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# Methionine regulates self-renewal, pluripotency, and cell death of GIC through cholesterol—rRNA axis

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## Abstract

**Background:** Glioma-initiating cells (GICs) are the source of glioma cells that can self-renew, have pluripotency, and are treatment-resistant, so are the starting point for relapse and eventual death despite multimodality therapy. L-[methyl-<sup>11</sup>C] methionine PET has observed high accumulation at the time of recurrence, it is important to understand the mechanism of tumor cell activation caused by the reorganization of methionine metabolism.

**Methods:** We cultured cells in methionine-deprived culture medium for comprehensive analysis. Based on the obtained results, the possible target molecules were chemically inhibited and the respective markers were analyzed.

**Results:** Methionine depletion markedly decreased proliferation and increased cell death of GICs. Decreased S-adenosyl-methionine, which is synthesized intracellularly by catalyzed by methionine adenosyltransferase using methionine, triggered the following: (i) global DNA demethylation, (ii) hyper-methylation of signaling pathways regulating pluripotency of stem cells, (iii) decreased expression of the core-genes and pluripotent markers of stem cells including *FOXM1*, *SOX2*, *SOX4*, *PROM1*, and *OLIG2*, (iv) decreased cholesterol synthesis and increased excretion mainly through decreased *SREBF2*, and (v) down-regulation of the large subunit of ribosomal protein configured 28S and *ACA43*, small nucleolar RNA guiding the pseudouridylation of 28S rRNA, which is essential for translation. In addition, inhibition of cholesterol synthesis with statin resulted in a phenotype similar to that of methionine depletion and decreases in stem cell markers and small nucleolar RNA *ACA43*. Moreover, suppression of *FOXM1* decreased stem cell markers such as *SOX4* and *PROM1*. The gene expression profile for cholesterol production was obtained from the Ivy Glioblastoma Atlas Project database and compared between tumor cells with relatively low methionine levels in areas of pseudopalisading arrangement around necrosis and tumor cells in the infiltrating region, showing that cells in the infiltrating region have higher capacity to produce cholesterol.

**Conclusions:** Methionine metabolism is closely related with self-renewal, pluripotency, and cell death in GICs through modification of cholesterol biosynthesis, especially in the *SREBF2-FOXM1* and *ACA43* axis with modification of rRNA.

**Keywords:** Methionine, Cholesterol biosynthesis, rRNA, *ACA43*, GIC

## Introduction

Glioblastoma multiforme is the most common and deadly type of primary brain tumor with a median overall survival of less than 15 months despite aggressive treatment [1]. Glioblastoma multiforme can produce glioma

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cells and consequently self-renew, so glioma-initiating cells (GICs) are considered to be the root stem cell for tumorigenesis and recurrence. Cancer stem cells including GICs retain “plasticity” that changes dynamically as a result of metabolic reorganization.

Malignant transformation or recurrence of glioblastoma multiforme can be detected as increased methionine accumulation on PET before contrast enhancement is seen on MRI. Therefore, detailed understanding of the methionine metabolism of GICs may be important for tumor control. Methionine concentrations are expected to be high in areas of sparse tumor cells, but relatively low in areas of dense lesions, such as in the peri-necrotic pseudopalisading region, due to low blood supply. Therefore, glioblastoma multiforme may develop with nonuniform concentration of methionine within the same lesion. We hypothesized that reorganization of the methionine metabolism in the presence of restricted common source of methyl donors could modify the character of tumor cells as reflected by epigenetic status, and gene and protein expression profiles.

The present study showed that the methionine metabolism in GICs was closely related to cholesterol metabolism and rRNA expression, resulting in change in pluripotency, progression, and avoidance of cell death.

## Materials and methods

### Cell lines and culture conditions

Human GICs (MZGC1, MZGC2, MZGC3, MZGC4, MZGC5, MZGC6, MZGC7 and MZGC8 cells) were isolated and established as cell lines from the surgical tissues of patients treated in our institution from March 2015 to April 2021. GICs were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham's liquid supplemented with SUBSER-ESrF<sup>®</sup> (Cell Science & Technology Institute, Inc., Sendai, Japan), CELRENA<sup>®</sup> medium (Cell Science & Technology Institute, Inc.), in 2-hydroxyethyl methacrylate-coated T75 flasks in a humidified incubator at 37 °C under an atmosphere of 5% CO<sub>2</sub> and 95% air. For the methionine depletion studies, cells were plated on *laminin-coated 10-cm culture dishes* and cultured with complete CELRENA<sup>®</sup> medium or methionine deficient CELRENA<sup>®</sup> medium (custom medium CST1 MFG No. cn2243a0) for 48–72 h. Cells were dispersed onto laminin-coated 96-well plates, replaced with treated media on day 1, and time-lapse photography was performed with Incucyte<sup>®</sup> (Sartorius, Osaka, Japan). Simvastatin and FDI-6 were purchased from Selleck (Tokyo, Japan).

### Dye exclusion test

Dye exclusion testing was performed to analyze cell viability. Fixed amounts of cells were seeded and cultured

in medium with (control) or without methionine for 3, 5 and 7 days. Harvested cells were suspended in phosphate buffered saline-containing trypan blue and then examined to determine the percentage of cells with blue clear cytoplasm (nonviable cells) versus total cells using a cell counter (LUNA, Logos Biosystems, Annandale, VA, USA). The relative rate against the control sample was calculated.

### Cell cycle analysis and detection of surface marker of GICs

Cells with or without treatment of methionine depletion/statin addition were collected using Accutase<sup>®</sup> solution (Sigma, USA). The Cell Cycle Phase Determination Kit (Cayman Chemical, Ann Arbor, MI, USA) was used for cell cycle analysis. In brief, collected cells were rinsed twice with buffer, then fixed at –20 °C overnight. Cells were washed twice with ice-cold phosphate buffered saline, and stained with propidium iodide/RNase staining buffer solution in the dark for 30 min at room temperature. Then, cells were analyzed with a flow cytometer (Guava<sup>®</sup> EasyCyte<sup>™</sup> Mini, Luminex Japan, Tokyo, Japan). A histogram of the cell cycle distribution was generated from 5000 events per sample and data were analyzed using Guava<sup>®</sup> Cell Cycle software. To detect CD133, the putative marker of GICs, by flow cytometry (FCM), 1 × 10<sup>6</sup> cells derived from tumorspheres were analyzed using fluorescent (FITC) labelled antibodies (CD133/1 (AC133)-VioBright FITC, Miltenyi Biotec, Germany) and BD FACSCalibur (BD Biosciences, USA).

### RNA extraction including microRNA (miRNA), cDNA synthesis, and quantitative PCR (qPCR) analysis

RNA from cultured tumor cells was extracted with the *mirVana* miRNA Isolation Kit (Ambion, Thermo Fisher Scientific K.K., Tokyo, Japan) or RNeasy Plus Mini Kit (QIAGEN, Germantown, MD, USA). For cDNA synthesis, RNA was reverse transcribed from random hexamers using the SuperScript<sup>™</sup> VILO<sup>™</sup> cDNA Synthesis Kit (Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Real-time qPCR was then performed in triplicate on the StepOne Plus or Quant3 (Applied Biosystems, Thermo Fisher Scientific) using SYBR<sup>™</sup> Green Realtime PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) to determine the mRNA levels. PCR was performed using a 20 µl volume containing 2 µl cDNA, 300 µM of each primer, and 10 µl of 2 × PCR master mix under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The data were normalized to the amount of human *18S* rRNA, and the values are represented as the mean ± SD of 2<sup>–ΔΔCt</sup> in a triplicate assay.

The primers used in this study were described in Supplementary Table S2.

#### Colony formation assay

Cells were seeded into laminin-coated 12-well plates in triplicate at a density of 500 cells/well in 2 ml of medium with or without treatment medium. After 120 h, formed colonies were trypsinized, reseeded and cultured for further 72 h. After 192 h, the cell colonies were stained for 15 min with a solution containing 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove excess dye. Each well was observed with Olympus IX71 microscope (Olympus, Tokyo, Japan).

#### Gene and miRNA expression analysis

Gene and miRNA expression was analyzed with a GeneChip™ System with a Human Genome Clariom™ D Array and GeneChip™ miRNA 4.0 Array (Thermo Fisher Scientific) according to the manufacturer's instructions. mRNA expression analysis used amplification and biotin labeling of fragmented cDNA with the biotin labeling system (GeneChip™ WT PLUS Reagent Kit, Thermo Fisher Scientific) in duplicate. miRNA expression analysis used biotin-labeled total RNA 1 µg including miRNA from tissue using the FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix®, Thermo Fisher Scientific). Labeled probes were hybridized to the Human Genome Clariom™ D Array (Thermo Fisher Scientific) or GeneChip™ miRNA 4.0 Array (Thermo Fisher Scientific) with the GeneChip™ Scanner 3000 7G (Affymetrix®, Thermo Fisher Scientific). Expression data of mRNA were extracted from image files using GeneSpring GX 14.9.1 (Agilent Technologies, Santa Clara, CA, USA). Normalization and expression value calculations were performed using the GeneSpring system. Signal values of miRNA were calculated using the Transcriptome Analysis Console software (Affymetrix®, Thermo Fisher Scientific). Statistical analysis was performed using two-tailed, unpaired *t*-tests. Any change  $\geq$  twofold with  $P < 0.05$  was considered statistically significant.

