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PRECLINICAL STUDY

S-adenosylmethionine biosynthesis is a targetable metabolic vulnerability of cancer stem cells

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Abstract

Purpose Many transformed cells and embryonic stem cells are dependent on the biosynthesis of the universal methyl-donor *S*-adenosylmethionine (SAM) from methionine by the enzyme MAT2A to maintain their epigenome. We hypothesized that cancer stem cells (CSCs) rely on SAM biosynthesis and that the combination of methionine depletion and MAT2A inhibition would eradicate CSCs.

Methods Human triple (ER/PR/HER2)-negative breast carcinoma (TNBC) cell lines were cultured as CSC-enriched mammospheres in control or methionine-free media. MAT2A was inhibited with siRNAs or cycloleucine. The effects of methionine restriction and/or MAT2A inhibition on the formation of mammospheres, the expression of CSC markers ($CD44^{hi}$/$C24^{low}$), MAT2A and CSC transcriptional regulators, apoptosis induction and histone modifications were determined. A murine model of metastatic TNBC was utilized to evaluate the effects of dietary methionine restriction, MAT2A inhibition and the combination.

Results Methionine restriction inhibited mammosphere formation and reduced the $CD44^{hi}$/$C24^{low}$ CSC population; these effects were partly rescued by SAM. Methionine depletion induced MAT2A expression (mRNA and protein) and sensitized CSCs to inhibition of MAT2A (siRNAs or cycloleucine). Cycloleucine enhanced the effects of methionine depletion on H3K4me3 demethylation and suppression of Sox9 expression. Dietary methionine restriction induced MAT2A expression in mammary tumors, and the combination of methionine restriction and cycloleucine was more effective than either alone at suppressing primary and lung metastatic tumor burden in a murine TNBC model.

Conclusions Our findings point to SAM biosynthesis as a unique metabolic vulnerability of CSCs that can be targeted by combining methionine depletion with MAT2A inhibition to eradicate drug-resistant CSCs.

Keywords Methionine · Breast cancer · *S*-adenosylmethionine · Nutrition · Cancer stem cell · Therapeutics

Introduction

The essential sulfur-containing amino acid methionine plays a critical role in maintaining the pluripotency of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells [1,

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2]. Methionine is converted to *S*-adenosylmethionine (SAM) by a family of conserved methionine adenosyltransferase (MAT) enzymes whose catalytic subunits are derived from two genes, the liver-specific *Mat1a* and the ubiquitously expressed *Mat2a* genes [3]. SAM is the universal methyl-donor for a broad range of methyltransferases involved in DNA and histone methylation of the epigenome [4]. Depletion of SAM by a variety of strategies, including methionine deprivation, leads to demethylation of a specific histone modification (H3K4me3), a conserved epigenetic mark that transcriptionally activates gene networks that regulate pluripotency [1, 5, 6]. Transient methionine restriction induces differentiation of ES and iPS cells, while prolonged methionine restriction activates p53-dependent apoptosis in these cells [1]. In this way, methionine metabolism directly links the nutrient status of stem cells to the epigenetic regulation of pluripotency.

Intriguingly, many tumor cells are also dependent on methionine for cell proliferation and survival [7]. Methionine restriction activates cell cycle arrest and/or apoptosis in a broad range of transformed cells and inhibits tumor growth in diverse murine models [8–11]. These effects of methionine depletion are rescued by homocysteine supplementation in normal cells but not transformed cells [12]. However, the molecular mechanisms underlying the "methionine dependence" of cancer are poorly understood. Although clinical trials in advanced solid tumors have demonstrated the safety of dietary methionine restriction alone or in combination with cytotoxic agents, these studies have failed to demonstrate therapeutic efficacy [13–15]. We have recently demonstrated that methionine restriction "primes" triple (estrogen receptor, progesterone receptor and HER2)-negative breast tumors to respond to pro-apoptotic TRAIL receptor agonists by increasing cell surface expression of TRAIL receptor-2 (TRAIL-R2 or DR5) [16]. Dietary methionine restriction enhances the activity of TRAIL receptor agonists in a murine model of metastatic triple-negative breast cancer (TNBC). In principle, this "metabolic priming" approach could be used to target other stress response pathways activated by methionine restriction to selectively enhance the therapeutic efficacy of this dietary intervention.

Given the dependence of both stem cells and cancer cells on methionine, we postulated that cancer stem cells (CSCs), rare self-renewing cells within tumors that are likely responsible for treatment resistance and tumor progression [17], might be especially vulnerable to methionine depletion. Moreover, because methionine restriction induces expression of MAT2A as a homeostatic response to preserve SAM levels [1, 18], we hypothesized that methionine restriction primes CSCs to MAT2A inhibition. Here we report that methionine restriction inhibits the formation of CSC-enriched mammospheres and reduces the population of $CD44^{hi}/CD24^{low}$ CSCs. These effects are partly rescued by SAM supplementation. Methionine depletion induces MAT2A expression and sensitizes CSCs to inhibition of MAT2A expression or activity. The MAT2A inhibitor cycloleucine augments the effects of methionine depletion on H3K4me3 demethylation. Moreover, the combination of dietary methionine restriction and cycloleucine is more effective than either individual intervention at suppressing primary and lung metastatic tumor burden in a murine model. Taken together, our findings point to SAM biosynthesis as a novel metabolic vulnerability of CSCs and indicate that MAT2A inhibition selectively enhances the antitumor activity of methionine depletion.

Methods and materials

Cell culture and reagents

Human MDA-MB-231 and GILM2 TNBC cells stably expressing mCherry were cultured as described [19, 20]. BT20 TNBC cells were grown in MEM medium supplemented with 10% FBS, 1% sodium pyruvate, 1% NEAA, 2% sodium bicarbonate and 100 IU/mL penicillin/streptomycin (Thermo Fisher Scientific). Cell lines were authenticated by STR analyses. Cycloleucine and SAM were purchased from Sigma-Aldrich.

