Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia

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Besides the conventional carbon sources, acetyl-CoA has recently been shown to be generated from acetate in various types of cancers, where it promotes lipid synthesis and tumour growth. The underlying mechanism, however, remains largely unknown. We find that acetate induces a hyperacetylated state of histone H3 in hypoxic cells. Acetate predominately activates lipogenic genes ACACA and FASN expression by increasing H3K9, H3K27 and H3K56 acetylation levels at their promoter regions, thus enhancing de novo lipid synthesis, which combines with its function as the metabolic precursor for fatty acid synthesis. Acetyl-CoA synthetases (ACSS1, ACSS2) are involved in this acetate-mediated epigenetic regulation. More importantly, human hepatocellular carcinoma with high ACSS1/2 expression exhibit increased histone H3 acetylation and FASN expression. Taken together, this study demonstrates that acetate, in addition to its ability to induce fatty acid synthesis as an immediate metabolic precursor, also functions as an epigenetic metabolite to promote cancer cell survival under hypoxic stress.

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Acetyl-CoA, as a central metabolic intermediate, is widely used in macromolecule biosynthesis and energy production to support cell growth and proliferation. As a donor of acetyl group, acetyl-CoA is also dynamically associated with acetylation modification to modulate protein functions. Therefore, maintenance of cellular acetyl-CoA pool is essential for the regulation of various cellular processes.

In human, acetyl-CoA is mainly produced from oxidation of glucose and other conventional carbon sources, such as glutamine and fatty acids. However, in human brain cancers, glucose contributes <50% carbons to cellular acetyl-CoA pool, suggesting the existence of a substantive supply for acetyl-CoA. Subsequent studies reveal that cancer cells avidly capture acetate as their alternative carbon source to support cell survival and proliferation under stressed conditions, in particular hypoxia. Moreover, various human cancers show enhanced acetate uptake in [11C]-acetate PET studies. These findings suggest that cancer cells utilize acetate as an alternative carbon source to glucose to maintain cellular acetyl-CoA pool under stressed conditions.

Acetate has long been identified as a major carbon source in bacteria and yeasts. Yeast acetyl-CoA synthetases (Acs1p and Acs2p) fuel cell growth by converting acetate to acetyl-CoA. Very recently, acetate is also found to be an alternative carbon source besides glucose, glutamine and fatty acids in human cancer, attracting intensive investigations. Generally, mammalian acetyl-CoA synthesis from acetate is carried out by ACSS2 to support lipid synthesis in the cytosol, and by ACSS1 to fuel ATP production in mitochondria. Acetate is mainly acquired from diet, but can also be generated in ethanol metabolism or deacetylation processes. The function of acetate has long been overlooked due to its relative low physiological concentration (0.2–0.3 mM) in blood. Recent studies reveal that cancer cells show increased acetate uptake under hypoxia even in the presence of low acetate concentration to support tumour growth. However, how cancer cells utilize acetate under hypoxia in such an efficient manner remains unclear.

Histone acetylation is intimately coordinated with cellular acetyl-CoA pool in response to metabolic state. As the downstream metabolite of carbon sources, acetyl-CoA represents a pivotal metabolic signal of nutrient availability. Yeast, histone is specifically acetylated at genes involved in lipogenesis, aminoacid biosynthesis and cell cycle progression upon entry into growth, in tune with intracellular acetyl-CoA level. ATP citrate lyase (ACL), the enzyme converting glucose-derived citrate into acetyl-CoA, regulates histone acetylation by sensing glucose availability. Yeast acetyl-CoA Carboxylase (Acc1p) consumes acetyl-CoA to synthesize lipids and regulates global histone acetylation through competing for the same nucleocytosolic acetyl-CoA pool. Thus, the acetyl-CoA flux dynamically regulates gene expression profile by modulating histone acetylation state.

These observations led us to hypothesize that acetate induces a metabolic adaptation through modulating histone acetylation in hypoxic cancer cells. Consistent with this idea, we found that acetate predominately activates the expression of lipogenic genes through upregulating histone acetylation at their promoter regions, which in turn promotes lipid synthesis under hypoxia. Beyond a carbon source for macromolecular biosynthesis, our findings highlight an epigenetic role for acetate in metabolic adaptation of cancer cells to hypoxic stress.

Results

Acetate restores histone acetylation under hypoxia. Cancer cells demand distinctive extracellular nutrients and reprogram the metabolic pathways to survive and proliferate when facing harsh situation, such as hypoxia. Hypoxia stress is a critical player in tumorigenesis and tumour development. By performing exometabolome analysis based on $^1$H-NMR spectra, we found that cancer cells absorbed around 20% acetate from the culture medium under normoxia while more than 80% acetate was consumed under hypoxia (Fig. 1a; Supplementary Fig. 1a,b), suggesting that cancer cells take up more acetate under hypoxia than nomoxia. Moreover, we carried out the quantification of acetate from five pairs of hepatocellular carcinoma (HCC) and adjacent samples by NMR. As shown in Supplementary Fig. 1c, acetate concentration range was from 0.56 to 2.67 μmol g$^{-1}$ in wet tissue (left) and acetate concentration in tissue would be roughly estimated around from 0.56 to 2.67 mM (right). In most cases acetate concentrations ranged around 0.5 mM and in two HCC samples acetate levels reached 2.5 mM (Supplementary Fig. 1c). To explore acetate effect on cancer cell, we treated HepG2 cells with acetate under hypoxia and found that acetate counteracted the declined histone acetylation under hypoxia compared with normoxia (Fig. 1b). Of particular interest, acetate induced significant increase of H3K9, H3K27 and H3K56 acetylation levels, but not H3K14, H3K18, H3K23 and H3K36 acetylation levels (Fig. 1b). This indicates that acetate rescues hypoxia-reduced histone acetylation with certain specificity. More importantly, by using metabolic-labelling technique based on liquid chromatography tandem mass spectrometry in multiple reaction monitoring mode (LC-MRM MS), we identified $^{13}$C$_2$-labelled acetylated H3K9 (Supplementary Fig. 1d), H3K27 (Supplementary Fig. 1e) and H3K56 (Supplementary Fig. 1f) peptides in HepG2 cells treated with $^{13}$C$_2$-acetate, demonstrating that acetate-derived acetyl-CoA was indeed incorporated into histones. In addition, compared with normoxia, histone acetylation is more susceptible to acetate supplementation under hypoxia, even under low concentration, in HepG2, A549 and DU 145 cells (Fig. 1c; Supplementary Fig. 1g,h).