Enrichment analysis used Metascape (<http://metascape.org/>) and Gene Set Enrichment Analysis (GSEA) (<http://www.broad.mit.edu/GSEA>). Significance of biological processes was determined through *P*-values calculated on a hypergeometric distribution (log10). Metascape was used to conduct meta-analysis with generation of heat maps of gene ontology terms that hierarchically cluster together under experimental conditions. Statistical criteria for a differentially expressed gene included a change greater than 1.5 fold, and an overall false discovery rate smaller than 5% (change  $\geq \pm 1.5$  fold and false discovery rate  $\leq 0.05$ ) with Benjamini-Hochberg correction (*q*) to account for multiple comparisons.

GSEA using MSigDB (v7.4) was also used to determine the significance of a pre-defined gene set by comparing the correlation between expression and class distinction with other random situations. The significance threshold was set at nominal *P* value  $< 0.05$  or false discovery rate *q* value  $< 0.25$ .

#### Measurement of cholesterol, S-adenosyl-methionine (SAM), and S-adenosylhomocysteine (SAH) in tissue using ELISA

Measurement of cholesterol, SAM, and SAH used glioblastoma stem cells cultured with CELRENA medium or methionine-deprived CELRENA medium for 72 h and  $10 \times 10^6$  cells/cell pellets were homogenized by sonication in ice-cold phosphate buffered saline followed by centrifugation at 10,000 g for 15 min. Intracellular cholesterol, SAM, and SAH concentrations were measured using the Total Cholesterol Assay Kit (Colorimetric, Cell Biolabs, Inc., San Diego, CA, USA) and SAM and SAH ELISA Combo Kit (Cell Biolabs, Inc.) in accordance with the manufacturer's instructions under the indicated conditions. The standards were generated using the cholesterol standard and SAH/SAM-bovine serum albumin conjugate supplied in the kit. After the reaction was stopped, the OD450 and OD620 values were read using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). SAH/SAM ratios were calculated as the ratio of SAM to average SAH using the intracellular concentration (µg/mL) to determine the relative methylation potential.

#### Reduced Representation Bisulfite Sequencing (RRBS) and data analysis

RRBS libraries with single MspI digestion were constructed for glioblastoma stem cells. Briefly, 1 µg of genomic DNA was digested with 20U of MspI enzymes in 18 µl reaction mixtures at 37 °C for 2–3 h. After purification, the digested products were blunt-ended, and then dA added, followed by methylated-adaptor ligation using a NEXTflex Bisulfite-Seq Ligation Kit. To obtain DNA fractions of  $> 100$  bp, the MspI-digested products were purified using Agencourt AMPure XP (Beckman Coulter). Bisulfite conversion was conducted using an EX DNA Methylation Gold Kit following the manufacturer's instructions. The final libraries were generated by PCR amplification using NEXTflex Bisulfite-Seq Ligation Kit and sequenced using an Illumina HiSeqX to generate 150 bp pair-end reads (Supplementary Table S1).

Filtering sequence reads for both poor quality and adapters were performed via Trim Galore v0.6.4 (<https://github.com/FelixKrueger/TrimGalore>). Alignment to the human reference assembly hg19 and methylation calling was performed Bismark v0.21.0 (PMID:21,493,656).

CpG sites in the resulting RRBS data were then interrogated for methylation patterns and differential methylation ( $q$  value  $< 0.05$  and methylation percentage difference of at least 25%) using the methylKit. The differential methylation data were then queried for differentially methylated regions (DMRs) using annotatr (<https://bioconductor.org/packages/release/bioc/html/annotatr.html>) and GeneDMRs, R package for Gene-based DMR analysis (<https://www.biorxiv.org/content/10.1101/2020.04.11.037168v1.full>). The resulting DMR outputs were also visualized in the genome browser or in the Integrative Genomics Viewer (IGV) program (<https://software.broadinstitute.org/software/igv/>). To establish the relationship between histone mark, insulators, transcription factors, and DMRs, the RRBS data conjunction with the data acquired from the Database of ChIP-Atlas Peak browser ([https://chip-atlas.org/peak\\_browser](https://chip-atlas.org/peak_browser)) were projected on to the IGV.

**In silico analysis**

Gene expression data obtained by the RNA sequencing technique involved in cholesterol metabolism in different

anatomical regions (infiltrating tumor, pseudopalisading cells) of the glioblastoma multiform were identified using the Ivy Glioblastoma Atlas Project (Ivy GAP) database (<http://glioblastoma.alleninstitute.org/>).

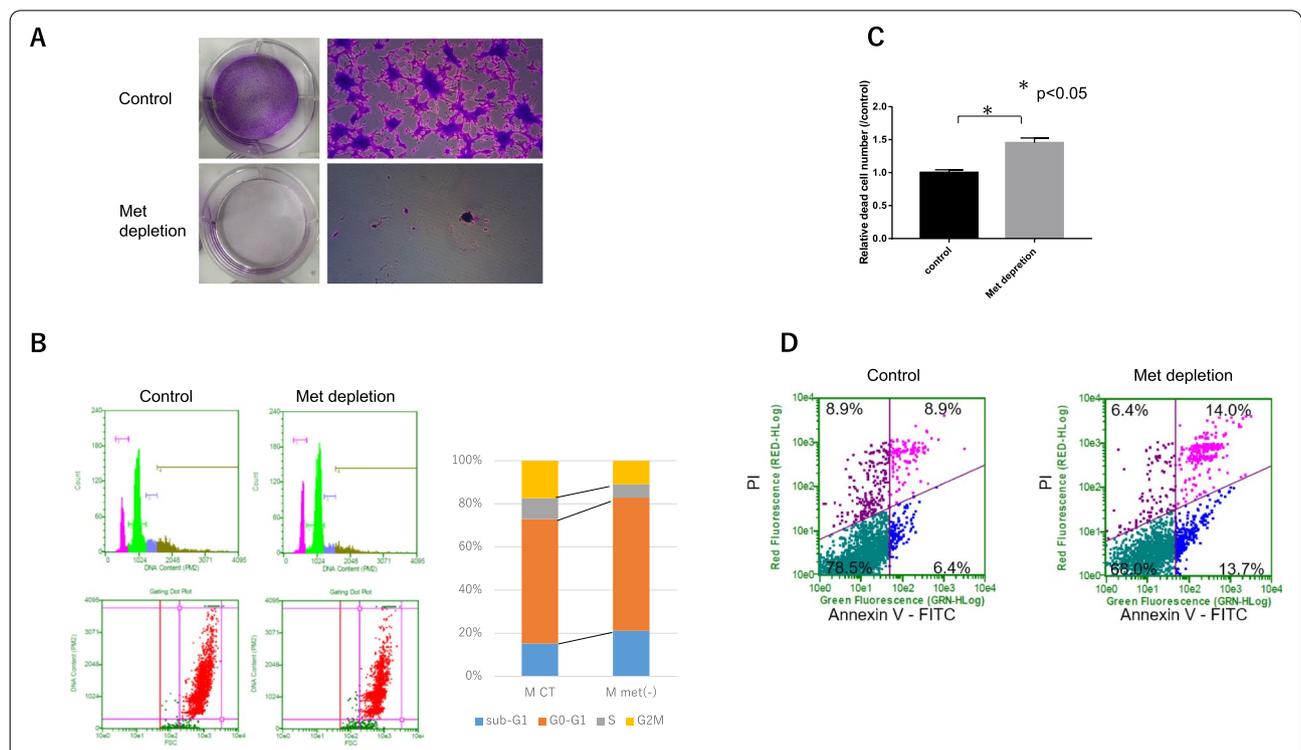
**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Data are expressed as means  $\pm$  SD of experiments performed in triplicate. One-way analysis of variance with Tukey’s post-hoc test or unpaired Student t-test was performed for comparisons of the quantitative data between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

**Results**

**Methionine depletion decreased cell proliferation and increased cell death**

Clonogenic assay showed marked decreases in cell growth and sphere formation, suggesting reduced self-renewal capacity induced by the depletion of methionine (Fig. 1A). Fluorescence-activated cell sorter analysis