Mammosphere assay

TNBC cells (1×10^4 cells per well) were seeded in 6-well ultra-low attachment plates (Corning) in mammosphere medium composed of serum-free RPMI containing 1% methylcellulose, 10 ng/mL basic fibroblast growth factor (bFGF/FGF2), 20 ng/mL epidermal growth factor (EGF), 2% B-27 supplements, 10 µg/mL human insulin and 100 IU/mL penicillin/streptomycin (Thermo Fisher Scientific). Experiments were performed in control or methionine-free (0% Met) mammosphere media with or without SAM (100 µM) and cycloleucine (50 mM). Three hundred µl of fresh media was added to each well every day (without removing the old media). Mammospheres ≥ 50 µm in diameter were manually counted in ten randomly selected 10 × fields per well (Nikon Eclipse TS100 Inverted Microscope) after the indicated number of days.

CD24 and CD44 cell surface expression

To measure cell surface expression of CD24 and CD44, TNBC mammospheres cultured for 6 days were collected, washed with PBS and then enzymatically dissociated with 0.05% trypsin/0.25% EDTA. Cells were pelleted by centrifugation at 500 × g for 5 min and resuspended in 10 µL fluorescein isothiocyanate (FITC)-conjugated mouse anti-human

CD24 monoclonal antibody and allophycocyanin (APC)-conjugated mouse anti-human CD44 monoclonal antibody (BD Pharmingen). After incubating cells for 30 min at 4 °C in the dark, cells were washed and then analyzed by flow cytometry.

MAT2A silencing

siRNAs targeting the sequences CACACAAGCUAAAUG CCAA (si-MAT2A-1) or CAGUUGUGCCUGCGAAAUA (si-MAT2A-2) in the human *Mat2a* gene and non-silencing control siRNA were purchased from Sigma-Aldrich. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Immunoblotting

Whole-cell lysates were immunoblotted as described [21] using primary Abs against MAT2A, MAT1A, β-actin (Sigma-Aldrich), Sox9 and KLF4 (Cell Signaling).

Real-time PCR

Real-time PCR for MAT2A and GAPDH was performed as described previously [16]. Primers for MAT2A (forward 5-GACATTGGTGCTGGAGACCA, reverse 5-ACTCTG ATGGGAAGCACAGC) were purchased from Integrated DNA Technologies, and real-time PCR was performed using a comparative Ct method to normalize RNA expression in samples to the controls in each experiment.

Annexin V labeling

For the Annexin V apoptosis assay, TNBC mammospheres cultured for 6 days were collected, washed with PBS and then enzymatically dissociated with 0.05% trypsin/0.25% EDTA. Cells were pelleted by centrifugation at 500 × g for 5 min and then analyzed by flow cytometry using the Annexin-PE Apoptosis Detection Kit (BD Bioscience).

Immunoblotting for histones and histone modifications

Mammospheres were incubated on ice in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% Na-DOC, 0.4 mM phenylmethanesulfonyl fluoride, 2X protease inhibitor cocktail) and the lysates spun at 21,000 × g. The supernatant protein concentration was quantified (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific). Pellets (containing histones) were resuspended and boiled in 2X SDS loading dye at amounts proportional to the supernatant protein concentration. Equal amounts of pellet lysate were separated on 15% SDS–PAGE gels and transferred onto nitrocellulose membrane in 1X Towbin/20% methanol. Membranes were blocked in 5% milk/TBS-T and incubated overnight in primary Abs against K4me3 (Active Motif), K9me3 (Abcam), K27me3 (Millipore), H3 [22] or H4 [23] in 5% milk/TBS-T. Membranes were washed with TBS-T, incubated with goat anti-rabbit IgG-HRP (BioRad 1706515) in 5% milk/TBS-T, washed with TBS-T, and HRP was visualized by chemiluminescence (SuperSignal™ West Pico, Thermo Fisher Scientific).

Murine model of metastatic TNBC

GILM2-mCherry TNBC cells (2×10^6) were resuspended in Matrigel (BD Biosciences) and injected into the lactiferous ducts of both 4th mammary glands in 4- to 5-week-old female NOD *scid* IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory). Mice were then randomly divided into four treatment groups (10 mice per group): control diet plus vehicle, control diet plus cycloleucine (25 mg/kg twice weekly for 4 doses), methionine-free diet (0% methionine, Teklad TD.140119) plus vehicle, or methionine-free diet plus cycloleucine (25 mg/kg twice weekly for 4 doses). Mice were started on their respective diets 2.5 weeks after tumor cell inoculation, and the diets were continued throughout the treatment period. Mammary tumor volume was calculated using calipers as described [24]. Lung metastatic burden was visualized by fluorescence microscopy in isolated whole lungs and scored using NIH ImageJ analysis as described [24]. For immunoblots and mass spectrometry analysis, a separate cohort of female NSG mice with orthotopic mammary xenograft tumors ($n = 3$ mice per group) were placed on a control diet or methionine-free diet for 2 weeks, after which time the mice were euthanized and tumors were harvested and frozen for immunoblot analysis. Blood was collected into EDTA tubes at the end of the experiment; samples were centrifuged for 10 min at 2000 × g at 4 °C. All animal experiments were carried out as part of an IACUC-approved protocol at the University of Wisconsin-Madison.

Metabolite sample preparation and mass spectrometry analysis

Frozen plasma samples (50 µl) were combined with 200 µL cold acetone and 5 µL tris(2-carboxyethyl)phosphine (TCEP, 2 mM), vortexed for 5 min, and centrifuged for 15 min at 13,400 × g at 4 °C. Metabolite-containing extract (80 µL) was combined with 20 µL dilution buffer (60% acetonitrile, 1 mM TCEP), and 2 µL internal standard (5 µM heavy methionine, 1 µM heavy SAM). Standard curves for each metabolite were prepared with equivalent addition of internal standard. Targeted LC-MS/MS experiments were performed using a TSQ Quantiva Triple Quadrupole

mass spectrometer and Ultimate 3000 RSLC Binary Pump (Thermo Fisher Scientific). Analytes were injected and separated on an ACQUITY BEH Amide column (Waters) heated to 35 °C. Mobile phase A consisted of 10 mM ammonium formate with 0.1% formic acid and mobile phase B was 95% acetonitrile with 10 mM ammonium formate with 0.1% formic acid. An 11-min gradient starting with 95% B and ending with 40% B was employed with an 18-min total method runtime. Selected reaction monitoring (SRM) scans were taken of selected metabolites using 2–3 transitions per metabolite using previously optimized transitions for each target. The total cycle time was 1 s. The mass spectrometer was equipped with a heated electrospray ionization source operated in positive mode using a spray voltage of 3500 V. Sheath, auxiliary, and sweep gases were held at 25, 13, and 1 arbitrary units, respectively. The ion transfer tube was heated to 342 °C, and the vaporizer temperature was set to 358 °C. Q1 and Q3 resolution were set to 0.7 and 2 full width at half maximum. CID gas was set to 1.5 mTorr, and 16 V was used for the source fragmentation parameter. LC-MS/MS data were processed using TraceFinder software version 4.0 (Thermo Fisher Scientific). Metabolite peak areas were normalized using internal standards, and metabolites were quantified with standard curves.

