearing liver in vivo under MALDI-MS analyses.

Based on these data, we compared differences in GSO$_3^-$ and GSSO$_3^-$ between cancer xenografts treated with non-target shRNA and shCBS#2. As seen in Fig. 9c that indicated the same microscopic field shown in Fig. 9a, HCT116-derived metastatic tumours exhibited strong MS signals of GSO$_3^-$ and GSSO$_3^-$ compared with the parenchymal region in the same tissue section. HCT116-derived tumours indicated greater GSO$_3^-$ signals with smaller GSSO$_3^-$ signals than those treated with non-target control shRNA. The ratio image of GSSO$_3^-$/GSO$_3^-$ suggested that endogenous GSS$^-$ generation appears smaller in tumours derived from the shCBS-transfected HCT116 cells than in those derived from the control HCT116 cells (Fig. 9c). These results suggested that endogenous H$_2$S/HS$^-$ generation is smaller in the shCBS-treated xenografts in vivo, implicating a role of CBS in the cancer cells for endogenous H$_2$S/HS$^-$ generation in situ.

Discussion

We have shown a novel molecular mechanism by which CO acts as a regulator for the directional biotransformation of glucose through the arginine methylation of PFKFB3. Metabolome analyses revealed that effects of the HO-1 induction in U937 cells on a shift of glucose biotransformation towards PPP flux were approximately threefold greater than the basal flux, while those on suppression of glycolysis were ~50% of the basal control (Fig. 1). Mechanisms appear to involve CO-dependent inhibition of CBS and subsequent alterations of remethylation metabolism that resultantly suppress PFKFB3 methylation. Furthermore, the results provide evidence for the first example indicating a crucial role of site-specific asymmetric dimethylarginine modification of the carbohydrate-metabolizing enzyme in modulating directional biotransformation of glucose towards PPP in the cells.

PFKFBs are bifunctional enzymes that generate and degrade F-2,6-BP, a potent allosteric effector of PFK-1 (ref. 36). Previous studies showed that the expression levels of two inducible isozymes of PFKFBs play important roles in rapid proliferation of cancer cells$^{57-59}$. One is a PFKFB variant, TIGAR (Tp53-induced glycolysis and apoptosis regulator); because of its absence of kinase activity, this variant has been shown to protect cancer cells against oxidative stress through PPP-dependent NADPH generation in glioma and multiple myeloma cells$^{40-42}$. Another is PFKFB3, the isozyme lacking phosphatase activities, which is upregulated by hypoxia and AMPK signalling$^{43,44}$. PFKFB3 possesses higher kinase activity for production of F-2,6-BP, expressing predominantly in embryonic cells and cancer cells$^{36,43}$.

Considering the structural proximity of the R131/R134 region of PFKFB3 to other functional domains of the enzyme (Supplementary Fig. 6), the current results provided evidence for critical roles of the R131/134-specific methylation for regulating sensitivity to K142-specific polyubiquitination and enzymatic ability to generate F-2,6-BP. First, recent studies have revealed that polyubiquitination of PFKFB3 is a critical step leading to degradation through the E3 ubiquitin ligase APC/C-cdh1 (ref. 26), although stabilizing mechanisms for PFKFB3 remain largely unknown. Considering that the R131/134 region is spatially close to K142, the target residue for polyubiquitination (Fig. 3d and Supplementary Fig. 6), it is not unreasonable to suggest that methylation of these arginine residues contributes to constitutive PFKFB3 stabilization in cancer cells. Second, previous studies showed that R133, another arginine residue proximal to methylated arginine residues (R131/134) on the same helix, is able to interact with fructose-6-phosphate (F-6-P), the substrate of PFKFB3 with the covalent bond$^{45}$. The current observation that R131/134K mutant-transfected cells displayed lower PFKFB3 activity than the WT-transfected cells (Fig. 5) is in good agreement with a hypothesis that R131/134 methylation facilitates F-6-P binding to the enzyme. Of interest is that the cells overexpressing the R131/134L mutant displayed more potent suppression of glycolytic conversion than those overexpressing the R131/134K mutant; these results suggest that interference with PFKFB3 methylation by the double R/L-mutant results in downregulation of glycolysis (Fig. 6), presumably because of its potent inhibitory action on the PFKFB3 activity. A potential steric effect of the lysine residue of the mutant PFKFB3 on the enzyme activity should further be examined. Collectively, these observations are consistent with a notion that asymmetric dimethylarginine modification of R131/134 does not only interfere with the recruitment of ubiquitin ligase for the enzyme degradation but also maintain glycolysis in cancer cells. There are several therapeutic strategies targeting PFKFB3 for cancer treatment$^{46,47}$. The current results shed light on a novel molecular basis for controlling activities and degradation of PFKFB3 through modulation of methylation that might benefit for the refinement of anti-cancer therapeutics.