To characterize the manner of epigenetic regulation by acetate, we treated cancer cells with acetate for different time points under hypoxia. We found that exogenous acetate supplementation rapidly increased H3K9, H3K27 and H3K56 acetylation levels in a time-dependent manner under hypoxia in different cancer cell lines (Fig. 1d; Supplementary Fig. 1i,j). Acetate supplementation dramatically increased H3K9, H3K27 and H3K56 acetylation levels within 2 h (Fig. 1d; Supplementary Fig. 1i,j). Furthermore, acetate was found to increase histone H3K9, H3K27 and H3K56 acetylation levels in a dose-dependent manner under hypoxia in HepG2 (Fig. 1e), A549 (Supplementary Fig. 1k), DU145 (Supplementary Fig. 1l), SKBr3 (Supplementary Fig. 1m) and HT29 cells (Supplementary Fig. 1n). Low concentration of acetate (1.25 mM) slightly increased histone acetylation levels, while higher concentrations of acetate (2.5–5 mM) significantly increased histone acetylation levels under hypoxia (Fig. 1e; Supplementary Fig. 1k, l, m, n). Collectively, these data imply that acetate is implicated in the regulation of histone acetylation under hypoxia.

Acetate epigenetically activates de novo lipogenesis. It is well known that histone acetylation is associated with transcription activity. To investigate the biological effect on cancer metabolism induced by the acetate-mediated histone acetylation, we detected the mRNA expression changes of metabolic genes. We designed quantitative PCR (qPCR) primers targeting 139 metabolic genes, covering a wide range of metabolic pathways (that is, glycolysis, TCA cycle, pentose phosphate pathway, glycogen metabolism, ketone body metabolism, aminoacid metabolism, fatty acid synthesis and β-
oxidation, cholesterol synthesis, sphingolipid metabolism, and metabolism-related transporters) (Supplementary Table 1) and conducted qPCR to screen potential metabolic pathway(s) affected by acetate-induced epigenetic regulation. Scatter plot of mRNA expression data of 139 genes in HepG2 cells was shown in Fig. 2a, by comparing cell culture under hypoxia with normoxia. The expression of VEGF and LDHA, which were included as positive controls for verifying hypoxic effect, increased by more than two-fold under hypoxia (Fig. 2a). We then tested the effect of acetate on gene expression under either normoxia (Fig. 2b) or hypoxia (Fig. 2c), respectively. Compared with the normoxia group, the upregulated genes by two-fold in hypoxia group and hypoxia with acetate group showed a lot overlap (Supplementary Fig. 2a). Besides, FASN and ACSS2 were the unique genes in hypoxia with acetate group, indicating that acetate played an important role in lipid synthesis under hypoxia (Supplementary Fig. 2a). In line with this, FASN and ACACA mRNA levels were activated by more than two-fold with acetate under hypoxia.
Figure 2 | Acetate predominately activates lipid synthesis pathway through epigenetic regulation under hypoxia. (a) Scatter plot of mRNA expression data of 139 metabolic genes in HepG2 cells, comparing cells treated with hypoxia (y-axis) to normoxia (x-axis). The mRNA expression value of triplicate experiments was shown on a log 2 scale. Grey lines indicated two-fold differences in the mRNA expression levels between two groups. Upregulated genes (＞2-fold change, P<0.05) were shown in magenta. Downregulated expressed genes (＞2-fold change, P<0.05) were shown in blue. Two-tailed unpaired Student’s t-test was used. (b) Scatter plot of mRNA expression data of 139 metabolic genes in HepG2 cells under normoxia, comparing cells treated with 2.5 mM acetate (y-axis) to acetate-free (x-axis). The mRNA expression value of triplicate experiments was shown on a log 2 scale. Grey lines indicated two-fold differences in the mRNA expression levels between two groups. Downregulated genes (＞2-fold change, P<0.05) were shown in blue. Two-tailed unpaired Student’s t-test was used. (c) Scatter plot of mRNA expression data of 139 metabolic genes in HepG2 cells under hypoxia, comparing cells treated with 2.5 mM acetate (y-axis) with acetate-free (x-axis). The mRNA expression value of triplicate experiments was shown on a log 2 scale. Grey lines indicated two-fold differences in the mRNA expression levels between two groups. Upregulated genes (＞2-fold change, P<0.05) were shown in magenta. Two-tailed unpaired Student’s t-test was used. (d) Fold-change analysis of the mRNA expression of 139 genes in HepG2 cells treated with or without 2.5 mM acetate under hypoxia. (e,f) FASN (e) and ACACA (f) mRNA levels in HepG2 cells treated with indicated concentrations of acetate for 12 h under normoxia or hypoxia were quantified by qPCR. The results were presented as mean ± s.d. of triplicate experiments. (g,h) ChIP-qPCR assays showing H3K9, H3K27 and H3K56 acetylation enrichment at FASN (g) and ACACA (h) promoter regions in HepG2 cells treated with indicated concentrations of acetate under normoxia or hypoxia for 4 h. Rabbit IgG was included as a negative control. Each histogram was presented as mean ± s.d. of triplicate experiments (*P<0.05; **P<0.01; NS, not significant; by two-tailed unpaired Student’s t-test).
while no activation effect was observed under normoxia (Fig. 2b,c). Both FASN and ACACA are in the top-10 list of upregulated metabolic genes induced by acetate under hypoxia, indicating that acetate predominantly affects de novo lipid synthesis compared with other metabolic pathways (Fig. 2c,d). Interestingly, ACSS1 and ACSS2 mRNA levels were decreased under hypoxia compared with normoxia (Fig. 2a), which was discrepant with the other studies reported that ACSS2 was upregulated under hypoxia. This may be due to the experimental conditions, such as different cell lines used and treating time points. Moreover, mRNA level of ACSS1 and ACSS2, rather than ACLY, were also upregulated on acetate treatment under hypoxia (Fig. 2c,d). The rescue of hypoxia-reduced ACSS1 and ACSS2 expression by acetate may indicate that ACSS1 and ACSS2 play an important role in cancer cells adapting to hypoxia.