Tumor apoptosis assay

The percentage of tumor cells that were positive for active caspase-3 was determined by immunohistochemistry of formalin-fixed, paraffin-embedded tumor tissue sections using an Ab against cleaved caspase-3 (Cell Signaling) as described [24].

Statistics

The statistical significance of differences between groups was determined by ANOVA with Bonferroni posttests using GraphPad Prism 4 software.

Results

Methionine restriction inhibits CSCs by a SAM-dependent mechanism

To explore the role of methionine metabolism in CSCs, we grew TNBC cells in ultra-low attachment plates in normal mammosphere growth media or methionine-free mammosphere growth media with or without supplemental SAM. Methionine depletion resulted in a robust decrease in the number of mammospheres in three different human TNBC cell lines (Fig. 1a). Moreover, SAM supplementation largely rescued the effects of methionine restriction in these mammosphere assays. Consistent with these findings, mammospheres grown in methionine-free media exhibited a reduction in the population of $CD44^{hi}/CD24^{low}$ cells that are enriched for CSCs (Fig. 1b). SAM supplementation at least partly rescued the effects of methionine restriction on the $CD44^{hi}/CD24^{low}$ population. Collectively, these results indicate that methionine depletion inhibits CSCs by a SAM-dependent mechanism.

Methionine restriction induces MAT2A and potentiates the effect of MAT2A silencing on CSCs

Consistent with prior reports [1, 18], methionine restriction increased MAT2A protein and mRNA levels (Fig. 2a, left and right panels, respectively) in TNBC mammospheres, but did not alter MAT1A protein levels. To determine the functional relevance of the observed induction in MAT2A in mammospheres, we silenced MAT2A expression in mammospheres by transfecting them with one of two different siRNAs targeting MAT2A (si-MAT2A-1 or si-MAT2A-2). Both siRNAs robustly reduced the expression of MAT2A protein in all three TNBC cell lines compared to a non-silencing control (C) siRNA (Fig. 2b). Silencing MAT2A modestly inhibited mammosphere formation and potentiated the effects of methionine restriction on mammospheres (Fig. 2c). Taken together, these results indicate that SAM biosynthesis is required for CSC survival and point to this pathway as a targetable metabolic vulnerability.

Methionine restriction potentiates the effect of the MAT2A inhibitor cycloleucine on CSCs

Based on our siRNA results, we postulated that cycloleucine, an amino acid analog that competitively inhibits MAT activity [25], would enhance the inhibitory effects of methionine restriction on CSCs. Consistent with our hypothesis, methionine restriction or cycloleucine individually inhibited mammospheres, but the combination of methionine restriction and cycloleucine virtually eradicated mammospheres (Fig. 3a). Cycloleucine augmented apoptosis induction by methionine restriction in TNBC mammospheres with effects ranging from additive (MDA-MB-231 cells) to synergistic (GILM2 and BT20) (Fig. 3b). Moreover, cycloleucine potentiated the cytotoxicity of methionine depletion against adherent TNBC cells (Fig. S1). To determine the specific role of SAM depletion in the observed effects, we added SAM to TNBC mammospheres treated with methionine restriction, cycloleucine or the combination. Notably, SAM supplementation at least partly rescued the inhibitory effects of methionine depletion and cycloleucine on TNBC mammospheres (Fig. 3c). Collectively, these results indicate that