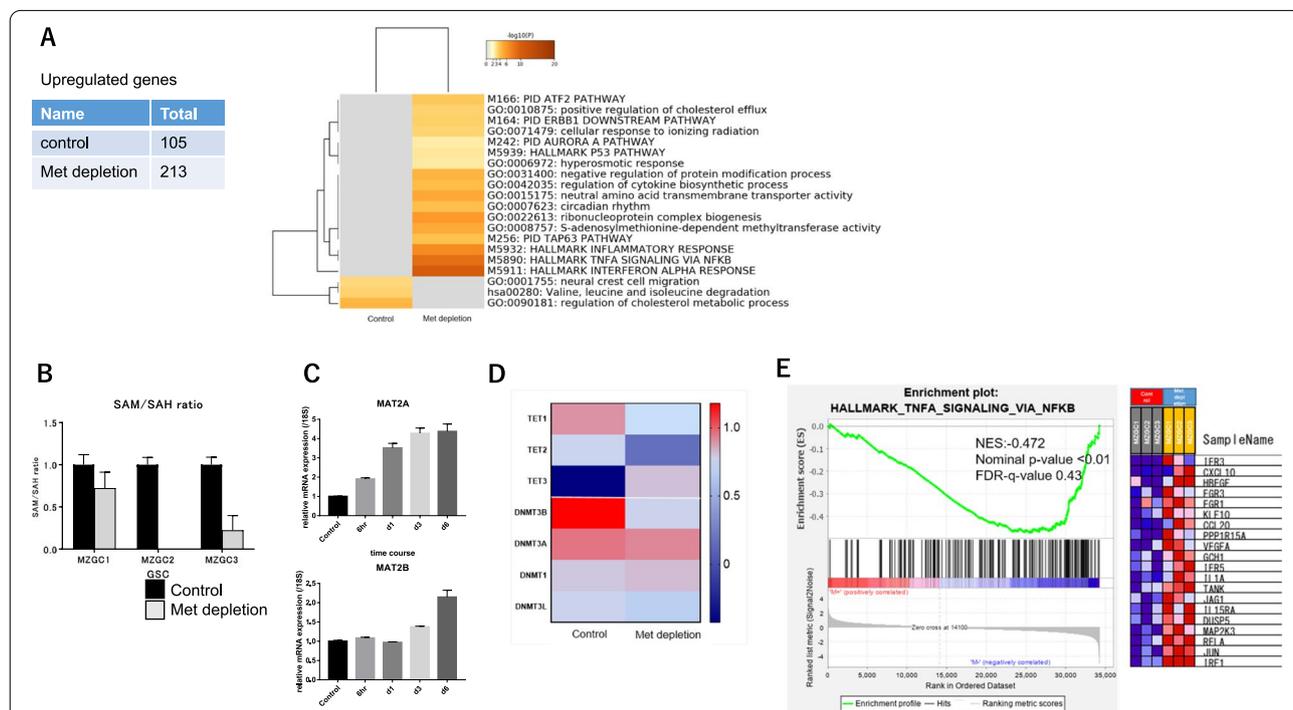
**Fig. 1** **A** Clonogenic assay: Methionine depletion for 5 days then reseeded for 3 days markedly decreased colony formation of MZGC1 cells. Quantitative analysis of colony areas in response to methionine depletion. **B** Fluorescence-activated cell sorter analysis to detect cell cycle distribution. Propidium iodide (PI) staining assay using a Cell Cycle Phase Determination Kit was performed on MZGC1 cells. Cell cycle analysis showed that methionine depletion for 3 days increased sub-G1 and decreased S, G2M populations. **C** Dye exclusion test showed that methionine depletion for 3 days increased dead cell rate. **D** Fluorescence-activated cell sorter analysis using annexin-V kit showed that methionine depletion induced apoptotic cell death

and dye exclusion test were also performed to assess cell death or cell cycle arrest. Methionine depletion for 3 days resulted in increased sub-G1 population and cell death (Fig. 1B and C). Further fluorescence-activated cell sorter analysis using annexin-V showed apoptosis induced by methionine depletion, although the time to apoptosis varied from cell to cell (Fig. 1D, Supplementary Figure S1).

**Methionine depletion impaired SAM-dependent methyltransferase pathway**

A comprehensive gene expression analysis was performed using a microarray to detect the characteristics of changes in specific molecular networks and canonical signaling pathways induced by modified methionine metabolism. A total of 213 genes were upregulated in the methionine withdrawal group, which were enriched in 17 Metascape terms/pathways, including 1) SAM-dependent methyltransferase pathway, 2) hallmark TNFA

signaling via the nuclear factor-kappa B (NFKB), 3) regulation of cytokine biosynthetic process, 4) neutral amino acid transmembrane transporter activity, 5) circadian rhythm, 6) ribonucleoprotein complex biogenesis, 7) negative regulation of protein modification process, and 8) positive regulation of cholesterol efflux (Fig. 2A, Supplementary Figure S2). In contrast, 115 genes downregulated in the methionine withdrawal group were enriched in terms, regulation of cholesterol metabolic process and neural crest cell migration. SAM is a methionine derivative that is essential for the catalytic reaction of methylase. Intracellular SAM levels were reduced by methionine depletion (Fig. 2B). SAM is synthesized intracellularly by methionine adenosyltransferase (MAT) using the essential amino acids methionine and ATP as substrates. SAM is a methyl group donor and the dependent pathways include *MAT2A*, *MAT2B*, *DNMT1*, *DNMT3A*, and *DNMT3B*. qPCR and expression array were performed to examine whether methionine depletion in GICs



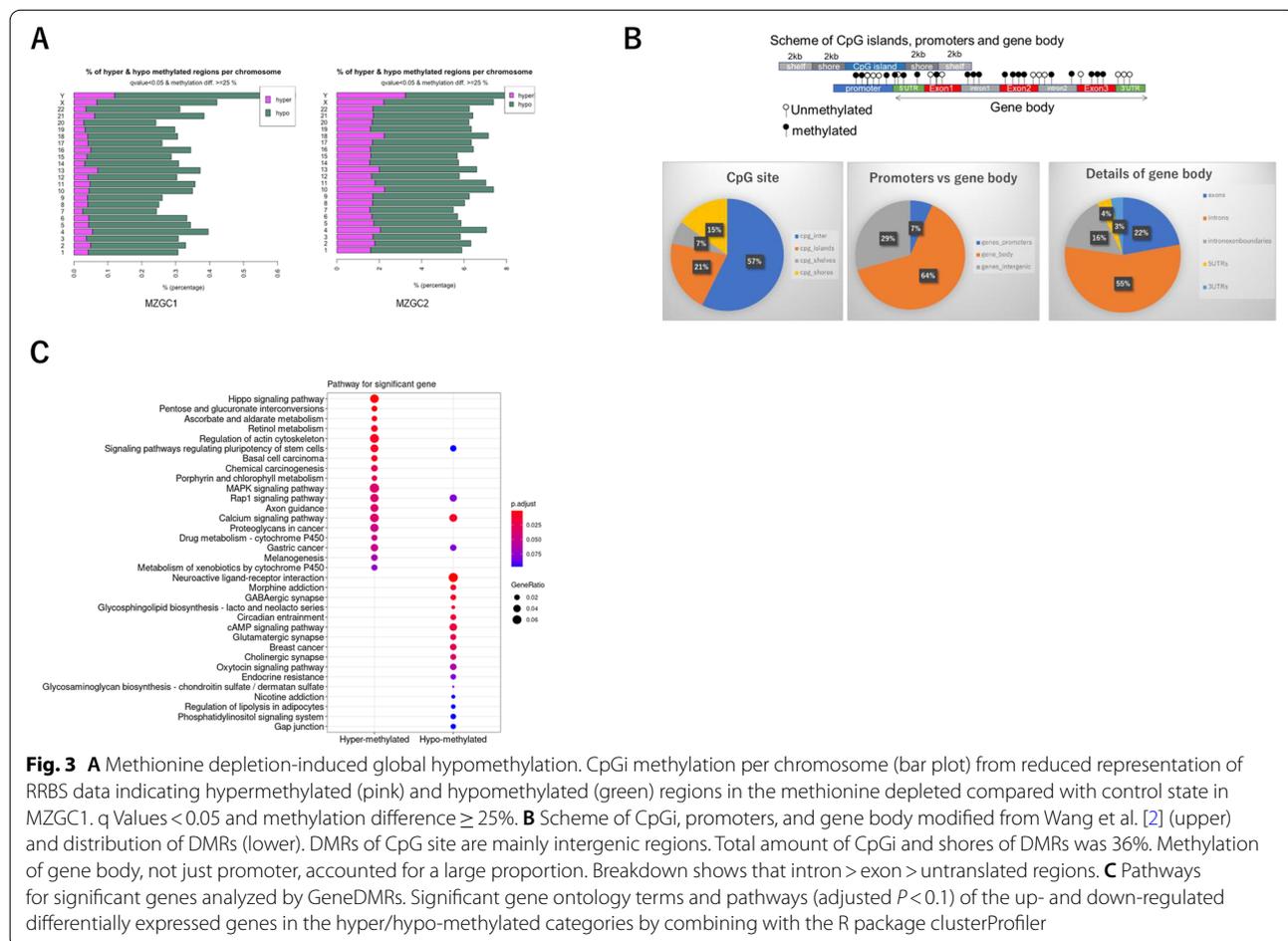
**Fig. 2** **A** Microarray analysis using Metascape. Heat map showing commonly altered pathways in the control and methionine-depleted states of three GICs (*MZGC1*, *MZGC2*, and *MZGC3*). Statistically enriched terms (gene ontology/Kyoto Encyclopedia of Genes and Genomes terms, canonical pathways, hall mark gene sets) are shown with their *P* value. The 20 families of pathways with the lowest  $-\log_{10}$  adjusted *P* value are depicted. **B** Methionine depletion decreased (SAM/SAH ratio), which was measured using an ELISA Combo Kit in MZGC1 and MZGC2. **C** Methionine depletion increased *MAT2A* and *MAT2B* mRNA expression in time-dependent manners. Expression of MAT11, alpha (*MAT2A*) and beta (*MAT2B*), was measured by qPCR after methionine depletion for 6 h, 24 h (day 1), 72 h (day 3), and 144 h (day 6) in MZGC3. **D** Heat map showing average interested gene mRNA expression level from expression array of MZGC1, MZGC2, and MZGC3 using Prism 7. Methionine depletion for 5 days downregulated *TET1*, *TET2*, *DNMT3A*, and *DNMT3B* mRNA expression. **E**: GSEA showing that methionine depletion significantly activated hallmark TNFA signaling via the NFKB pathway. GSEA of differential expression between control and methionine depletion states demonstrated that methionine-depleted GICs were enriched in hallmark TNFA signaling via the NFKB pathway. Heat map of top 20 genes from GSEA analysis is shown on the right. Red column corresponds to upregulated genes and blue column corresponds to downregulated genes