We further investigated whether acetate-induced histone acetylation was associated with acetate-promoted gene transcription on lipogenesis. We treated cancer cells with gradient concentrations of acetate and found that FASN and ACACA mRNA expression were upregulated in a dose-dependent manner under hypoxia (Fig. 2c,d). Intriguingly, change in FASN and ACACA mRNA expression are more prone to low concentration of acetate under hypoxia, compared with normoxic control (Fig. 2e,f). To characterize whether the upregulation of lipogenic genes is caused by histone acetylation induced by acetate supplementation, we treated cancer cells with trichostatin A (TSA), a histone deacetylase inhibitor, as a positive control. We found that TSA and acetate increased both the global acetylation level of histone H3 and the mRNA expression of FASN and ACACA, although TSA and acetate had no synergic effect (Supplementary Fig. 2b). In addition, acetate treatment led to time-dependent enhancement of H3K9, H3K27 and H3K56 acetylation levels (Supplementary Fig. 2c). In line with the increased histone H3 acetylation, FASN and ACACA mRNA expressions were also elevated in a time-dependent manner (Supplementary Fig. 2c). Furthermore, we carried out ChIP–qPCR assays to define the underlying mechanism of acetate-induced FASN and ACACA expressions. We found that acetate markedly enhanced H3K56 acetylation level at the promoters of FASN and ACACA (Supplementary Fig. 2d,e), but not ACLY (Supplementary Fig. 2f). Furthermore, we found that histone acetylation (H3K9ac, H3K27ac and H3K56ac) at the promoters of FASN and ACACA responded to acetate treatment in a dose-dependent manner and histone acetylation was more prone to be induced by low concentration (≤2.5 mM) of acetate under hypoxia, compared with normoxia (Fig. 2g,h). These results demonstrate that acetate is capable of epigenetically regulating lipogenic genes at pathobiological concentration.

To rule out the possibility that promoter acetylation of FASN and ACACA reflects increased transcription, we alternatively performed ChIP–qPCR and tested promoter histone acetylation of LDHA and VEGF (Supplementary Fig. 2g, h, i, j). The acetylation levels (H3K9ac, H3K27ac and H3K56ac) at LDHA and VEGF promoters were increased under hypoxia, which was further upregulated by acetate supplementation (Supplementary Fig. 2g, h, i, j). Consistently, mRNA expression of LDHA and VEGF was also activated by acetate (Supplementary Fig. 2k,l). These observations suggest that acetate contributes to histone acetylation at the promoters of hypoxia-induced genes. Collectively, these data support our notion that acetate promotes lipid synthesis pathway through epigenetic regulation.

Acetate-induced lipogenesis does not reflect lipid demands. Cancer cells show increased demands for lipids, through scavenging extracellular lipids or de novo lipid synthesis. We tried to figure out whether acetate-induced expression of FASN or ACACA is affected by the extracellular lipids under hypoxia. Consistent with data in Fig. 2, FASN and ACACA mRNA expression was more prone to be induced by acetate under hypoxia, compared with normoxia (Fig. 3a,c) and acetate-activated promoter histone acetylation of FASN and ACACA more pronouncedly under hypoxia (Fig. 3b,d). Furthermore, lipid depletion in the serum did not alter acetate-induced expression of FASN and ACACA in HepG2 cells (Fig. 3e; Supplementary Fig. 3a). Similarly, FASN and ACACA promoter histone acetylation was not affected by lipid depletion as well (Fig. 3f; Supplementary Fig. 3b). Moreover, palmitate supplementation did not change the expression of FASN, VEGF and ACACA (Fig. 3g; Supplementary Fig. 3c,d). Consistently, histone acetylation at the promoter regions of FASN and ACACA did not respond to palmitate supplementation (Fig. 3h; Supplementary Fig. 3e). These results demonstrate that acetate increases histone acetylation at promoters of lipogenic genes and activates their mRNA expression, without reflecting lipid demand of hypoxic cells.