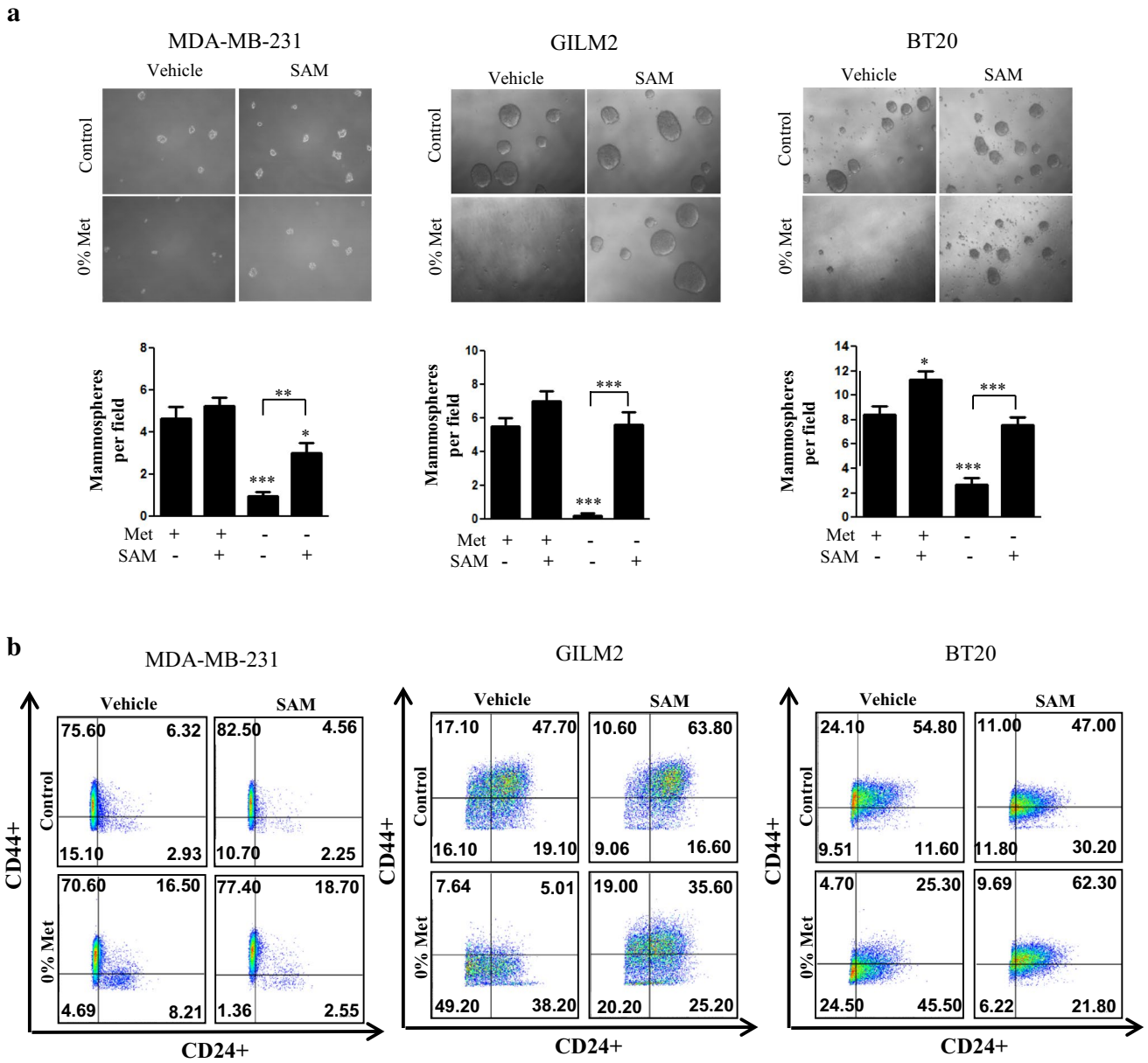
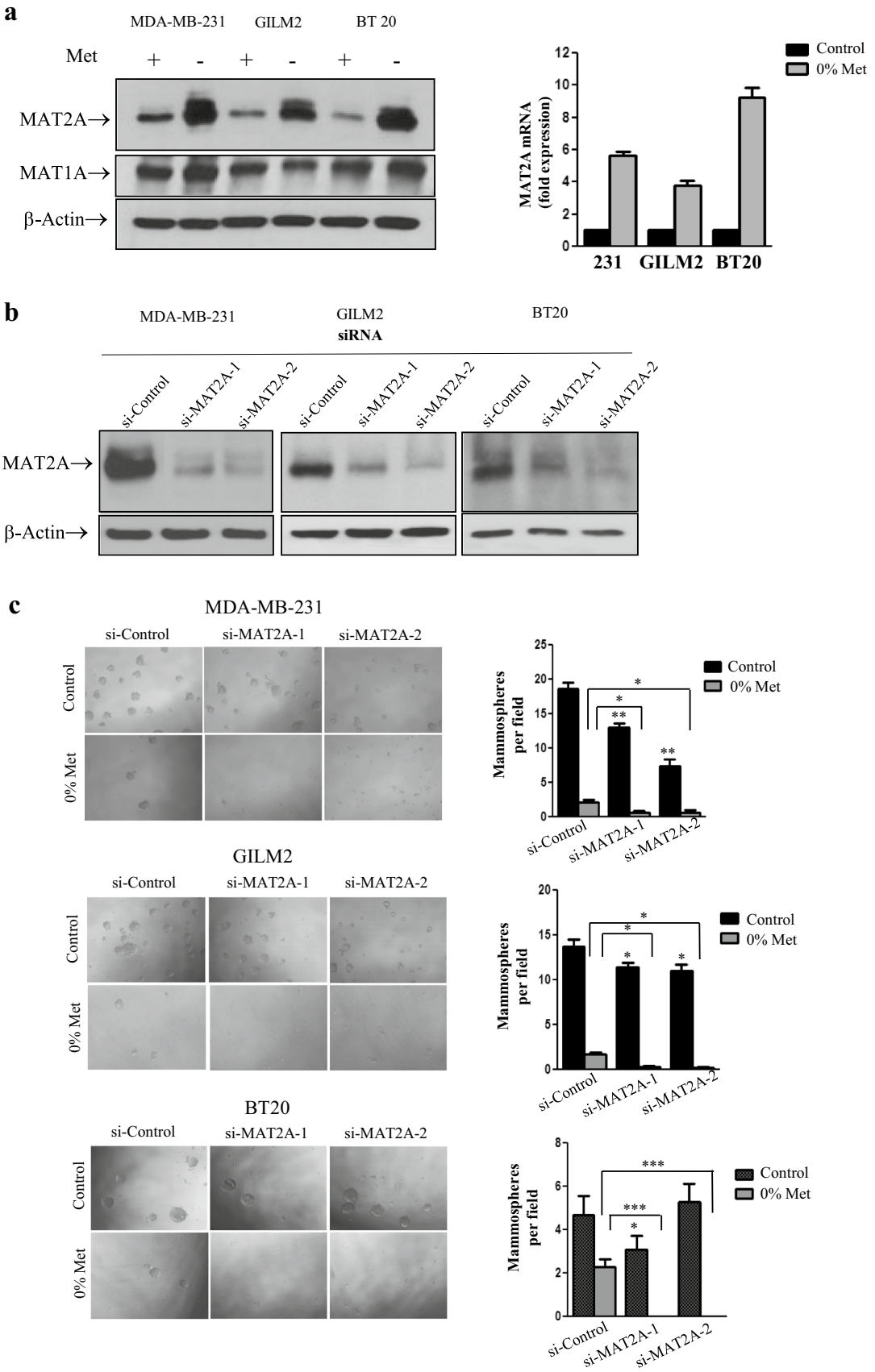


Fig. 1 Methionine restriction inhibits CSCs by a SAM-dependent mechanism. **a** Mammosphere assay in TNBC cells. MDA-MB-231, GILM2 and BT20 TNBC cells were cultured in ultra-low attachment plates in normal (control) or methionine-free mammosphere growth media (0% Met) with or without SAM (100 μM). Top panel, representative images showing mammospheres at day 7. Bottom panel, mammospheres were scored in ten fields from each well (mean ± SEM, $n = 3$) $^{*}P < 0.05$, $^{***}P < 0.001$ versus mammospheres cultured in control media without SAM. **b** Flow cytometry analysis of CD44 and CD24 expression in primary mammospheres from TNBC cells grown for 6 days in control or methionine-free media with or without SAM (100 μM)

cycloleucine potentiates the effects of methionine restriction on CSCs by a SAM-dependent mechanism.