could functionally regulate histone and DNA methylase. Methionine depletion up-regulated *MAT2A* and *MAT2B* in time-dependent manners (Fig. 2C). *DNMT3B*, *DNMT3A*, *TET1*, and *TET2* were down-regulated (Fig. 2D). As shown above, hallmark TNFA signaling via the NFKB is activated in the methionine-depleted state, as we confirmed in GSEA (Fig. 2E). Cell death via apoptosis might result from the activated TNFA signaling pathway (Figs. 1B–D, 2A, and E).

**Methionine depletion leads to pathway-dependent changes in methylation status**

We tried to assess the relationship between transcriptome and DNA methylation status. Epigenetic analysis using RRBS revealed that depletion of methionine promoted genome wide hypomethylation in both promoter and gene body in many cases (Fig. 3A). We analyzed DMRs to identify DMR networks associated with affected pathways using the R package “GeneDMRs.” GeneDMRs can separately analyze the methylation levels within the gene, gene body, CpG island regions (CpGi)

mainly localized in the promoter, and overlapping regions (Fig. 3B). Although there is some degree of variation between cell lines (Fig. 3A), analysis of raw data using GeneDMRs allowed statistically significant pathways to be picked up according to the degree of methylation. Pathway analysis of CpGi revealed that signaling pathways such as regulating pluripotency of stem cells, porphyrin and chlorophyll metabolism, retinol metabolism, regulation of actin cytoskeleton, and MAPK signaling pathway are hypermethylated (Fig. 3C) in the presence of methionine depletion. In contrast, regulation of lipolysis in adipocytes, circadian entrainment, neuroactive ligand-receptor interactions, glutamatergic synapse, cholinergic synapse, and phosphatidylinositol signaling system pathways were hypomethylated. CpGi analysis often classifies pathways as either hyper- or hypomethylated, but often overlap by gene body analysis (Supplementary Figure S3). Pathways classified as either by gene body analysis included focal adhesion and Kyoto Encyclopedia of Genes and Genomes glioma pathway which were hypomethylated (Supplementary Figure S3).



**Fig. 3** **A** Methionine depletion-induced global hypomethylation. CpGi methylation per chromosome (bar plot) from reduced representation of RRBS data indicating hypermethylated (pink) and hypomethylated (green) regions in the methionine depleted compared with control state in MZGC1. q Values < 0.05 and methylation difference ≥ 25%. **B** Scheme of CpGi, promoters, and gene body modified from Wang et al. [2] (upper) and distribution of DMRs (lower). DMRs of CpG site are mainly intergenic regions. Total amount of CpGi and shores of DMRs was 36%. Methylation of gene body, not just promoter, accounted for a large proportion. Breakdown shows that intron > exon > untranslated regions. **C** Pathways for significant genes analyzed by GeneDMRs. Significant gene ontology terms and pathways (adjusted *P* < 0.1) of the up- and down-regulated differentially expressed genes in the hyper/hypo-methylated categories by combining with the R package clusterProfiler

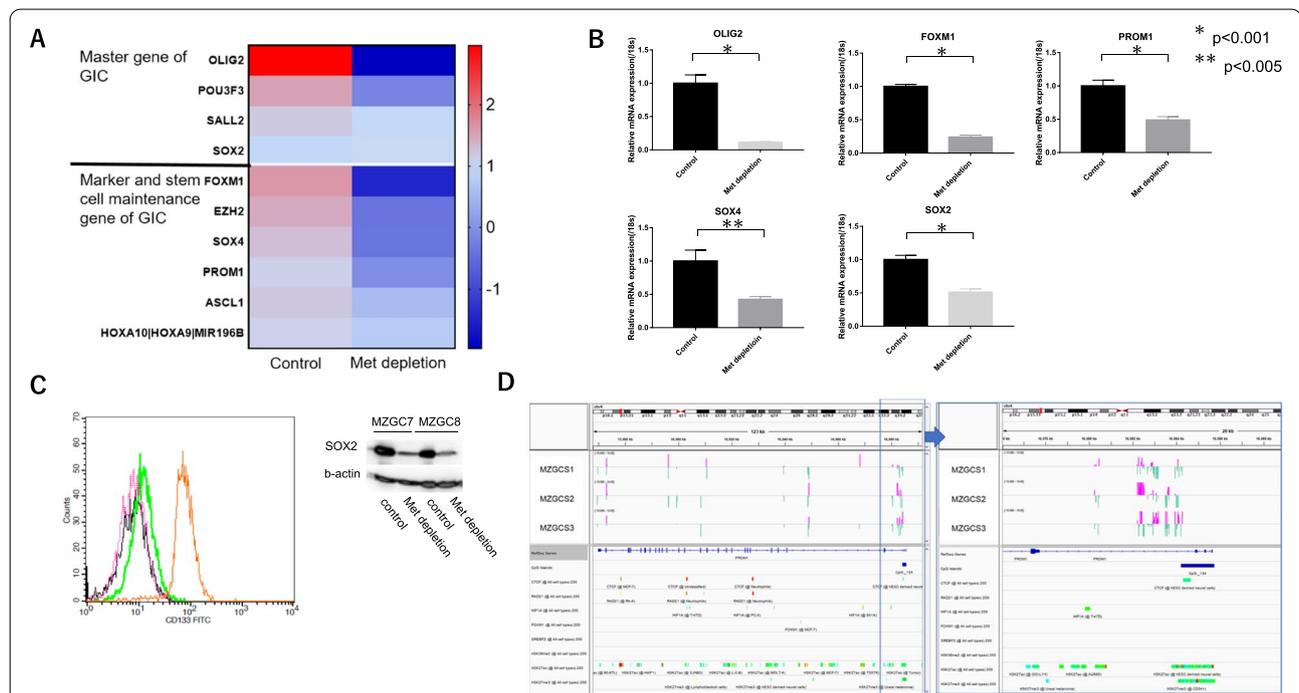
**Methionine depletion changed GIC pluripotency**

RRBS demonstrated the regulating pluripotency of stem cell pathways (Fig. 3C), so the expression of genes related to GIC pluripotency was investigated. Expression array analysis observed down-regulation of OLIG2 gene expression, one of the four core genes of GICs [3, 4], and down-regulation of FOXM1, PROM1, and other marker/maintenance genes of GICs (Fig. 4A). qPCR, FCM and immunoblotting experiments confirmed down-regulation of these markers (Fig. 4B and C). We investigated epigenetic changes of those genes. The CpGi in the promoter and genebody region of PROM1 gene are mainly hypomethylated by the removal of Methionin, while the shore region is mainly hypermethylated. Interestingly, almost the same locus of the gene body was changed in three GICs (Fig. 4D).