In microenvironments in vivo, cancer cells have been thought to be exposed to incomplete oxygenation that paradoxically causes oxidative stress through multiple mechanisms involving suppressed mitochondrial electron transfer and increased NADH (refs 48,49). Accumulation of inflammatory cells surrounding tumours and exposure to therapeutic interventions such as radiation and anti-cancer reagents serve as other resources of oxidative stress in vivo$^{27,50}$. Under proliferative conditions, cancer cells in G2/M phase exhibit greater extents of O$_2$ consumption, mitochondrial respiration and ROS generation than those in G1 phase, suggesting that the cells are exposed to oxidative stress periodically during the proliferative cycle$^{28}$. To protect against several resources of oxidative stress, cancer cells turned out to accumulate relatively high concentrations of GSH versus GSSG in vivo and in vivo$^{30,51,52}$. Considering metabolic pathways in non-malignant cells under physiological conditions, CO-dependent CBS inhibition might downregulate trans-sulphuration pathway to suppress glutathione synthesis. As seen in experiments in U937 cells, CO application or HO-1 induction by haemin actually decreased total amounts of glutathione (Fig. 5c). However, such an effect was surpassed by the potency of CO to shift towards PPP and to increase NADPH and the GSH/GSSG ratio. In HCT116 cells, which highly express CD44 variant and possess the ability to uptake cystine from extracellular...
space through glutamate–cystine transporter (xCT)\textsuperscript{33}, the cultured cells (Fig. 7) as well as xenograft tumours \textit{in vivo} (Figs 8 and 9) appear to compensate glutathione synthesis, even under CBS knockdown conditions. Actually, HCT116 cells undergoing CBS knockdown increased GSH/GSSG without decreasing total glutathione contents both \textit{in vitro} and \textit{in vivo} (Figs 7–9), supporting the aforementioned notion.

In summary, cancer cells appear to utilize two discernible forms of PFKFB3, an asymmetrically di-methylated form that increases F-2,6-BP for PFK-1 activation and an unmethylated form that causes a shift of glucose biotransformation directionally towards PPP (Fig. 10). Under exposure to anti-cancer reagents, radiation therapy, oxidative stress or prolonged hypoxia (Fig. 10 and Supplementary Fig. 12), cancer cells upregulate HO-1/CO system. Under these circumstances, the cancer cells utilize CBS to suppress PFKFB3 methylation to ensure synthesis of reducing equivalents such as NADPH and GSH for survival at an expense of glycolytic energy management. The CO/CBS-assisted signalling system does not only serve as a switch for decreasing methylated PFKFB3 but also accounts for a mechanism for H\textsubscript{2}S/HS\textsuperscript{-} mediated bioregulation\textsuperscript{34}. So far as judged by a decrease in the GSSO\textsubscript{3}/GSO\textsubscript{3} ratio in CBS knockdown tumours \textit{in vivo}, endogenous H\textsubscript{2}S\textsuperscript{-} suppression is likely to coincide with cancer proliferation. However, by developing a reliable quantitative method for short-lived H\textsubscript{2}S\textsuperscript{-} and persulphide metabolites to investigate biological actions of these species\textsuperscript{34}, roles of tumour-derived H\textsubscript{2}S/HS\textsuperscript{-} for cancer survival should carefully be examined \textit{in vivo}. Mechanisms for regulating tenuous balance between methylated and unmethylated forms of PFKFB3 for cancer survival deserve further studies, provided that therapeutic interventions targeting the methylation switch lead to the suppression of cancer proliferation \textit{in vivo}.