Methionine restriction augments the effects of cycloleucine in suppressing histone methylation

Nutrient availability of Met and SAM has been directly linked to changes in histone H3 K4, K9 and K27 trimethylation [1, 5, 26]. Although some TNBC mammospheres cultured in the presence of cycloleucine or in methionine-free media exhibited modest reductions in H3K4me3, H3K9me3 and H3K27me3 levels (Fig. 4a), the combination of methionine restriction and cycloleucine led to marked demethylation of H3K4me3 in all of the TNBC mammospheres. We next examined the effects of these interventions on the expression of the breast CSC transcriptional


a
MDA-MB-231
GILM2
BT 20
Met
+ - + - + -
MAT2A→
MAT1A→
β-Actin→
MAT2A mRNA (fold expression)
Control
0% Met
231
GILM2
BT20
b
MDA-MB-231
GILM2
BT20
siRNA
si-Control
si-MAT2A-1
si-MAT2A-2
MAT2A→
β-Actin→
c
MDA-MB-231
GILM2
BT20
si-Control
si-MAT2A-1
si-MAT2A-2
Control
0% Met
Mammospheres per field

◂**Fig. 2** Methionine restriction induces MAT2A expression and potentiates the effect of MAT2A silencing on CSCs. **a** Methionine deprivation increases MAT2A protein and mRNA levels. Left panel, immunoblot analysis of MAT2A and MAT1A protein expression in TNBC mammospheres grown in control or methionine-free mammosphere media for 4 days. Right panel, real-time PCR analysis of MAT2A mRNA levels in TNBC mammospheres grown in control or methionine-free mammosphere media for 72 h. MAT2A mRNA levels were normalized to expression in TNBC cells cultured in control media. **b** Immunoblot analysis of MAT2A expression in TNBC cells transfected with a scrambled control siRNA or two different siRNAs targeting MAT2A (MAT2A-1 and MAT2A-2) 48 h after transfection. **c** Mammosphere assay in TNBC cells transfected with a scrambled control siRNA or two different siRNAs targeting MAT2A (MAT2A-1 and MAT2A-2). The next day, cells were collected, seeded in ultra-low attachment plates in control or methionine-free mammosphere media (0% Met) and grown for 4 days. Left panel, representative images showing mammospheres at day 4. Right panel, mammospheres were scored in 10 fields from each well (mean ± SEM, $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the indicated comparisons

regulators Sox9 and KLF4 [27, 28]. Notably, methionine restriction and the combination of methionine restriction and cycloleucine consistently reduced Sox9 protein levels but had more variable effects on KLF4 levels (Fig. 4b). Taken together, these findings suggest that the combination of methionine restriction and cycloleucine robustly induces demethylation of H3K4me3 and suppresses expression of a subset of breast CSC transcriptional regulators.

Dietary methionine restriction enhances the antitumor effects of cycloleucine in vivo

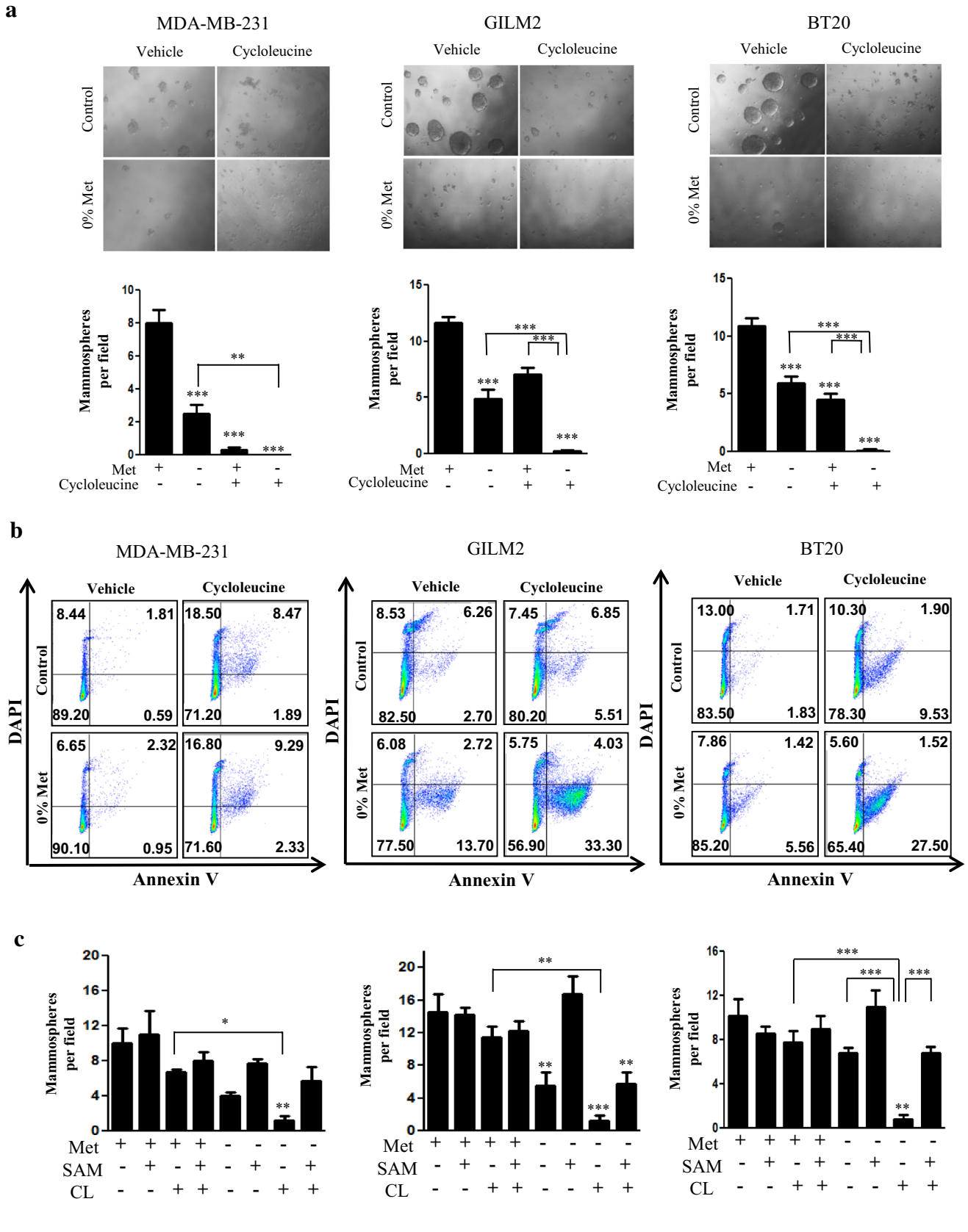
To examine the antitumor activity of dietary methionine restriction, cycloleucine or the combination in vivo, female NSG mice with orthotopic GILM2-mCherry mammary tumors were fed a control or methionine-free diet and treated with vehicle or cycloleucine (25 mg/kg twice weekly for 4 doses). Both the methionine-free diet and cycloleucine alone inhibited mammary tumor growth compared to vehicle-treated mice on a control diet, but the combination of methionine-free diet plus cycloleucine was more effective than either individual intervention (Fig. 5a, left panel). Mice on the methionine-free diet receiving vehicle or cycloleucine exhibited modest weight loss at the end of the study, while cycloleucine treatment itself did not result in weight loss (Fig. 5a, right panel). Plasma levels of methionine, but not SAM or SAH, were significantly reduced in all three intervention groups (Fig. 5b), validating the efficacy of each intervention. Consistent with our in vitro findings, dietary methionine restriction increased protein levels of MAT2A in mammary tumors (Fig. 5c). The combination of dietary methionine restriction and cycloleucine was more effective than either individual treatment at suppressing lung metastases (Fig. 5d) and inducing apoptosis in primary tumors and lung metastases as determined by active caspase-3 immunostaining (Fig. 5e). These findings indicate that dietary methionine restriction enhances the antitumor effects of cycloleucine by inducing its molecular target MAT2A.