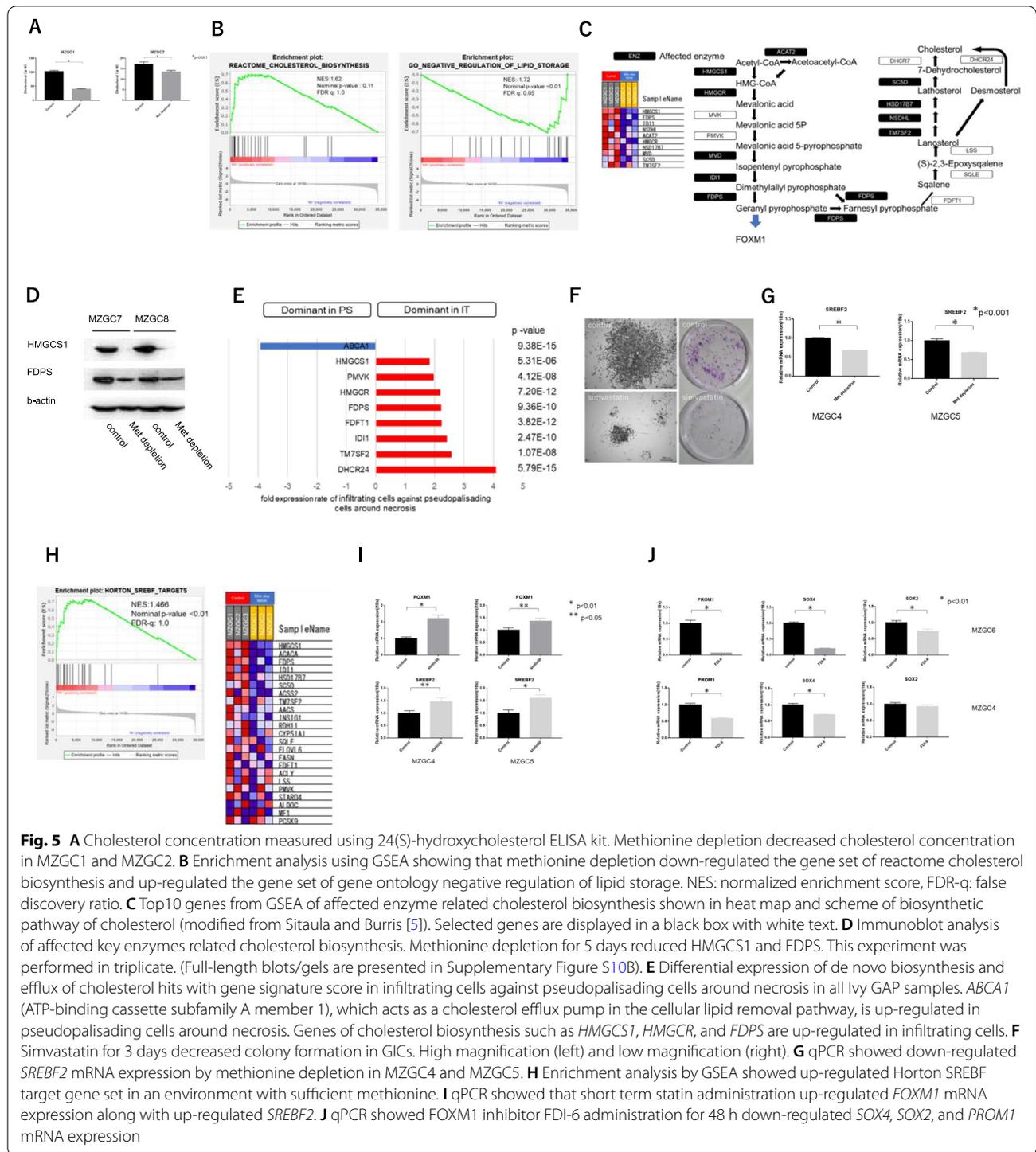
**Methionine targets cholesterol metabolism: methionine removal and statin addition caused similar phenotypic changes in GICs**

Metascape analysis (Fig. 2A) suggested that methionine depletion affects cholesterol metabolism, so a detailed study was conducted. First, we analyzed cholesterol consumption in GICs. Methionine depletion reduced cholesterol in GICs (Fig. 5A). GSEA showed that methionine depletion reduced cholesterol biosynthesis and increased cholesterol excretion pathways (Fig. 5B). The 10 enzymes of cholesterol biosynthesis (HMGCS1, FDPS, ID11, NSDHL, ACAT2, HMGCR, HSD17B7, MVD, SC5D, and TM7SF2) showing greatest decreases are shown in the schema (Fig. 5C) We also confirmed reduced protein level by methionine depletion (Fig. 5D).

Next, we compared the expression levels of genes of cholesterol metabolism within the tumor mass (perinecrotic pseudopalisading area) with the infiltrating area using Ivy GAP database. The genes related to de



**Fig. 4** **A** Microarray results of top 10 master, maintenance, and marker genes changed by methionine depletion. Averages of the interested gene mRNA expression from the expression array are depicted using Prism7. **B** Validation with qPCR of master, maintenance, and marker genes of GICs. Methionine depletion down-regulated OLIG2, SOX2, SOX4, FOXM1, and PROM1 mRNA gene expression in MZGC4. **C** FCM (left panel) and immunoblot analysis (right panel). Methionine depletion decreased markers of GICs CD133 and SOX2. Left panel: CD133 expression in cultured medium with (green, pink) / without (black) methionine in MZGC7. Methionine depletion for 5 days (Green) and 7 days (black) reduced CD133 expression to the same level of isotype control (pink). Orange overlay showed CD133 positive cells as positive control. Right panel: Methionine depletion for 5 days reduced SOX2 expression in MZGC7 and 8. Each experiment was performed in triplicate. (Full-length blots/gels are presented in Supplementary Figure S10A). **D** IGV showing gene locus of PROM1 with DMRs of MZGC1, MZGC2, and MZGC3 along with gene map from Reference Sequence (RefSeq), CpGi, binding site of insulator, transcriptional factors, and histone markers from ChIP-seq database. DMRs by methionine depletion were localized in almost the same locus, where CpGi and gene body occur, in all cell lines. Close-up of the promoter region shows DMRs are localized around CpGi and shores (right). FOXM1 binds to intron of PROM1 gene body, whose histone mark is H3K27ac. Hypermethylated (pink) and hypomethylated (green) in the methionine depleted compared to control states are indicated. Hg19 used as reference genome



novo synthesis of cholesterol were predominantly up-regulated in infiltrating tumor cells compared with tumor cells in the perinecrotic pseudopalisading area (Fig. 5E, Supplementary Figure S4). However, *ABCA1*, which acts as the efflux pump of cholesterol, showed

the opposite distribution. Simvastatin was added to assess whether chemical inhibition of *HMGCR* had same effect with methionine depletion. Simvastatin decreased colony formation, showing a very similar phenotypic change to methionine depletion (Fig. 5F,

Supplementary Figure S5) and down-regulation of the stem cell markers *FOXM1*, *PROM1*, *OLIG2*, and *SOX2* (Supplementary Figure S6A, B and S10C). These results indicated that methionine metabolism is deeply involved in the survival, proliferation, and pluripotency of GICs through cholesterol metabolism.

IGV investigation of the methylation status of each gene found no significant change in gene body or CpGi as in *HMGCS1* (Fig. S7A). We think that the cholesterol metabolic genes might be mainly regulated by another mechanism. We analyzed the transcription factor candidates and insulator binding sites that are likely to bind to the promoter region from the ChIP-seq database. CpGi in the promoter of *HMGCS1* had no DMRs, but *CTCF* and *SREBF2* binding are reported (Fig. S7A). Since *SREBF2* is known to be an important regulator of cholesterol metabolism, we checked the expression of *SREBF2*. *SREBF2* was down-regulated by methionine depletion (Fig. 5G). Moreover, we found multiple genes in the Horton SREBF target gene set were down-regulated by methionine depletion (Fig. 5H). Among the top 10 affected genes related to cholesterol biosynthesis (Fig. 5C), *HMGCS1*, *FDPS*, *ID1L*, *SC5D*, *TM7SF2*, and *HSD17B7* are *SREBF* target genes. Therefore, we thought that *SREBF2* was one of the key molecules linking methionine and cholesterol metabolism. To assess the methylation status of *SREBF2*, we applied the data of RRBS to IGV. DMRs were mainly found in the gene body including 3'-untranslated region (Fig. S7B).