ACSS1 and ACSS2 are both involved in acetate-induced lipogenesis. In mammals, two main enzymes involve in acetyl-CoA production from acetate: the cytosolic ACSS2 and its mitochondrial parologue ACSS1. To dissect which enzyme is responsible for mediating the acetate-induced increase of histone acetylation, we knocked down ACSS1 or ACSS2 individually and assessed the corresponding histone acetylation levels. To our surprise, knockdown of either ACSS1 or ACSS2 was incapable of blocking acetate-induced increase in acetylation levels at H3K9, H3K27 and H3K56 sites (Supplementary Fig. 4a,b). Though discrepant with the other studies reporting that ACSS2, but not ACSS1, appeared to be the key enzyme involved in metabolizing acetate for lipid synthesis, our findings were consistent with those by Yun et al. and Bjornson et al., which reported that ACSS1 was also vital for acetate-dependent lipid synthesis. These results indicate that ACSS1 and ACSS2 are functionally redundant. We then established ACSS1/2 double knockdown stable cell pools. Concurrent knockdown of ACSS1/2 by using two different sets of shRNAs dramatically inhibited acetate-induced increase in acetylation levels of H3K9, H3K27 and H3K56 under hypoxia (Fig. 4a; Supplementary Fig. 4c). Moreover, we conducted quantification based on LC-MRM MS to see whether ACSS1/2 double knockdown could reduce the abundance of histone acetylation peptides derived from acetate by using [U-13C2]-acetate tracer. Acetate-derived acetyl-CoA was found actively incorporated into H3K9, H3K14 and H3K27 acetylation sites, compared with H3K18, H3K23 and H3K56 acetylation sites (Fig. 4b; Supplementary Fig. 4d). These results demonstrate that acetate could function as an acetyl donor to induce histone acetylation. ACSS1/2 double knockdown induced a more significant decrease in [13C2]-labelled acetylated H3K9, H3K27 and H3K56 peptides derived from [U-13C2]-acetate (Fig. 4b; Supplementary Fig. 4d). As for the discrepancy of H3K56 acetylation between western blot data and MS quantification, it might be due to the fact that concentration of [13C2]-labelled acetylated H3K56 peptide was too low to meet the limit of quantification. In addition, we designed experiments to test whether ACSS1/2 double knockdown could block acetate-induced histone acetylation at FASN and ACACA promoter regions. ACSS1/2 knockdown efficiency was verified by qPCR (Supplementary Fig. 4e). We found that acetate-increased H3K9, H3K27 and H3K56 acetylation levels at FASN (Fig. 4c) and ACACA (Fig. 4d) promoter regions were blocked by ACSS1/2 double knockdown.
Figure 3 | Acetate epigenetically activates lipogenic genes without reflecting cellular lipid demands. (a) FASN expression in HepG2 cells treated with indicated concentrations of acetate for 12 h under normoxia or hypoxia were quantified by qPCR. The results were presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); NS, not significant; by Student’s t-test). (b) ChIP-qPCR assays showing histone acetylation enrichment at FASN promoter region in HepG2 cells treated with indicated concentrations of acetate under normoxia or hypoxia for 4 h. Rabbit IgG was included as a negative control. Each histogram was presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); NS, not significant; by Student’s t-test). (c) ACACA expression in HepG2 cells treated as in panel (a) were quantified by qPCR. The results were presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); NS, not significant; by Student’s t-test). (d) ChIP-qPCR assays showing histone acetylation enrichment at ACACA promoter region in HepG2 cells treated as in b. Each histogram was presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); NS, not significant; by Student’s t-test). (e) Quantification of FASN expression in HepG2 cells treated with or without 2.5 mM acetate in media plus 10% FBS or 10% LPDS for 12 h under hypoxia by qPCR. The results were presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); by Student’s t-test). (f) ChIP-qPCR assays showing histone acetylation levels at FASN promoter region in HepG2 cells treated as in e for 4 h under hypoxia. The results were presented as mean ± s.d. of triplicate experiments (\(^*P<0.01\); by Student’s t-test). (g) Quantification of FASN expression in HepG2 cells treated with 10% LPDS plus the indicated concentration of palmitate (PA) for 12 h under normoxia or hypoxia by qPCR. The results were presented as mean ± s.d. of triplicate experiments (NS, not significant; by Student’s t-test).
Figure 4 | Both ACSS1 and ACSS2 are involved in acetate-induced epigenetic regulation of de novo lipogenesis. (a) Stably concurrent knockdown of ACSS1 and ACSS2 totally blocks acetate-induced increase of histone acetylation levels. Histone acetylation levels in shscr or shACSS1/2 HepG2 cells treated with or without 2.5 mM acetate under normoxia or hypoxia were analysed by western blot. (b) 13C2-labelled H3K9ac and H3K27ac levels are decreased by ACSS1/2 knockdown. Quantification of 13C2-labelled histone H3 acetylation levels in shscr or shACSS1/2 HepG2 stable cell line treated with [U-13C2]-acetate for 4 h under hypoxia was analysed by LC-MRM MS. The percentage indicates the ratio of H3K[13C2-ac]/total H3Kac in each site. The results were presented as mean ± s.d. of triplicate experiments (*P < 0.05; ***P < 0.001; by Student’s t-test). (c, d) ACSS1/2 knockdown totally blocks acetate-induced histone acetylation association with FASN and ACACA promoter. ChIP-qPCR assays were performed to determine H3K9, H3K27 and H3K56 acetylation enrichment at FASN (c) and ACACA (d) promoter region in shscr or shACSS1/2 HepG2 stable cell lines treated with or without acetate under hypoxia for 4 h. IgG was included as a negative control. The results were presented as mean ± s.d. of triplicate experiments (**P < 0.01; ***P < 0.001; NS, not significant; by Student’s t-test). (e) ChIP-qPCR assays were performed to determine H3K9, H3K27 and H3K56 acetylation enrichment at ACly promoter region in shscr or shACSS1/2 HepG2 cells treated as in d. IgG was included as a negative control. The results were presented as mean ± s.d. of triplicate experiments (NS, not significant; by Student’s t-test).
under hypoxia. ACSS1/2 knockdown had no effect on histone acetylation levels at ACLY promoter region (Fig. 4e). Consistently, ACSS1/2 knockdown blocked acetate-induced FASN expression (Supplementary Fig. 4f). Collectively, our data demonstrate that acetate contributes to histone acetylation in an ACSS1/2-dependent manner under hypoxia.

Previous study linked ACLY-dependent glucose metabolism to histone acetylation. To dissect contributions of different carbon sources to histone acetylation, we performed qPCR and ChIP–qPCR assays in ACSS1/2-knockdown and/or ACLY-knockdown cells. The knockdown efficiency was verified (Supplementary Fig. 4g). Depletion of either ACSS1/2 or ACLY reduced gene expression of LDHA and VEGF, which were further decreased by knockdown of both ACSS1/2 and ACLY (Supplementary Fig. 4h,i). Consistently, either ACSS1/2 or ACLY knockdown reduced histone acetylation at the promoter regions of LDHA and VEGF (Supplementary Fig. 4j,k,l). These results demonstrate that both ACSS1/2 and ACLY contribute to histone acetylation under hypoxia.

Acetate-induced lipogenesis promotes cell survival. To test whether acetate would affect lipid pool in cancer cells, we cultured HepG2 stable cells in media with isotopically labelled [U-13C2]-acetate for 24 h under hypoxia and measured the isotope enrichments of fatty acids pool by using gas chromatography coupled with mass spectrometry (GC-MS). ACSS1/2 double knockdown indeed decreased the fractional contribution of acetate to palmitate and stearate synthesis (Fig. 5a). These results are consistent with the model that acetyl-CoA derived from acetate stimulates de novo lipid synthesis. Furthermore, we knocked down FASN expression to the unstimulated state by using the low-efficiency siRNA as in Supplementary Fig. 5a, and treated cells with isotopically labelled [U-13C2]-acetate under hypoxia. The de novo lipid synthesis from acetate-derived carbon was reduced in FASN knockdown cells, demonstrating that acetate functions as an acetyl donor to promote lipogenesis and acetate-induced FASN expression plays an important role in lipogenesis (Fig. 5b).