Discussion

We have demonstrated that CSCs are dependent on SAM biosynthesis for self-renewal and survival. Several lines of evidence support this conclusion. First, methionine depletion inhibits mammosphere formation and reduces the population of $CD44^{hi}/CD24^{low}$ CSCs. Strikingly, SAM supplementation at least partly rescues the effects of methionine restriction on CSCs, indicating that CSCs rely on SAM rather than methionine *per se* for self-renewal and survival. Second, methionine depletion leads to induction of the *Mat2a* gene and increased MAT2A protein levels as a homeostatic response to attempt to preserve SAM levels in the context of reduced methionine availability. Our findings are consistent with prior reports describing MAT2A induction in response to methionine restriction [1, 18]. Third, dual targeting of SAM biosynthesis by combining methionine restriction with MAT2A inhibition using siRNAs or cycloleucine results in more robust elimination of CSCs, apoptosis induction and demethylation of H3K4me3 than either intervention alone. Fourth, SAM supplementation partly rescues the effects of combined methionine depletion and MAT2A inhibition on CSCs, underscoring the essential role of SAM in CSC survival and methylation of H3K4me3.

Mechanistically, inhibition of SAM biosynthesis activates apoptosis in a subset of CSCs and inhibits CSC self-renewal, which may reflect at least partly the observed reduction in H3K4me3 levels, an epigenetic mark linked to activation of pluripotency gene networks that is highly sensitive to nutrient availability of methionine [1, 5, 26]. The observed robust reduction in levels of Sox9, a transcriptional regulator of CSC self-renewal, in response to inhibition of SAM biosynthesis may contribute to the depletion of CSCs, although we have not specifically assessed the functional relevance of this alteration in these studies. By targeting a metabolic liability of CSCs, inhibition of SAM biosynthesis has a major therapeutic advantage over cytotoxic drugs, which are inactive against CSCs [17].

We have also demonstrated that dual inhibition of SAM biosynthesis inhibits mammary tumor growth and lung metastases more robustly than methionine restriction or cycloleucine individually in a murine TNBC model that recapitulates many clinical features of this disease. Dual targeting also augmented apoptosis induction in primary and metastatic tumors. Consistent with our findings in cell-based models, dietary methionine restriction induced the expression of MAT2A in mammary tumors, providing additional mechanistic insights into the enhanced efficacy


a
MDA-MB-231
GILM2
BT20
Vehicle
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Control
0% Met
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Met
Cycloleucine
b
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Vehicle
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Control
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7.86
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85.20
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5.60
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65.40
27.50
c
Mammospheres per field
Met
SAM
CL

◂**Fig. 3** Methionine restriction enhances the effects of the MAT2A inhibitor cycloleucine on CSCs. **a** Mammosphere assay of TNBC cells grown in ultra-low attachment plates in control or methionine-free mammosphere media (0% Met) with or without cycloleucine (CL, 50 mM). Top panel, representative images showing mammospheres at day 7. Bottom panel, mammospheres were scored in ten fields from each well (mean ± SEM, $n = 3$) ***$P < 0.001$ versus mammospheres grown in control media without cycloleucine. **b** TNBC cells were cultured in ultra-low attachment plates in control or methionine-free mammosphere media (0% Met) with or without CL (50 mM) for 5 days. Apoptosis was measured by Annexin V labeling using flow cytometry. **c** TNBC cells were cultured in ultra-low attachment plates in control or methionine-free mammosphere media (0% Met) with or without SAM (100 μM), CL (50 mM), or both for 7 days. Mammospheres were scored in ten fields from each well (mean ± SEM, $n = 3$) *$P < 0.05$, ***$P < 0.001$ versus mammospheres grown in control media or the indicated comparisons

of dual targeting of SAM biosynthesis. Notably, dietary methionine restriction, cycloleucine treatment and the combined treatment reduced plasma methionine levels but did not significantly affect plasma SAM or SAH levels. Although cycloleucine was largely abandoned as a chemotherapy drug due to CNS toxicity, we used much lower doses (50 mg/kg twice weekly × 4) than the reported LD50 in mice (120–140 mg/kg/d × 1 week) [29–31]. At the doses used in these studies, cycloleucine was well tolerated and did not cause weight loss, although methionine restriction did result in modest weight loss. Additional higher affinity MAT2A inhibitors have recently been described [32], and one such MAT2A inhibitor (AG-270) is currently being evaluated in a phase I clinical trial in patients with lymphoma or solid tumors (NCT03435250, Clinical trials.gov). Hence, additional MAT2A inhibitors that may have an improved therapeutic index will be available for future clinical translation. Moreover, dietary methionine restriction reduces visceral fat and improves glucose homeostasis [33, 34], suggesting that these systemic effects may also contribute to the antitumor activity of this intervention. Taken together, our results