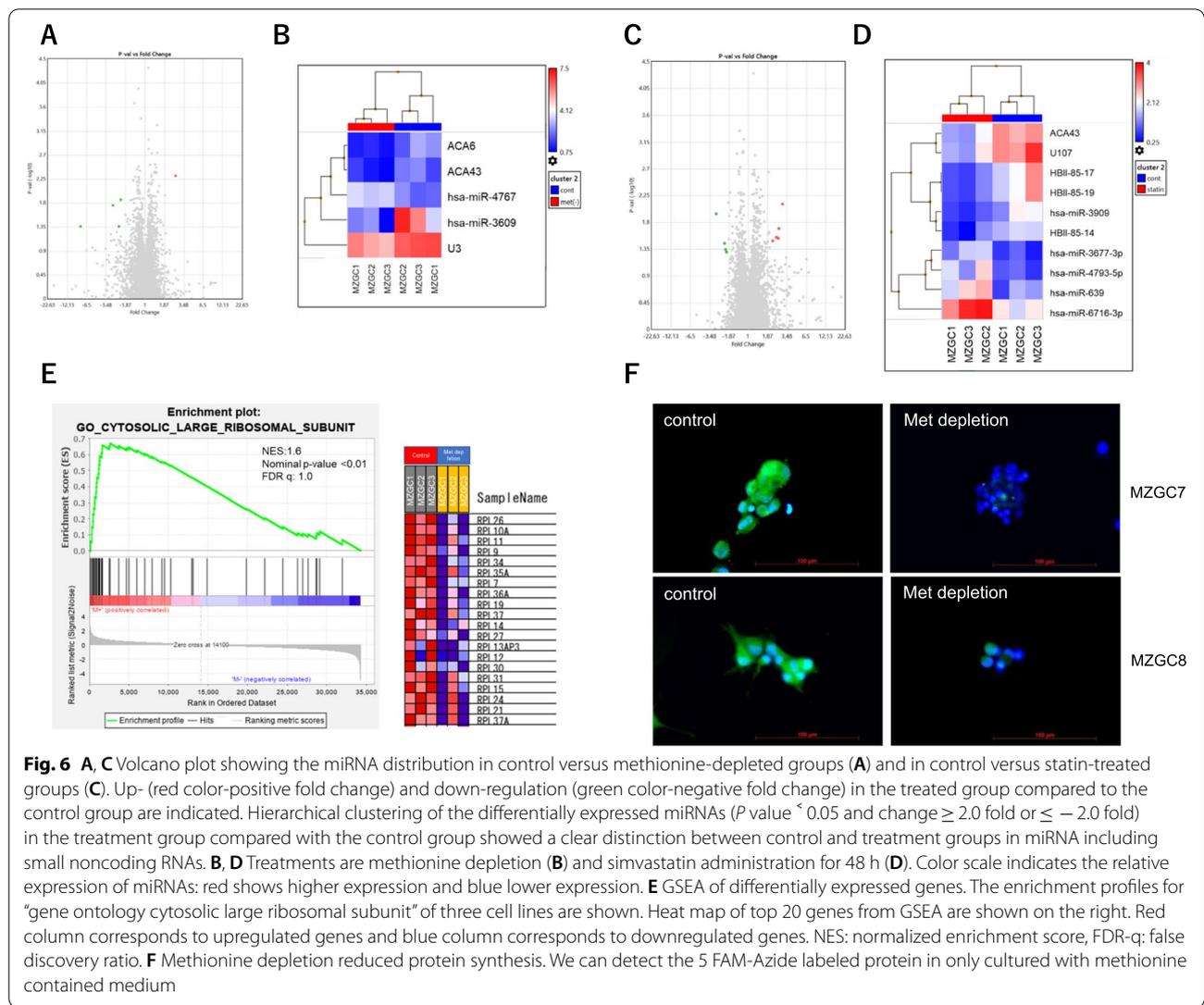
We then considered whether cholesterol metabolism is related to the pluripotency of GICs. *FOXM1*, a maintenance gene of GICs, is also associated with cholesterol metabolism [6]. Thus, we investigated the relationship between cholesterol metabolism and *FOXM1*. *FOXM1* was down-regulated with *SREBF2* by methionine depletion (Figs. 4A, B, and 5G). *SREBF2* increased transiently in the short time after statin administration with feedback that cholesterol production was reduced, but then decreased in the longer term (Fig. 5I). *FOXM1* was up-regulated together with up-regulated *SREBF2* (Fig. 5 I). IGV showed many hypomethylated DMRs in the gene body and CpGi in the promoter of *FOXM1*, which includes the *CTCF* binding site (Fig. S7C). Interestingly, this site has been reported to bind with *SREBF2* (Fig. S7C). To assess whether *FOXM1* regulates the core/marker genes of GICs, we used FDI-6 as a chemical inhibitor of *FOXM1*. FDI-6 down-regulated *SOX4*, *SOX2*, and *PROM1* (Fig. 5J). This observation suggests that methionine metabolism altered the pluripotency of GICs through the cholesterol metabolism to the *FOXM1* axis.

### Methionine also targets protein synthesis

*hsa-miR-33A* has a DNA locus presenting many DMRs, adjacent to the *SREBF2* locus (Fig. S7B), and is known to cause post-translational modifications of genes involved in cholesterol metabolism [7], so we analyzed non-coding RNAs using miRNA arrays. Exhaustive analysis of methionine depletion and statin administration identified one common *SNORA17B* (*ACA43*), but not *hsa-miR-33A* (Fig. 6A–D). *ACA43* is predicted to induce pseudouridylation of U4938 of a 28S rRNA. Most pseudouridylation sites are located within functionally important ribosomal domains, which may affect the functional features of the ribosome. We thought that another common target of methionine and cholesterol metabolism might be a ribosomal protein. Entry of the mRNA expression data into GSEA using ontology gene sets found that gene ontology cytosolic large ribosomal subunit gene expression was extremely upregulated in an environment with adequate methionine (Fig. 6E). Identified RPL genes such as *RPL26*, *RPL10A*, *RPL11*, *RPL9*, and *RPL34* are all constituent proteins of a large subunit of 28S, with the function to bind tRNA during the synthesis of polypeptides. We also confirmed that methionine depletion reduces protein synthesis with OPP assay (Fig. 6F). Since epitranscriptome modifications such as pseudourinalization include methylation, we searched for genes involved in epitranscriptome methylation affected by methionine metabolic reorganization. The transcriptome Venn diagram showed methyltransferase-like protein 16 (*METTL16*), which is related to *MAT2A*, and methyltransferase-like protein 20 (*METTL20*) were predominantly up-regulated by methionine depletion (Table 1).

### Discussion

SAM is the key molecule of transmethylation reactions and polyamine biosynthesis using methionine [8]. The biosynthesis of SAM is catalyzed by three major MATs, I, II, and III [9]. MATI and MATIII are encoded by *MAT1A*. MATII consists of an  $\alpha$ 2 catalytic subunit encoded by *MAT2A* and the  $\beta$ -regulatory subunit encoded by *MAT2B*. MATII functions as a transcriptional corepressor in the oxidative stress response, forming a SAM-integrating transcription regulation module that influences histone methyltransferase activity [10, 11]. We demonstrated that methionine depletion caused decreased SAM and increased *MAT2A* and *MAT2B* in time-dependent manner. As a result, some genes are hypomethylated and others are hypermethylated, although the genome as a whole tends towards hypomethylation. Analysis of signaling pathways to determine what groups of genes are altered also shows a clear distinction between hyper- and hypo- methylation for each pathway. We speculate that



**Fig. 6** **A, C** Volcano plots showing the miRNA distribution in control versus methionine-depleted groups (**A**) and in control versus statin-treated groups (**C**). Up- (red color-positive fold change) and down-regulation (green color-negative fold change) in the treated group compared to the control group are indicated. Hierarchical clustering of the differentially expressed miRNAs ( $P$  value  $\leq 0.05$  and change  $\geq 2.0$  fold or  $\leq -2.0$  fold) in the treatment group compared with the control group showed a clear distinction between control and treatment groups in miRNA including small noncoding RNAs. **B, D** Treatments are methionine depletion (**B**) and simvastatin administration for 48 h (**D**). Color scale indicates the relative expression of miRNAs: red shows higher expression and blue lower expression. **E** GSEA of differentially expressed genes. The enrichment profiles for “gene ontology cytosolic large ribosomal subunit” of three cell lines are shown. Heat map of top 20 genes from GSEA are shown on the right. Red column corresponds to upregulated genes and blue column corresponds to downregulated genes. NES: normalized enrichment score, FDR-q: false discovery ratio. **F** Methionine depletion reduced protein synthesis. We can detect the 5 FAM-Azide labeled protein in only cultured with methionine contained medium

this is probably where the reorganisation takes place, with the intention of gene expression to escape methionine depletion and allow the cell to survive. Although we did not search for the mechanism in this study, we believe that a search for the methylation status of histones and gene expression will be necessary in the future, as there are reports that SAM metabolism affects genome-wide histone methylation and is involved in the maintenance of pluripotent stem cells [12]. Since we expected decreased SAM to result in genomic hypomethylation of the promoter and gene body of *MAT2A*, we entered the RRBS data into IGV. Epigenetic methylation status of *MAT2A* and *MAT2B* were not so changed in spite of the marked increase in mRNA gene expression (Fig. S8). Thus, we searched for genes that affect the epi-transcriptome modulation using microarray results to understand the mechanism that causes the increase in key molecule

*MAT2A* under the methionine-depleted state. We found two up-regulated N6-adenosine-methyltransferases, *METTL16* and *METTL20*, which are involved in the post-transcriptional methylation of internal adenosine residues in eukaryotic mRNAs to form N6-methyladenosine). In agreement with our data, increased *METTL16* occupancy on *hp1* increased *MAT2A* splicing under SAM-limiting conditions [13]. Cells induce *MAT2A* expression by enhanced splicing of a retained intron. Interestingly, *METTL16* also targets *ACA43*, one of the key factors shared by cholesterol and methionine metabolism, as discussed below [14].