**Methods**

**Antibodies and chemicals.** All experiments requiring gene recombination and treated with 25 μM haemin for 6h. The cells (5 × 10\textsuperscript{6}) treated with AdOx for 16h, or 5 μM CO for 30 min or 25 μM haemin for 6 h were washed with PBS twice, and then frozen in liquid nitrogen.

**Cell culture.** U937 cells were obtained from the American Type Culture Collection (ATCC), and cultured in RPMI1640 (Invitrogen) supplemented with 10% FBS (SIGMA-Aldrich), penicillin–streptomycin (Invitrogen). Stable transfectants of WT HO-1 or mutant HO-1 (H25A) in U937 cells were established as described previously\textsuperscript{25}. Adherent cell lines, Hek293, HeLa and HCT116 cells were cultured with DMEM (Invitrogen) containing with 10% FBS, penicillin streptomycin. When previously\textsuperscript{25}, Adherent cell lines, HEK293, HeLa and HCT116 cells were cultured with DMEM (Invitrogen) containing with 10% FBS, penicillin streptomycin. When necessary, PBS is saturated with 100% CO gas (Nippon MEGACARE, Tokyo, Japan) for 20 min on ice. CO-saturated PBS (around 1 mM) was diluted with RPMI1640 to 5 μM (final concentration). For haemin treatment, the cells were cultured with 25 μM haemin for 6h. The cells (5 × 10\textsuperscript{6}) treated with AdOx for 16h, or 5 μM CO for 30 min or 25 μM haemin for 6 h were washed with PBS twice, and then frozen in liquid nitrogen.

**Determination of F-2,6-BP content.** F-2,6-BP contents were measured using a coupled spectrophotometric assay by monitoring the rate of F-6-P formation from F-6-P and ATP\textsuperscript{43}. Approximately 1 × 10\textsuperscript{6} cells were sonicated in 0.2 mL of lysate buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM dibuthylthiotetrol (DTT), 1 mM EDTA, 1 mM EGTA, 0.02% phenylmethylsulfonyl fluoride and centrifuged at 20,000g for 30 min, then the supernatants were used for the assay. Relative FPK-1 activities were expressed as the ratio of FPK-1 activity obtained from control lysate derived from non-treated cells as 100% activity. F-2,6-BP was determined in supernatants by its ability to activate pyrophosphate-dependent FPK-1 (F2288, SIGMA-Aldrich) from potato tubers according to the previous method\textsuperscript{55}.
Dihydroxyacetone phosphate 15N-labelled cystathionine, The CBS-silencing U937 cells (1 × 10⁶ cells) were obtained from ATCC, and 5% CO₂. After centrifugation at 20,000g for 4°C for 15 min, the supernatants were filtered through a 5-kDa cut-off filter (Millipore). Metabolites including those that had at least one 1³C atom in the molecule were collectively indicated as 1³C-labelled metabolites. For fluoximes analysis, Glycolysis was calculated as the sum of intermediates of glycolysis (glucose-6-phosphate + fructose-6-phosphate + fructose-1,6-bisphosphate + glyceraldehyde-3-phosphate + dihydroxyacetone phosphate + phosphoglycerate + 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate), while 2ZPPP was the sum values of the metabolites in PPP (glyceraldehyde-3-phosphate + fructose-6-phosphate + 6-phosphogluconate + ribulose-5-phosphate + ribose-5-phosphate + sedoheptulose-7-phosphate + erythrose-4-phosphate + glyceraldehyde-3-phosphate). The energy charge (EC) was calculated using the values for ATP, ADP, and AMP, as the following.