Given that elevated lipid synthesis could promote tumour cell survival under hypoxia, we carried out CCK8 assay and found that acetate supplement enhanced cell survival under hypoxia, but not normoxia (Fig. 5c). To interrogate whether acetate promoted cancer cell survival through regulating lipogenic genes, we employed specific siRNAs targeting FASN or ACACA, of which the relative low knockdown efficiencies are capable of suppressing acetate-simulated FASN and ACACA mRNA expression to the level similar to unstimulated state (Supplementary Fig. 5a). We found that acetate-induced cell survival under hypoxia was inhibited by knockdown of either FASN or ACACA, indicating that acetate-promoted cell survival was dependent on lipogenesis and the induction of lipogenic gene expression was vital for cell survival (Fig. 5d). Strikingly, we found that inhibition of either FASN or ACACA to the untreated level had no effect on acetate-induced FASN promoter histone acetylation (Supplementary Fig. 5b). We attempted to further dissect whether acetate-induced de novo lipid synthesis is vital for cancer cell cultured under hypoxia. We found that HepG2 and SkBr3 cells were more sensitive to C75, a chemical inhibitor of FASN, when cultured under hypoxia (Supplementary Fig. 5c), consistent with previous report that cancer cells were dependent on de novo lipid synthesis under hypoxia. Moreover, C75 treatment abrogated acetate-induced cancer cell survival without affecting FASN and ACACA mRNA levels (Supplementary Fig. 5d,e,f). Consistently, ACSS1/2 knockdown blocked the increased cell survival induced by acetate supplement, indicating that acetate-induced cell survival was ACSS1/2-dependent (Fig. 5e).

In addition, we performed Matrigel assay and found that HepG2 cells supplied with acetate exhibited growth advantage over the control group, and this effect was blocked by ACSS1/2 double knockdown (Fig. 5f). Taken together, beyond functioning as a precursor for lipogenesis, acetate also serves as acetyl donor to induce histone acetylation and mRNA expression of lipogenic genes, contributing to the adaptation of cancer cells to hypoxia.

ACSS1/2 positively correlates with FASN expression in HCC. ACSS2 is reported to be upregulated in various human cancers. ACSS1 is also reported to be increased in hepatocellular carcinoma. Through an analysis of 190 human HCC samples data from the Cancer Genome Atlas (TCGA), we found a significant correlation between FASN and ACSS2 mRNA levels and no significant correlation between FASN and ACSS1 mRNA levels (Supplementary Fig. 6a). Our aforementioned results demonstrated that acetate could epigenetically regulate FASN expression, which prompts us to examine ACSS1/2, FASN, and H3 acetylation levels and their correlation in human HCC. We collected 53 pairs of primary human HCC samples with adjacent normal tissues, and determined protein changes (ACSS1, ACSS2 and FASN) and six histone acetylation marks (H3K9ac, H3K14ac, H3K18ac, H3K23ac, H3K27ac and H3K56ac) in all these samples (n=53) (Fig. 6a; Supplementary Fig. 6b). To perform a statistical analysis of the association between ACSS1/2 and histone acetylation, we divided tumour samples into two groups based on ACSS1/2 expression (high versus low). Tumours with 1.5-fold higher expression of ACSS1 or ACSS2 or both than that of adjacent normal control tissue are grouped into ACSS high-expression tumours (tumour/normal ≥1.5, n=26), while ACSS low-expression tumours (tumour/normal <1.5, n=27) express both ACSS proteins at 1.5-fold lower than its normal control (Fig. 6b). We found 16 cases with ACSS1 high expression, 8 cases with ACSS2 high expression and 2 cases with both ACSS1 and ACSS2 high expression (Fig. 6b). The higher percentage of ACSS1 high-expression cases than that of ACSS2 fully proves the importance of ACSS1 in acetate utilization by cancer. Our results demonstrate that four out of six histone acetylation marks, including H3K9ac (P=0.0220), H3K14ac (P=0.0289), H3K27ac (P=0.0034) and H3K56ac (P=0.0410), are significantly stronger in ACSS high-expression tumours than that from ACSS low-expression tumours with two-tailed unpaired Student’s t-test (Fig. 6c). In line with this observation, FASN protein level is also significantly upregulated in ACSS high-expression tumours (Fig. 6d). Consistently, we performed immunohistochemistry (IHC) analysis and found that ACSS1/2 expression was positively correlated with H3 acetylation and FASN expression in human HCC. A representative case was shown in Fig. 6e. By using Spearman correlation analysis, we have evaluated the relationship between histone acetylation and FASN expression. Surprisingly, each and every histone acetylation mark in our analysis exhibits significant positive correlation with FASN expression (P<0.0001 for H3K9ac, P=0.0003 for H3K14ac, P=0.0009 for H3K18ac, P=0.0385 for H3K23ac, P<0.0001 for H3K27ac and P<0.0001 for H3K56ac; Supplementary Fig. 6c). Collectively, these results strongly support a positive correlation between ACSS1/2, FASN and histone acetylation in human HCC.