Another up-regulated N6-methyladenosine methyltransferase, *METTL20* (also known as electron transfer flavoprotein subunit beta lysine methyltransferase), is a mitochondrial lysine protein methylase that modifies Lys-199 and Lys-202 of the electron transfer flavoprotein

**Table 1** Genes significantly up-regulated with change cut-off at 2.0 fold and P value cut-off at 0.05 in all MZGC1, MZGC2, and MZGC3 during methionine depletion

ISG15	PCNXL2	CDC42E3	JADE2	TIAM2	RP11-513M16.7	SLC3A2	NFKBIA	PTRH2	porvy
forreybo	SMYD3	MREG	EGR1	TAP2	LOC286297RP11-381O7.3vzaby	LL18BP	WARSS	SLC25A19	FP671120.2
RNU11	RP11-309G3.3	IRAK2	VTRNA1-1	IPCEF1	BANCR	werma	CIPCC	<b>METTL16</b>	MIR3687-11MI R3687-2
GMEB1	SNAI4	FAM212	VTRNA1-3	cheeblo	ABCA1	MOB2	SNO RD11.5	C18orf2	FP671120.1
GADD45A	ERRF1	RP11-80H18.4	ARHGA	MAFK	PTPN3	RHOG	LAC TB	CCDC102B	FP236383.8
CHI3L2	TMO4	RP11-362K14.7	KCNIP1	GNPMB	pleyseyby	SPT Y2D	SGF2	skorsar	MAP3K7C
RNU1-120P	ARHGFE2	RP11-255C15.3	F2RL2	RP4-725G10.3	TRUB2	TRMT12	CYL	TCONS	AF129408.17
PRPF3	GEMIN6	RTP4	IRF1	POP7	ASB6	C11orf68	NIP7	ELAC1	PWP2
CHTOP	SMYD5	CHCHD4	fawstoyby	KCND2	MT-TM	CREBZF	KIAA051	CTB-25B13.5	ASPHD2
AURKAP1	MAT2A	OSBPL1	NRG2	RP11-390E23.6	MT-TD	SSS CA1	RP11-473M20.16	ADGRE5	OSBP2
KLHL21	TANK	RYBP	STC2	neegloyby	MT-TK	PYR OX D1	UBALD1	GDF15	snarvaw
niroma	UNC580	RP11-572C15.3	MFAP3	TPK1	MT-TP	<b>METTL2</b>	RSL1D1	PPP1R15A	HIF0
RNU5E-4P	ZFAND2B	RP11-15L13.4	RIOK1	KCTD7	SHROOM2	ATP11	RNR3	DAPK3	MIR6371
SLC6A9	KCNK4	LAMP3	TRIM38	STAG3L5P-PVIRIG2P-PILRB	ZNF674-AS1	RP11-603K19.1	CTD-E14.7	TMED1	RNU12
JUN	RHBDD1	LSG1	AL021918.2	SEMA3A	RGAG1	ELK1	SLC7A5	warjaw	TFP11
RP11-38614.4	SP14OL	CCDC80	AL021918.1	AC100802.3	FUNDC2	NOP	RNR33P2	AKAP8	darves
wabee	SP100	ETV5	PPT2EGFL8	shorshor	sarno	BHLHE41	RNR33P3	SERTA	AL353644.9
RNVU1-19	LINC01173	CHRNA9	MADZL1BP	RP11-705O24.1sswakor	RAB39B	SLC38A1	RNM TL1	STRN4	MIR3687-11MI R3687-2hsa-mir-3687-1
MIR5087	RP11-111J6.2	LINC01091	sherjey	NOV	RP11-131M6.1	yanemi	MIR4521	CCDC130	AL592188.2
narreebo	ZNF512	shoykloyby	NCOA7	PVT1MIR1204	MYPN	ITG A5	ADP RM	PVR	SURF6
PI4KB	RP11-78114.1	TDO2	ULBP1	DUSP4	IFT2	DUSP6	MAP2K3	GRWD1	
S100A3	RNU2-63P	TLR3	SYNJ2	BRF2	IFT3	NUAK1	HEXIM1	SLC39A4	
S100A2	AC108463.2	woysperb	C6orf52	THAP1	IFT5	RAB35	ITGA4	U2AFIL4	
SMG5	NMI1A	PPARGC1A	AL021917.1	rawarby	C10orf2	ANKLE2	RP11-199F11.2	ABALON	
GPATC4	ABC A12	SLC7A11	HER3	AC024995.1	DUSP5	USP LI	NRI1	AAR2	
DEDD	RNF25	TMEM154	GPANK1	KB-1460A15	NPS	KLF5	mir	AC005220.3	
RABIF	SCG2	TLR1	RP3-437C15.1	UBAP1	RP11-479G22.8	NXT1P1	CTD-3014M21.1	OGFR	
RBBP5	SP110	RP11-79P5.10	NDUFA4	PRPF4	SVILP1	LIG4	ETV4	TRMT6	
LAMB3	PER2	RP11-455B3.1	ARHGA	TOR1B	PNPLA2	C14orf19	ATX N7L3	JAG1	
TAF5L	RSA D2	CTC-487M23.6	gublor	ketora	TMEM138	TRMT6-1A	RP11-1072C15.4	BCL2L1	

METTL16 and METTL20 are depicted in red

subunit beta, which is a mobile electron carrier coupling mitochondrial fatty acid oxidation to respiration [15, 16]. Investigation using the knockout mouse showed *METTL20* is a positive regulator of beta-oxidation. Beta-oxidation is known as a metabolic node involved in glioma-genesis [17], and also provides an alternate source of ATP in nutrient unfavorable conditions. Our observation about *METTL20* may represent the steps to obtain ATP.

Acetyl CoA is an important intermediate in the metabolism of lipids for energy storage and supply, from which fatty acids and cholesterol are synthesized. As mentioned above, beta-oxidation of fatty acids may be affected by methionine deprivation, but the metabolism of cholesterol was also greatly affected. Methionine depletion downregulates cholesterol biosynthesis and increases excretion. Furthermore, methionine depletion and statin administration showed similar phenotypic changes in GICs. These observations suggest that cholesterol metabolism is necessary to maintain the pluripotency of GICs. Biosynthesis of cholesterol is tightly regulated by feedback pathways because cholesterol is critical for cell growth and function. Cholesterol is important as a component of the plasma membrane and lipid rafts, and as a precursor for steroid hormones, bile acids, and vitamin D [18, 19]. Other organs are more dependent on cholesterol supply from the bloodstream, whereas the brain obtains cholesterol exclusively from de novo synthesis, which involves the mevalonate and Bloch and Kandutsch-Russell pathways [20–22].

Within a tumor mass, imbalances in methionine concentration as well as oxygen concentration should exist. Using the Ivy GAP database, we compared the expression of genes involved in cholesterologenesis in the pseudopalisading cells and infiltrating tumor portions [23]. Our finding that cholesterologenesis was higher in the infiltrating tumor than in the pseudopalisading cell areas was consistent with the results of in vitro experiments. De novo synthesis is mediated by the transcriptional activity of sterol regulatory element binding proteins (SREBPs), which promote the transcription of enzymes involved in the cholesterol and fatty acid biosynthetic pathways, such as *FDPS* and *HMGCR*. When cholesterol levels fall below homeostatic levels, SREBP cleavage activating protein is isolated from insulin-inducible genes (*INSIG1*, *INSIG2*) resident in the endoplasmic reticulum [18, 24]. The SREBP is then transported to the Golgi, where the active site is cleaved and activated as a transcription factor [25]. Among the three types (SREBP1a, SREBP1c, and SREBP2), SREBP2, which is encoded by the *SREBF2* gene, mainly regulates genes involved with cholesterol biosynthesis. *SREBF2* is upregulated with statin treatment by feedback but down-regulated by methionine depletion

(Fig. 5G and J). Such downregulation of *SREBF2* under methionine depletion is thought to be the main cause of the downregulation of SREBF target genes of cholesterol biosynthesis.