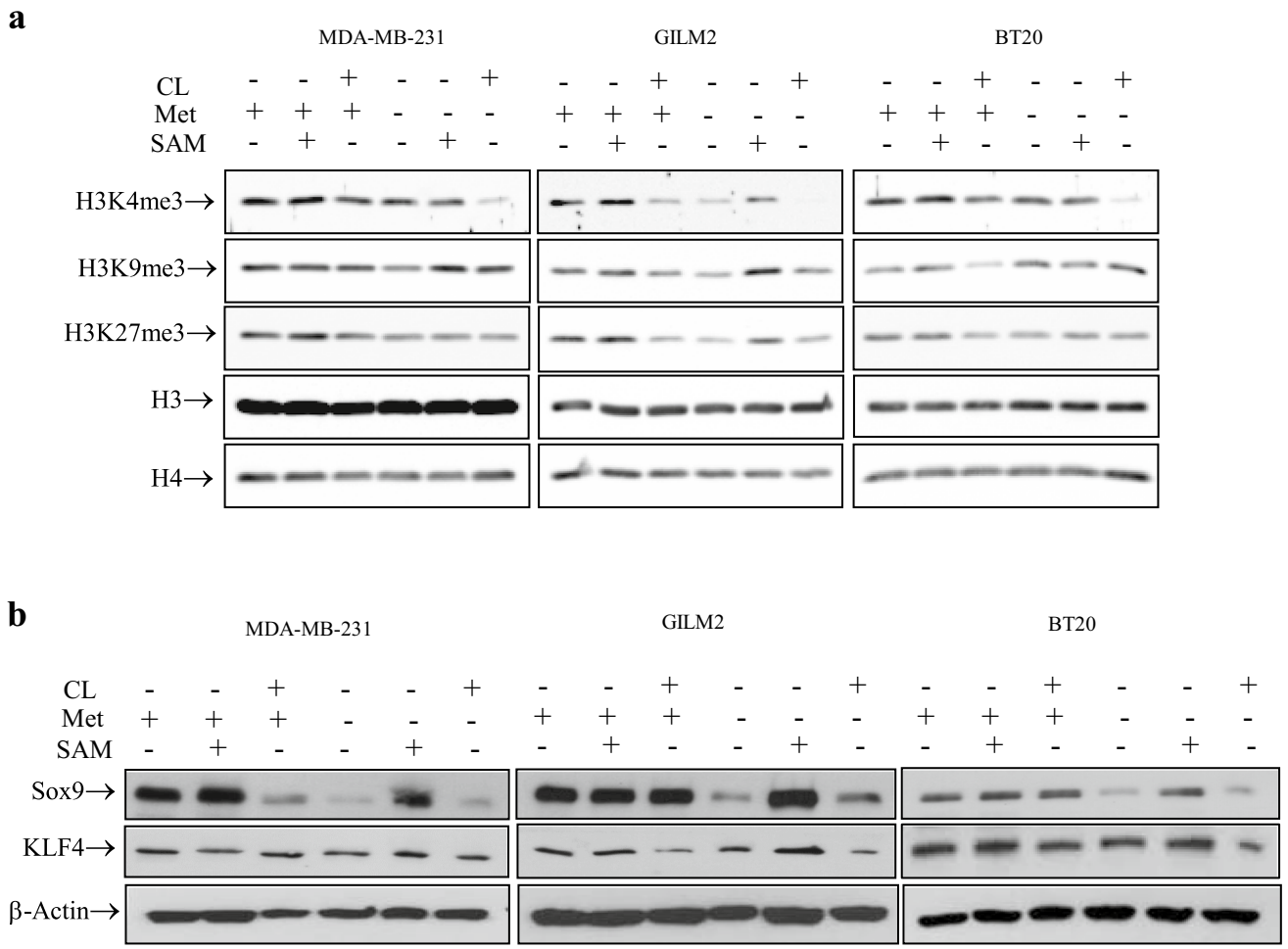
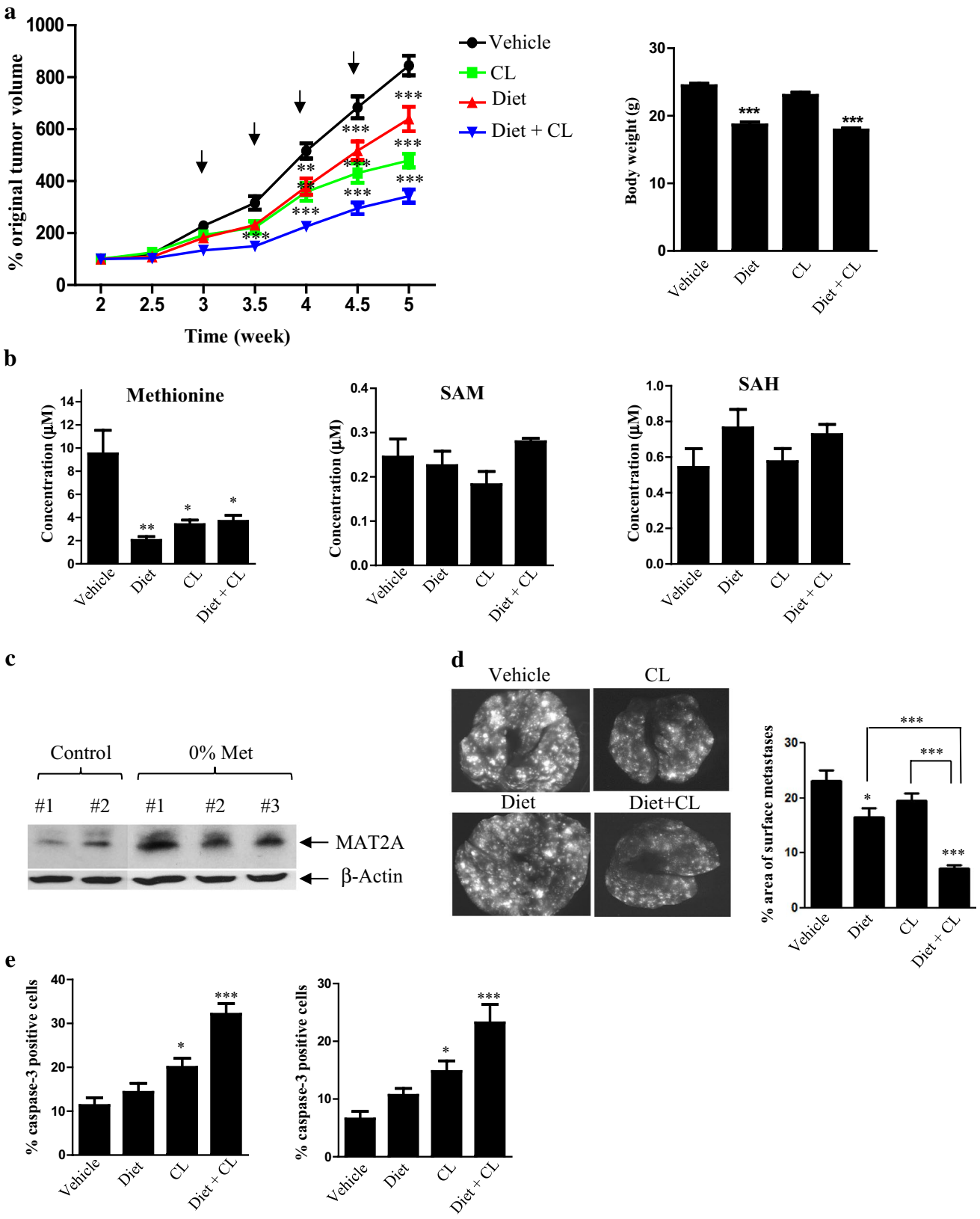


