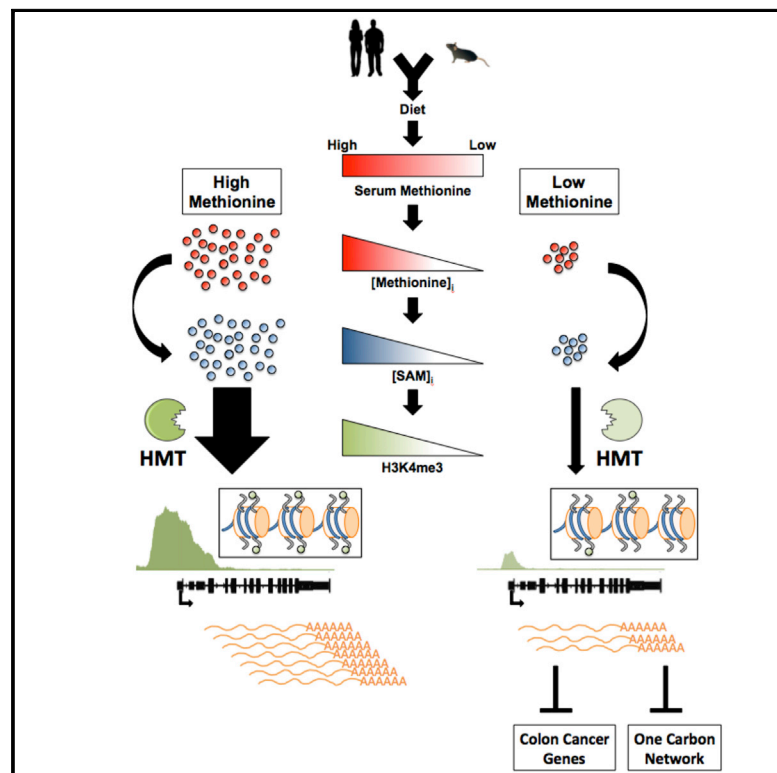


Cell Metabolism

Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism

Graphical Abstract



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In Brief

Mentch et al. show that modulation of methionine metabolism, by altering nutrient availability, regulates SAM and SAH levels to drive specific histone methylation events that affect gene expression. Methionine cycle alterations can be sustained by diet in vivo and modulate histone methylation in the liver.

Highlights

- Histone methylation dynamics occur in response to changes in SAM and SAH
- Metabolism-regulated histone methylation affects gene expression
- Diet alters methionine metabolism and histone methylation in the liver
- Variation in human serum MET occurs at levels needed to alter histone methylation

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Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism

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SUMMARY

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) link one-carbon metabolism to methylation status. However, it is unknown whether regulation of SAM and SAH by nutrient availability can be directly sensed to alter the kinetics of key histone methylation marks. We provide evidence that the status of methionine metabolism is sufficient to determine levels of histone methylation by modulating SAM and SAH. This dynamic interaction led to rapid changes in H3K4me3, altered gene transcription, provided feedback regulation to one-carbon metabolism, and could be fully recovered upon restoration of methionine. Modulation of methionine in diet led to changes in metabolism and histone methylation in the liver. In humans, methionine variability in fasting serum was commensurate with concentrations needed for these dynamics and could be partly explained by diet. Together these findings demonstrate that flux through methionine metabolism and the sensing of methionine availability may allow direct communication to the chromatin state in cells.

INTRODUCTION

Alterations in the methylation status of proteins, nucleic acids, and metabolites contribute to the pathogenesis of many of the major human pathophysiological conditions including cancer, obesity, and aging (Bergman and Cedar, 2013; Greer and Shi, 2012; Kraus et al., 2014). When these changes affect the methylation of histones and nucleic acids that determine the epigenetic status in cells, they can affect the expression of thousands of

genes (Barth and Imhof, 2010). Changes in methylation status occur because of differences in the enzyme activity of methyltransferases and demethylases. Genes that encode these enzymes are frequently altered in pathological states, leading to alterations in methylation (Chi et al., 2010; Dawson and Kouzarides, 2012). It has also been long established that S-adenosylmethionine (SAM) is the universal methyl donor for these enzymes that transfer its methyl group to yield S-adenosylhomocysteine (SAH) and a methylated substrate (Finkelstein, 1990). The methylation of this substrate provides a link between the metabolism that regulates SAM and SAH, which may act through product inhibition of a methyltransferase, and the epigenetic status of cells (Gut and Verdin, 2013; Katada et al., 2012; Teperino et al., 2010).