Discussion

Acetyl-CoA is a central metabolite derived mainly from glucose, glutamine and fatty acids in mammals. However, the capability of cancer cells to produce acetyl-CoA from these conventional carbon sources is dramatically decreased under hypoxia. Cancer cells, therefore, need alternative carbon sources and...
Figure 5 | Epigenetic regulation of de novo lipogenesis by acetate is vital for cell survival under hypoxia. (a) ACSS1/2 mediates acetate-induced palmitate and stearate production. Fractional abundance of palmitate (left) and stearate (right) isotopologues in ACSS1/2 stably double-knockdown HepG2 cells (designated as magenta) compared with that of control cells (designated as black) after 24 h of culture under hypoxia with [U-13C2]-acetate. Each histogram was presented as mean ± s.d. of triplicate experiments. The inset shows the percentage of lipogenic acetyl-CoA derived from acetate carbon (**P < 0.01; by Student’s t-test). (b) Fractional abundance of palmitate (left panel) and stearate (middle panel) isotopologues in scramble (sicr) and siFASN HepG2 cells treat as in a. Each histogram was presented as mean ± s.d. of triplicate experiments. The inset shows the percentage of lipogenic acetyl-CoA derived from acetate carbon. The knockdown efficiency was detected by PCR with reverse transcription (right panel) (***P < 0.001; NS, not significant; by Student’s t-test). (c) Acetate promotes cell survival under hypoxia. Viability of HepG2 cells treated with indicated concentrations of acetate under normoxia or hypoxia was determined via CCK8 assay. The results were presented as mean ± s.d. of triplicate experiments (***P < 0.001; **P < 0.01; NS, not significant; by Student’s t-test). (d) Viability of control or siFASN or siACACA HepG2 cells treated with or without 2.5 mM acetate under hypoxia was determined via CCK8 assay. The results were presented as mean ± s.d. of triplicate experiments (***P < 0.001; by Student’s t-test). (e) ACSS1/2 mediates the promotion of cell survival by acetate. Viability of shscr or shACSS1/2 HepG2 stable cell lines treated as indicated was determined via CCK8 assay. The results were presented as mean ± s.d. of triplicate experiments (**P < 0.01; NS, not significant; by Student’s t-test). (f) ACSS1/2 mediates cells growth advantage by acetate in Matrigel. Shscr or shACSS1/2 HepG2 cells were plated into the Matrigel bed and cultured with DMEM with 10% FBS, and 2% Matrigel for 12 days before the images were taken (left). Scale bar, 0.25 mm. The quantitative data were presented as mean ± s.d. (n = 3) (right) (***P < 0.001; NS, not significant; by Student’s t-test).
Figure 6 | ACSS1/2 positively correlates with histone acetylation and FASN expression in human hepatocellular carcinoma. (a) FASN, ACSS1 and ACSS2 protein levels (upper panels, normalized against β-actin) and H3K9, H3K14, H3K18, H3K23, H3K27 and H3K56 acetylation levels (lower panels, normalized against histone H3) in 53 pairs of HCC (each paired with cancerous tissue (designated as C) and adjacent normal tissue (designated as N)) were analysed by western blot. Two pairs of representative samples were shown. For the other 51 pairs of samples, please refer to Supplementary Fig. 6b. (b) Heatmap of protein expression (ACSS1, ACSS2 and FASN) and histone acetylation levels (H3K9ac, H3K14ac, H3K18ac, H3K23ac, H3K27ac and H3K56ac) in all 53 pairs of HCC. Data were presented as Z-score of relative protein expression or histone acetylation. Tumours with 1.5-fold higher expression of ACSS1 or ACSS2 or both than that of adjacent normal control tissue are grouped into ACSS-high tumours (tumour/normal ≥1.5, n = 26), while ACSS-low tumours (tumor/normal <1.5, n = 27) express both ACSS proteins at 1.5-fold lower than its normal control. (c) H3K9ac (P = 0.0220), H3K14ac (P = 0.0289), H3K27ac (P = 0.0034), and H3K56ac (P = 0.0410), are significantly stronger in ACSS-high tumours than that in ACSS-low tumours. Statistical analyses were performed with a two-tailed unpaired Student’s t-test (**P < 0.01; *P < 0.05; NS, not significant). (d) FASN protein level is significantly upregulated in ACSS-high tumours (P = 0.002). Statistical analyses were performed with a two-tailed unpaired Student’s t-test (**P < 0.01). (e) Representative immunohistochemical staining results for ACSS1, ACSS2, FASN and H3K9/K27/K56 acetylation in adjacent normal tissue (N) and hepatocellular carcinoma (C). Scale bar, 100 μm.
conduct metabolic reprogramming. Clinical studies report the increase in $[^{13}]$-acetate uptake in multiple types of cancers including prostate, liver, lung and brain tumours. Recently, new studies further demonstrate that various tumours consume exogenous acetate to generate acetyl-CoA for lipid synthesis$^{17}$. These studies support that acetate serves as an important carbon source for cancer cells under unfavourable conditions. The underlying mechanism of how acetate functions as carbon source to fuel tumour growth, however, remains as an open question.

In this study, we elucidate that acetate functions as an epigenetic regulating metabolite to enhance lipid synthesis and to promote tumour survival under hypoxia. First, histone acetylation at H3K9, H3K27 and H3K56 sites can be stimulated by acetate in both time- and dose-dependent manners under hypoxia in multiple cancer cell lines. Notably, acetate-derived acetyl-CoA is found to be incorporated not only into fatty acid synthesis but also into acetylated histone peptides. Second, acetate-induced histone acetylation is then associated with FASN and ACACA promoter regions, which activates FASN and ACACA expressions under hypoxia. Third, acetate-driven epigenetic regulation is mediated by ACS1 and ACS2. Last and most important, acetate-induced lipogenic genes expression is found to promote de novo lipid synthesis and cell survival under hypoxic stress. Collectively, besides its ability to induce fatty acid synthesis as an immediate metabolic precursor, our study reveals a new mechanism of epigenetic regulation by acetate to increase lipid synthesis and promote cell survival under unfavourable conditions (Fig. 7). However, we assess effects of acetate using limited set of cell lines in vitro, it needs further confirmation of the findings using more cell lines. In addition, the experiments in vivo would provide more validation.

Alterations in histone modifications play important roles in transcriptional regulation in cancer cells$^{35}$. H3K9ac, H3K27ac and H3K56ac, three epigenetic marks of active gene transcription, are found to be altered in various cancers. H3K9 hyperacetylation is associated with specific genes in breast cancer$^{36}$. Similarly, H3K27 acetylation is upregulated in colorectal cancer$^{37}$. Intensive investigations also show that H3K56 acetylation is closely related with epigenetic activation and is increased in multiple types of cancer$^{38-42}$. In our work, we found that H3K9, H3K27 and H3K56 acetylation levels were increased in ACS1/2-overexpressed human HCC, showing a significantly positive correlation between ACS1/2 expression, histone acetylation and FASN expression. ACS1/2 upregulation suggests that these cancer cells utilize acetate as both epigenetic metabolite and carbon source to meet their growth needs. These findings validate our model in vivo: ACS1/2 links histone acetylation with FASN expression and acetate-induced epigenetic regulation may play a pivotal role in human HCC. As the major enzymes in acetate metabolism, ACS1 and ACS2 may merit further explorations as a therapeutic target for cancer.