Other groups have evaluated the effects of SREBPs on glioblastoma multiform development as a key transcription factor in the regulation of sterol homeostasis [26, 27]. Under hypoxia and serum deprivation conditions, SREBP is upregulated to maintain the expression of fatty acid and cholesterol biosynthesis genes in glioblastoma multiform cells, and inhibition of SREBP activity under hypoxia results in the death of glioblastoma multiform cells [18, 27]. So how does *SREBF2* decrease under methionine depletion? We cannot determine the detailed mechanism, but the gene body of *SREBF2*, especially the 3' side including the hsa-miR-33A locus, is dramatically hypomethylated, and this might be the cause of downregulation of *SREBF2*. The *hsa-miR-33A* is also known as regulator of cholesterol efflux through *ABCA1* gene expression [28].

To identify which miRNA contributes to the association between cholesterol metabolism and methionine depletion, we decided to explore the commonalities of changes in the miRNA array with methionine depletion and statin administration. Unexpectedly, only *ACA43* but not *hsa-miR-33A* was downregulated in both arrays. *ACA43* belongs to the H/ACA box class of small nucleolar RNAs based on the predicted hairpin-hinge-hairpin-tail structure and a conserved H/ACA-box motif. *ACA43* is predicted to induce pseudouridylation of U4938, a 28S rRNA. Pseudouridylation is the isomerization of the nucleoside uridine to a different isomer, pseudouridine. Interestingly, *ACA43* was originally cloned from HeLa cells [29] and is associated with the GAR1 protein, one of the components of the telomerase complex [30]. Taken together, these reports and our findings may indicate that cholesterol metabolism and methionine metabolism are also involved in cell immortalization via *ACA43*. At least, cholesterol and methionine metabolism altered the translation of mRNA through downregulated *ACA43*. Translation was also suppressed through down-regulation of the large subunit of ribosomal protein 28S by methionine depletion (Fig. 6E and F). The novel anticancer drug rocaglamide A, which inhibits translation by binding to the translation initiation factor eIF4A, inhibits the proliferation of target cancer cells, suggesting that translation inhibition due to methionine depletion may have inhibited the growth of GICs [31, 32].

The final question is whether cholesterol metabolism is related to the apoptosis and pluripotency of GICs. Pharmacological inhibitors acting downstream of the mevalonate pathway induce cell death in glioma and pluripotent stem cells [33, 34]. Inhibition of *FDPS* by

pharmacological inhibitors and small interfering RNA alters the self-renewal of glioma stem cells [35]. *HMGCS1* contributes to gastric cancer progression through activating *Oct4* and *SOX2* promoters [36]. These observations support our findings that methionine depletion promotes down-regulation of *SOX2*, *OLIG2*, *PROM1*, and *FOXM1* along with down-regulation of cholesterol de novo biosynthesis. *FOXMI* had a positive correlation with mevalonate pathway-related genes including *HMGCR* and *SREBP2* in patients with hepatocellular carcinoma. Moreover, knockdown of *HMGCR* reduced *FOXMI* expression in hepatocellular carcinoma [6]. We observed *FOXMI* was increased by statin administration for a short period and decreased by methionine depletion along with *SREBF2*. Moreover, IGV showed first hypo-methylated gene body and CpGi of the *FOXMI* promoter by methionine depletion and the *SREBF2* binding site in CpGi of the *FOXMI* promoter. These findings strongly suggest that methionine down-regulates *FOXMI* through *SREBF2*. Since *FDPS* and *HMGCS1* are targets of *SREBF2*, *SREBF2* might be a key molecule-connecting cholesterol metabolism and pluripotency of stem cells. Finally, we found that the chemical inhibitor of *FOXMI* downregulates core and marker genes of GICs. Taken together, we thought *SREBF2-FOXMI* are important regulators of the core/marker genes in GICs (Fig. S9).

In conclusion, we found that methionine metabolism is closely related to cholesterol metabolism and ribosomal function, resulting in alteration of pluripotency, proliferation, and avoidance of apoptosis in GICs.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12885-022-10280-5>.

**Additional file 1: Supplementary Table S1.** RRBS libraries and Sequence data generated in this study.

**Additional file 2: Supplementary Table S2.** Primers sequence used in this study.

**Additional file 3: Supplementary Figure S1.** A Fluorescence-activated cell sorter analysis to detect cell cycle distribution. Propidium iodide (PI) staining assay using a Cell Cycle Phase Determination Kit was performed on MZGC6 cells. Cell cycle analysis showed that methionine depletion for 3 days increased sub-G1 and decreased S, G2M populations. B Dye exclusion test showed that methionine depletion (for 5 and 7 days) and statin treatment (for 1 and 2 days) increased dead cell rate in MZGC7. C Fluorescence-activated cell sorter analysis using annexin-V kit showed that methionine depletion induced apoptotic cell death. **Supplementary Figure S2.** Microarray analysis using Metascape. Heat map showing commonly altered pathways in the control and methionine-depleted states of three GICs (MZGC1, MZGC2, and MZGC3). Statistically enriched terms (gene ontology/Kyoto Encyclopedia of Genes and Genomes terms, canonical pathways, hallmark gene sets) are given with their *P* value. The 100 families of pathways with the lowest  $-\log_{10}$  adjusted *P* value are depicted. **Supplementary Figure S3.** Significant gene ontology terms and pathways for significant genes analyzed with gene body methylation status by GeneDMRs. Significant gene ontology terms and pathways (adjusted *P* < 0.1) of the up- and down-regulated differentially expressed

genes in the hyper/hypo-methylated categories by combining with the R package clusterProfiler. **Supplementary Figure S4.** Z-score of gene expression from Ivy GAP. Gene expression related de novo synthesis of cholesterol was upregulated in infiltrating tumor cells compared with pseudopalisading cells around necrosis. In contrast, the gene expression pattern related to efflux of cholesterol was exactly the opposite. **Supplementary Figure S6. A** qPCR of stem cell markers in MZGC4 and MZGC5. Statin treatment for 120 h down-regulated mRNA expression of *FOXM1*, *PROM1*, *OLIG2*, and *SOX2*. **B** Immunoblotting analysis showed that statin treatment for 2 days reduced SOX2 protein level as well as methionine depletion for 7 days in MZGC7 and 8. (Full-length blots/gels are presented in **Supplementary Figure S10C**). **Supplementary Figure S7. A** IGV showed few DMRs in promoter and gene body of HMGCS1. CTCF/RAD21, HIF1a, and SREBF2 binding site are reported in the CpG island of promoter, where histone marks are H3K27Ac and H3K27me3. **B** IGV showed the DMRs of SREBF2. DMRs of promoter were not found, but the binding of insulator CTCF and HIF1a were identified. DMRs were mainly localized in the gene body in the 3' side of the gene body, especially near the hsa-miR-33A locus. **C** IGV showed DMRs of *FOXM1*. Many DMRs are found in both gene body and promoter. CTCF, HIF1a, and SREBF2 are reported to bind to the lesion of DMRs in promoter, and H3K27ac may be a histone mark. **Supplementary Figure S8.** IGV showed few DMRs in the promoter and gene body of *MAT2A* and *MAT2B*. **Supplementary Figure S9.** Graphical summary. Interaction of the major factors and events by reorganization of methionine metabolism. **Supplementary Figure S10.** Full-length blots/gels and cropped lesions of Fig. 4C (A), Fig. 5D (B) and **Supplementary Figure S6B (C)** were indicated.

**Additional file 4.** Supplementary methods.

**Additional file 5:** Supplementary video **Figure S5**.

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#### Authors' contributions

Conceptualization and design of the study: K.Y., Data analysis and interpretation: K.Y., T.K., T.W., Y.N., S.Y., A.M. and H.T. Investigation: K.Y., T.W., Y.N. Validation: S.Y., A.M., Resources: K.Y. and T.K. Writing—original draft: K.Y. Writing—review and editing: T.K., T.W., Y.N., S.Y., A.M. and H.T. The author(s) read and approved the final manuscript.

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#### Availability of data and materials

The raw sequencing data have been deposited to the DNA Data Bank of Sequence Read Archive under the BioProject PRJDB12471 (<https://www.ebi.ac.uk/ena/browser/view/PRJDB12471>) with run accession numbers DRR322659, DRR322660, DRR322661, DRR322662, DRR322663 and DRR322664. (Supplementary Table S1) The data discussed in this publication are also available in GEA under the accession number E-GEAD-458.

#### Declarations

##### Ethics approval and consent to participate

This study was performed under the approval of the Institutional Review Board of Miyazaki University Hospital (Nos. 2015–518-4 and 09–0130-3), and all experiments were performed in accordance with relevant guidelines and regulations. This study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients for the usage of samples.

##### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

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