\[
EC = \frac{(\text{ATP} + 1/2 \text{ADP})}{(\text{ATP} + \text{ADP} + \text{AMP})}
\]

The determinations of sulfur-containing amino acids were carried out with API3000 LC-MS² systems (Applied Biosystems) equipped with a turbo ion spray source as described previously. In brief, U937 cells (1 × 10⁶ cells) were treated with 100 μM Ruthenium (III) chloride (RuC₃) or CORM-2-treated U937 were incubated with RPMI1640 medium containing 75 mg ¹³C-labelled methane (6094/¹³C) (Sigma-Aldrich; WOTTOC) for 5 min. The metabolites were separated with methanol/chloroform method. After centrifugation at 20,000 g for 4°C for 15 min, the supernatants were filtered through a 5-kDa cut-off filter (Millipore); to examine the metabolites in the filtrate it was analysed by using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 1100 series MSD mass spectrometer.

Cellular NADPH contents were determined by colorimetric determination method using NADP/NADPH determination kit according to the manufacturer’s manual (Biovision, Milpitas, CA). The U937, HEK293, HeLa and HCT116 cells were obtained from ATCC, and 5 × 10⁶ cells for each cell lines were used for the NADPH determination. Values were normalized by the amount of total protein. Intracellular reduced and oxidized glutathione contents were determined with CoulArray multi-electrode array detector system (Model 5600A; ESA Inc, Chelmsford, MA). Briefly, 1 × 10⁶ cells were homogenized in the extraction buffer (10% trichloroacetate, 100 mM NaCl) with PBS twice and frozen quickly by liquid nitrogen. For the determination of ¹³C-labelled cystathionine, The CBS-silencing U937 cells (1 × 10⁶ cells) were treated with 100 μM Ruthenium (III) chloride (RuC₃) or CORM-2-treated U937 were incubated with RPMI1640 medium containing 75 mg ¹³C-labelled methane (6094/¹³C) (Sigma-Aldrich; WOTTOC) for 5 min. The metabolites were separated with methanol/chloroform method. After centrifugation at 20,000 g for 4°C for 15 min, the supernatants were filtered through a 5-kDa cut-off filter (Millipore); to examine the metabolites in the filtrate it was analysed by using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 1100 series MSD mass spectrometer.

In vitro methylation assay. For in vitro methylation assay, recombinant GST-PFKFB3 (WT or each mutants, 2 μg each) incubated with GST-PRMT1, 3–8 μg each in PBS, containing 0.5 mM DTT, 1.65 μCi S-adenosyl-L-[methyl-³H] methionine (MP biomedical, Santa Ana, CA), and glutathione sepharose (GE healthcare) at 37°C for 2 h. The eluted products were separated by SDS-PAGE and subsequently analysed by Coomassie Brilliant Blue staining. The radioactive signals (exposure for 24 h) were analysed using a FUJIX Bio-Imaging Analysier BAS2500 (Fujifilm photo Film, Tokyo, Japan). The uncorrected gel images used in this assay are shown in Supplementary Fig. 13.

Detection of PFKFB3 polyubiquitination. Endogenous ubiquitinated proteins in U937 cells were enriched by ubiquitin Enrichment kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. In brief, whole-cell extracts (1 mg) were incubated with ubiquitin affinity resin for 4 h at 4°C. After washing with wash buffer, the resin was washed with elution buffer with 1 μl sample buffer. The eluted ubiquitinated proteins were separated by SDS-PAGE, and then immunoblotting was performed with anti-PFKFB3 polyclonal antibody. To detect PFKFB3 polyubiquitination, HEK293 cells transfected with FLAG-tagged WT PFKFB3 (WT) or K142A mutant PFKFB3 were used. To examine the effects of HO-1/CO-mediated arginine methylation of PFKFB3 on polyubiquitination levels of the enzyme, the cells were treated with 25 μM haemin for 6 h or co-transfected with human PRMT1. The cell lysates (250 μg) were immunoprecipitated with anti-FLAG antibody for 4 h at 4°C. The immunoprecipitates were analysed by SDS-PAGE, and then immunoblotting with anti-ubiquitin antibody (Thermo Fisher Scientific; 1:1000 dilution), the whole-cell extracts were analysed with anti-FLAG, anti-HO-1 and anti-GAPDH antibodies as input.