Disregulation of de novo lipid synthesis is a hallmark of cancer$^{44}$. Increased lipid synthesis fuels cancer engine and contributes to cancer cells survival when facing with stressed conditions, such as hypoxia$^{25,30,44-49}$. Upregulation of FASN is common in many cancers and FASN is targeted for cancer therapy$^{44,49-51}$. FASN inhibition with chemical inhibitors or RNAi can suppress tumour cell survival$^{52}$. FASN upregulation in cancers is in part due to the transcriptional activation by SREBPs$^{44,49,52,53}$. In addition, USP2a was reported to enhance FASN protein stabilization$^{44}$. Our study proves that acetate increases histone acetylation at promoters of lipogenic genes and activates their mRNA expression, without reflecting lipid demand of hypoxic cells. Interestingly, we data show that both ACS1/2-mediated acetate and ACLY-mediated glucose contribute to histone acetylation at the promoters of hypoxia-induced genes. VEGF or LDHA shows a similar response that occurs in hypoxia alone without acetate addition yet each remains responsive to acetate. This suggests the changes occurring at these promoters that lead to gene activation (presumably recruitment of co-activators) render them more sensitive to acetyl-CoA levels compared with other genes. How recruitment of co-activators modulates acetate-induced lipogenic genes expression is an interesting question.

In conclusion, our study adds a novel mechanism of epigenetic regulation to FASN upregulation. Besides glucose, cancer cells absorb more acetate under hypoxia. Besides functioning as an immediate metabolic precursor, acetate-derived acetyl-CoA increases H3K9, H3K27 and H3K56 acetylation levels at FASN and ACACA promoter regions, initiating epigenetic regulation of FASN and ACACA. Due to the limitation of our system in distinguishing the role of acetyl-CoA as a substrate rather than an epigenetic regulator, both pathways cannot be separated well and their relative contribution remains unclear. Histone acetylation modulated by acetate status might play a pivotal role in coordinating acetate and glucose availability with the intracellular level of FASN, hence fatty acid synthesis. Taken together, this study demonstrates that acetate, in addition to its ability to induce fatty acid synthesis as an immediate metabolic precursor, also functions as an epigenetic metabolite to promote cancer cell survival under hypoxic stress.

**Methods**

**Cell culture.** PLC-8024 cells were obtained from the Institute of Virology, Chinese Academy of Medical Sciences (Beijing, China). All the other cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy. HepG2, SKBr3, PLC-8024 and DU 145 cells were grown in DMEM medium.

![Figure 7](image_url) **Figure 7 | The model for acetate-induced epigenetic regulation of de novo lipogenesis.** In addition to its ability to induce fatty acid synthesis as an immediate metabolic precursor, acetate also functions as an epigenetic metabolite to induce H3 acetylations in both dose- and time-dependent manners under hypoxia, enhancing H3K9, H3K27 and H3K56 acetylation levels at FASN and ACACA promoter regions, which upregulates FASN and ACACA expression and increases de novo lipid synthesis to promote tumour cell survival.
ChIP–qPCR assays. ChIP–qPCR assays were performed as previously described. Briefly, 1 × 10⁶ HepG2 cells were cross-linked with 1% paraformaldehyde, lysed, and fragmented using high-output power setting for eight cycles (30 s on and 30 s off). Solubilized chromatin was immunoprecipitated with chip grade antibodies for H3K9 acetyl, H3K27 acetyl, H3K56 acetyl or rabbit IgG (negative control), following that the antibodies were preincubated with protein A sepharose overnight at 4 °C. Antibody–chromatin complexes were pulled down using protein A sepharose (Sigma-Aldrich), washed and then eluted. After crosslink reversal in a water bath at 65 °C overnight and proteinase K treatment for 2 h at 55 °C, the immunoprecipitated DNA was extracted with phenol–chloroform, and ethanol precipitated. The DNA fragments were detected by qPCR. Histone acetylation marks were mapped using primers spanning ~3 to 3 kb of target genes (ACACA, FASN, ALCY, VEGF and LDLA). Primers spanning the regions with peaks were adopted for ChIP–qPCR analysis. All tested primers targeting FASN, ALCY, ALCY, LDLA and VEGF are listed in Supplementary Table 1.

RNA interference. For RNA interference experiments, si ACSS1 #1 (EHU040311) and si ACSS2 #1 (EHU133541) were purchased from Sigma. The others were obtained from commercial synthetic siRNA oligonucleotide (GenePharma, Shanghai).

Western blotting. Standard western blot protocols were adopted. The quantification was carried out by subtracting background from the band intensity of western blots by using software ImageQuantTL (GE).

LC-MRM MS quantification of histone acetylation. Histone proteins were extracted and purified as previously described by Gao et al. Briefly, 3 × 10⁶ scramble (shsc) or shACSS1/2 HepG2 cells were cultured in DMEM medium (without glucose or glutamine, Sigma) by adding 10% dialysed FBS (BI), 2.5 mM [U-13C2]-acetate, 5 mM labeled-free glucose, 2 mM labeled-free glutamine for 4 h under hypoxia (1% O2). Followed by addition of hypoxic lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM KCl, 1.5 mM MgCl2, 1 mM DTT and protease inhibitors) to isolate nuclei. Crude histone was extracted with 0.4 N HCl overnight. After removal of HCl by ultrafiltration, the crude histone was processed to derivatization in a final condition of 100 mM NH4-propionylate ester (home-made), 25 mM NH4HCO3, 50% ACN, 50 °C for 30 min. The proteins were SpeedVac to dryness and digested with trypsin in 25 mM NH4HCO3 overnight. Finally, the digested peptides were concentrated to dryness again and derivatized for a second round of histone propionylation.

Stable isotope-labelled histone peptides containing various PTMs and chemical derivatization (>95%) were from New England Peptide LLC (Pennsylvania, USA). Certified ACS grade sodium hydroxide was purchased from Standard Chemical and hydrochloric acid was from VWR International LLC (Tewksbury, MA, USA). Certified acetic acid and BSTFA (with 1% TMCS) were purchased from Sigma-Aldrich. Methanol and chloroform were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Certified 1% O2, 94% N2, 5% CO2 was acquired by gas chamber (C42, Biospherix).

To detect acetate uptake in cancer cells, the cancer cells were grown in DMEM (Gibco, high glucose) supplemented with 10% FBS (BI) and penicillin/streptomycin. A volume of 200 μl of each medium was transferred into a 1.5-ml Eppendorf tube, then 600 μl of cold CD3OD with 0.09 mg ml⁻¹ methylation-propionate (TSP) was added to the sample (to prevent vescular stomatitis virus G). The virus in the culture media was harvested every 24 h for 48h after initial plasmid transfection. For infection, HepG2 cells were infected for 12 h with pMKO.1-shACSS1 and pMKO.1-shACSS2 retrovirus with 8 μg ml⁻¹ polybrene to increase infection efficiency and cultured with 100 μg ml⁻¹ hygromycin B for 1 week first. Then the stable shACSS1 or shACSS2 cells were infected with pMKO.1-shACSS1 and pMKO.1-shACSS2 retrovirus and selected in 1 μg ml⁻¹ puromycin for 1 week to obtain ACS1 and 2 double knockdown (shACS1/2) stable cell lines. The knockdown efficiency was verified by western blot.