Fig. 4 Methionine restriction augments the effects of cycloleucine in suppressing specific histone methylation. TNBC cells were grown in ultra-low attachment plates in control or methionine-free mammosphere media (0% Met) with or without SAM (100 μM) or CL (50 mM) for 4 days. **a** Immunoblot analysis of histone H3 methylation. **b** Immunoblot analysis of the breast CSC regulators Sox9 and KLF4


a
% original tumor volume
Time (week)
Vehicle
CL
Diet
Diet + CL
Body weight (g)
b
Methionine
SAM
SAH
Concentration (μM)
c
Control
0% Met
#1
#2
#3
MAT2A
β-Actin
d
Vehicle
CL
Diet
Diet+CL
% area of surface metastases
e
% caspase-3 positive cells

◂**Fig. 5** Dietary methionine restriction enhances the antitumor effects of cycloleucine in vivo. Female NSG mice with orthotopic GILM2-mCherry mammary tumors were randomized into four groups (10 mice per group): control diet plus vehicle, control diet plus cycloleucine (25 mg/kg twice weekly for 4 doses, indicated by arrows), methionine-free diet plus vehicle, or methionine-free diet plus cycloleucine (25 mg/kg twice weekly for 4 doses). Mice were started on their respective diets 2 weeks after tumor cell inoculation, and the diets were continued throughout the treatment period. **a** Left panel, percentage original mammary tumor volume (at 2 weeks) in each treatment group (mean ± SEM, $n = 10$ mice per group). Right panel, body weight of mice at the end of the study. **b** Plasma levels of Met, SAM and SAH in the mice in each treatment group after 2 weeks (mean ± SEM, $n = 3$ mice per group). **c** Immunoblot analysis of MAT2A expression in mammary tumors from mice receiving control diet or methionine-free diet for 2 weeks. **d** Left panel, representative whole lung images by fluorescence microscopy. Right panel, the percentage of the surface area occupied by lung metastases (mean ± SEM, $n = 10$ mice per group). **e** The percentage active caspase-3-positive tumor cells in mammary tumors (left panel) or metastatic lung tumors (right panel) at the end of the study (mean ± SEM, $n = 3$ tumors per group). In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated mice or the indicated comparison

provide proof-of-principle preclinical evidence in support of dual targeting of SAM biosynthesis by combining methionine restriction and MAT2A inhibition.

The proposed dual targeting of SAM biosynthesis aligns nicely with our recently described "metabolic priming" strategy. We reported previously that methionine restriction enhances cell surface expression of the cell death receptor TRAIL-R2, rendering triple-negative breast tumors more vulnerable to TRAIL-R2 agonists [16]. In an analogous manner, methionine stress activates MAT2A gene expression in TNBC cells, thereby "priming" them to respond to MAT2A inhibition by undergoing apoptosis or differentiation. MAT2A is often aberrantly expressed in human tumors in response to HIF-1α, IGF-1, Nrf2 and EGF and promotes cell growth and drug resistance [3, 35, 36]. Moreover, deregulated MAT2A expression has been linked to tamoxifen resistance in breast cancer [37]. Silencing MAT2A inhibits proliferation and induces apoptosis in carcinoma cells [38]. Collectively, these findings point to MAT2A as a promising therapeutic target in cancer.

In summary, we have identified SAM biosynthesis as a previously unrecognized metabolic vulnerability of CSCs. Methionine restriction induces MAT2A expression, thereby providing an explanation for the robust synergy between methionine depletion and MAT2A inhibition/silencing in suppressing CSC survival and SAM-dependent epigenetic alterations such as H3K4me3, which have been linked to pluripotency [6]. Dual targeting of SAM biosynthesis inhibits mammary tumor growth and lung metastasis in vivo more robustly than methionine restriction or cycloleucine alone. Our preclinical results lay the foundation for translating these insights into novel strategies to eradicate drug-resistant CSCs by targeting their unique metabolic requirements.

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Compliance with ethical standards

Conflict of interest JJC is a consultant for Thermo Fisher Scientific. The other authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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