Cell proliferation assay. The cells were seeded in six-well plates (5 × 10⁴ cells per well), and cell numbers were counted every 24 h over a 2-5-day period. Accurate cell number was measured every 24 h using a Countess automated cell counter analyser (Invitrogen).

Cell viability assay and human-derived cancer xenografts. Vulnerability of U937, HCT116 and HeLa cells to H₂O₂ or cisplatin was examined by APO- Cure-Alert TUNEL assay (Biocolor Ltd, Co Antrim, UK) or by Cell-based Assay Kit VEGF-C kit (Cayman Chemical). Animal experiments were carried out in accordance with the guidelines of Experimental Animal Committee of Keio University School of Medicine. Human colon cancer (HCT116) cells were transfected stably with non-targeting shRNA or shRNA-targeting CBS were injected into the spleen of immunodeficient male NOG (NOD/Shi-scid/IL2rg−/−) aged at 12–13 weeks old. Six weeks after transplantation, liver lobes of the mice fasted for 17 h were excised under sevoflurane anesthesia and
Quantitative imaging mass spectrometry. Using snap-frozen tissues collected from the non-targeting control- or shCBS-transfected HCT116 cell-derived tumour-bearing livers, Q-IMS was carried out according to our previous methods. For Q-IMS, 5 mm thick cryosections were stained with hematoxylin and eosin (H&E), serving as resources to analyse the distributions of compounds in liver tissues. To avoid saturation, liver sections that were thaw-mounted on water-coated glass slides (Braun Daltikon GmbH, Bremen, Germany) were imaged in a MALDI-TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan), we acquired the data in the negative ion mode with the scanning mass range from m/z 304.0 to 670 according to our previous methods. After selecting the region of interests by light observations, a series of repeated laser irradiation at 100 times per second was performed at 250 × 250 spots, giving 62,500 data points in total for each scan. Averaged spectra from each region of interest were generated and statistically analysed by house-made software SIMtools (Shimadzu Imaging Mass spectrometry toolbox for MALDI). By CE-MS analysis, we measured the concentrations of reduced and oxidized glutathione in a portion of tumour-bearing liver tissues that was adjacent to the corresponding IMS section according to our previous method. Then, the apparent contents of metabolites in the tumour region were calibrated as described previously. In brief, to calculate the apparent contents in tumour regions in tumour-bearing livers (OTumour, MS), the following equations were used after calculation of tumour occupancy rate (OTumour) in each liver with H&E-stained sections:

\[
I_{average} = \frac{I_{Liver \times O_{Liver}}}{100} + \frac{I_{Tumor \times O_{Tumor}}}{100}
\]

\[
C_{Tumor, MS} = \frac{C_{CE-MS} \times I_{Tumor}}{I_{average}}
\]

\[
C_i = \frac{C_{CE-MS}}{I_i / I_{average}}
\]

Visualization of glutathione S-sulphonate (GSSO₃⁻) in tumours. CBS is a hydrogen sulphide-generating enzyme that might help generation of persulphide compounds. We used MALDI-MS data acquired from CE-MS data from five mice in each group. To construct an ion-content map, MALDI-MS data was converted to analyse format, a common multidimensional biomedical imaging format file that contains MALDI signal intensities at each pixel i (x, y, z).

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Authors contributions

T.Y. designed, performed and analysed the experiments. N.T. and K.I. contributed to experimental design and performed the experiments. M.O., Y.N., T.M., T.N., A.Ku. and T.H. performed the experiments using CE-MS and Q-IMS and the related data analyses. A.Ka. and K.S. provided experimental support and edited the manuscript. T.Y. and M.S. designed the projects and wrote the manuscript.

Supplementary information

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