NMR spectroscopy. To detect acetate uptake in cancer cells, the cancer cells were grown in DMEM (Gibco, high glucose) supplemented with 10% FBS (BI) and penicillin/streptomycin. A volume of 200 μl of each medium was transferred into a 1.5-ml Eppendorf tube, then 600 μl of cold CD3OD with 0.09 mg ml⁻¹ methylation-propionate (TSP) was added to the sample (to prevent vescular stomatitis virus G). The virus in the culture media was harvested every 24 h for 48h after initial plasmid transfection. For infection, HepG2 cells were infected for 12 h with pMKO.1-shACSS1 and pMKO.1-shACSS2 retrovirus with 8 μg ml⁻¹ polybrene to increase infection efficiency and cultured with 100 μg ml⁻¹ hygromycin B for 1 week first. Then the stable shACSS1 or shACSS2 cells were infected with pMKO.1-shACSS1 and pMKO.1-shACSS2 retrovirus and selected in 1 μg ml⁻¹ puromycin for 1 week to obtain ACS1 and 2 double knockdown (shACS1/2) stable cell lines. The knockdown efficiency was verified by western blot.
The signal intensities were normalized against the intensity of the TSP signal at 0.00 p.p.m.

**GC–MS analysis of lipid pool.** HepG2 stable cells (5 x 10⁶) were cultured in DMEM medium (without glucose or glutamin, Sigma) by adding 10% dialysed FBS (BI), 25 mM label-free glucose, 4 mM label-free glutamine and 5 mM [U-13C]acetate for 24 h under 1% O₂. Cells were rapidly washed twice with 37 °C PBS, then 1 ml PBS was added to the dish and quickly detached from the dish using a cell lifter. The liquid-containing cells were transferred into a 2-ml tube and centrifuged at 15,294 g at 4 °C. The supernatant was discarded and the residue was added to 400 μl of cold (80/20 methanol: water) extraction solution for quenching. The sample was vortexed and then stored at −20 °C for 1 h. The samples will be vortexed and centrifuged at 15,294 g for 10 min at 4 °C. The supernatant will be moved to new tubes for drying and used for further detection. The sample preparation of the saponinified fatty acids has been described previously. Briefly, 1 ml MeOH/H₂O (1:1, v/v) solution with 0.1 M HCl and 1.6 p.p.m. internal standard (myristic acid-D27) in solution was added into cell debris at 0 °C, vortexed for 30 s, followed by addition of 0.5 ml chloroform, and vortexed for 1 min, then centrifuged at 12,000 g for 5 min at 4 °C. The resulting chloroform layer was transferred to a new glass vial, and the extract was dried under N₂. The desiccation was reconstituted into 1 ml MeOH/H₂O (9:1, v/v) containing 0.3 M NaOH, and incubated at 80 °C for 1 h to saponify the fatty acids. Then 0.1 ml of formic acid was added for acidification, followed by extraction twice with 1 ml hexane. The hexane layer was transferred to an Eppendorf tube for drying. The residue was then added to 20 μl of pyridine and 200 μl of BSTFA (with 1% TMCS) and vortexed for 30 s, then derivatized at 70 °C for 1 h. The mixture was vortexed for 30 s and centrifuged at 12,000 g for 5 min at room temperature before GC–MS analysis.

**Matrigel assay.** Matrigel assay was performed as previously reported with minor modifications. Briefly, or shACSS1/2 HepG2 cells were plated in 96-well plates at 5 x 10⁴ per well in 200 μl of medium treated with indicated conditions. The cell viability was measured after 48 h treatment by Cell Counting Kit-8 (Beyotime Biotechnology) according to the manufacturer’s instructions. CCK-8 solution was incubated with cells for 1 h at 37 °C. Absorbances at 450 nm were collected by ELx800 Universal Microplate Reader (BioTek).

**Hepatocarcinoma cancer samples.** Hepatocarcinoma cancer samples were obtained from the 10th People’s Hospital, Shanghai (affiliated to Tongji University), with consents from the patients. The procedure adhered to human subjects was approved by the Ethics Committee of the Institutes of Biomedical Sciences (IBS), Fudan University. Direct immunoblotting was performed as mentioned above.

**IHC staining.** Human HCC sections were de-paraffinized in xylene and hydrated in graded ethanol, followed by deionized water. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by boiling the sections in 0.01 M citrate buffer (pH 6.0) for 25 min in microwave oven. The sections were incubated with normal goat serum for 20 min to block non-specific staining and then incubated overnight at 4 °C with primary antibodies. After incubation for 45 min with a HRP-conjugated anti-rabbit secondary antibody, the sections were detected using diaminobenzidine (DAB) kit according to the manufacturer’s instructions, followed by counterstaining with hematoxylin. The primary antibodies were used as follows: anti-H3K9 acetyl (ab31219, Abcam, 1:500 dilution), anti-H3K27 acetyl (ab45173, Abcam, 1:80 dilution), anti-H3K56 acetyl (ab76307, Abcam, 1:60 dilution), anti-ACSS2 (ab133664, Abcam, 1:50 dilution), anti-ACSS1 antibody (HPA041014, Sigma, 1:50 dilution), and anti-FASN (C-20140, Santa Cruz, 1:40 dilution). The negative controls were performed by omitting the primary antibodies.

**Statistical analysis.** All data were statistically analysed with two-tailed unpaired Student’s t-test. The correlation analysis between FASN and ACSS1/2 mRNA levels in 190 human HCC samples from TCGA data base were carried out by using Pearson’s coefficient test and the correlation analysis between histone acetylation and FASN expression in human HCC samples by Spearman’s correlation test. The values of P < 0.05 were considered as statistically significant.

**Data availability.** The analysis result in Supplementary Fig. 6a is based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/ Data supporting the findings of this study are available within the article and its Supplementary Information files, and from the corresponding author upon request.
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