SAM and SAH are intermediate metabolites in a metabolic pathway that is a subset of a larger network collectively referred to as one-carbon metabolism (Locasale, 2013). One-carbon metabolism integrates nutrients from diverse sources such as glucose, serine, threonine, methionine, and choline and processes them into distinct outputs that achieve diverse biological functions. Whether the concentrations of SAM and SAH or their ratio ever reach values that could affect methyltransferase activity has been controversial. Some studies have concluded that their concentrations do not reach limiting values (Hoffman et al., 1979). Recent studies, however, have provided evidence that aberrant expression of NNMT, an enzyme that metabolizes SAM, has profound biological consequences resulting from changes in histone methylation (Kraus et al., 2014; Ulanovskaya et al., 2013). Others have found that only the levels of SAH correlated with methylation status (Caudill et al., 2001), including a recent finding that investigated threonine metabolism in mouse pluripotent stem cells and demonstrated that threonine catabolism affected both pyruvate and glycine metabolism and altered histone methylation (Shyh-Chang et al., 2013). Although this study was the first to our knowledge to document the influence of an amino acid on histone methylation, this effect was shown to occur through indirect pathways involving energy production

and acetyl-coA metabolism. Further studies have demonstrated in human pluripotent stem cells that a depletion of methionine, the precursor to SAM, could lead to changes in histone methylation (Shiraki et al., 2014). However, these changes are also accompanied by widespread induction of stress response pathways and cell death, confounding the interpretation of whether the changes in histone methylation occurred through the sensing of SAM/SAH status.

Given these previous findings, we hypothesized that there exists a direct mechanism whereby the status of one-carbon metabolism could alter the concentrations of SAM and SAH to confer, through their interaction with methyltransferases, the output of a defined methylation state. We focused on histones because key methylation modifications on their tails, such as trimethylation at lysine 4, are known to be required for the maintenance of defined cellular states (Benayoun et al., 2014; Ruthenburg et al., 2007) and have been shown to be modulated by metabolism (Shiraki et al., 2014; Shyh-Chang et al., 2013). We provide evidence in cells and mice that both SAM levels and the SAM/SAH ratio can be quantitatively tuned through changes in the metabolic flux of the methionine cycle to affect a critical component of chromatin status. This regulation occurred at physiologically relevant concentrations and appeared to control numerous physiological processes, including direct feedback regulation for the maintenance of homeostasis in one-carbon metabolism and the activity of genes involved in cancer and cell fate. Together these findings are consistent with a model whereby the status of one-carbon metabolism is in communication with the chromatin state of cells through its ability to modify the kinetics of enzymes that mediate histone methylation.

RESULTS

Methionine Metabolism Quantitatively Affects Histone Methylation

Because methionine is the closest substrate in the methionine cycle and the one-carbon metabolic cycle that affects SAM levels, we tested whether cells depleted of methionine have alterations in the methionine cycle and whether this would confer effects on the methylation of histones. We generated a media formulation in which methionine was restricted from the culture media. HCT116 cells were placed in this methionine-restricted (MR) (3 μ M) media for 24 hr. We then used a liquid chromatography, high-resolution mass spectrometry (LC-HRMS) metabolomics technology we have recently developed (Liu et al., 2014b) to generate a quantitative profile of more than 300 metabolites in response to methionine restriction that is visualized in a volcano plot (Figure 1A). It was found that metabolites in the methionine cycle exhibited dramatic changes in their concentrations, with only moderate compensation from other pathways that fuel the one carbon cycle (Figure 1B). Notably, both SAM and SAH are depleted under these conditions. To test whether these alterations were sufficient to induce changes in histone methylation, we considered the relative levels of several histone methylation marks involving trimethylation at lysines 4, 9, and 27 that are each known to have substantial roles defining chromatin states, most notably at active and inactive genes, and mediating gene expression (Shilatifard, 2006) (Figure 1C). In addition, the kinetic properties, such as SAM binding affinity (K_m), of these his-

tone methyltransferases (An et al., 2011; Chin et al., 2005; Horiuchi et al., 2013; Obianyo et al., 2008; Patnaik et al., 2004; Xiao et al., 2003) suggest that SAM concentration may play a role in their regulation and directly affect the rate of histone methylation reactions in the cell (Figure S1A). Each modification exhibited decreased methylation, with trimethylation at lysine 4 on histone H3 (H3K4me3) exhibiting the largest change. To investigate whether these changes were specific to methionine restriction, we considered the removal of several other amino acids (W, Q, K, H, and L) that include essential amino acids and glutamine, which has been shown to be essential for cell proliferation (Figure 1D). We found that in each case no change in histone methylation was observed. To further test the generality of this observation, we considered the response of H3K4me3 to methionine restriction on a panel of six human cell lines subjected to methionine restriction for 24 hr. In each case, H3K4me3 was responsive to methionine restriction (Figure 1E). Additionally, we observed decreases in H3K9ac after methionine restriction (Figure S1B). Having shown that extreme restriction of methionine is sufficient to alter histone methylation, we investigated the concentration of methionine needed to achieve this effect. We considered a titration of differing concentrations of methionine in the culture media ranging from 3–500 μ M. The relative levels of the methionine cycle and one-carbon metabolism-related metabolites, including methionine, SAM, SAH, homocysteine, betaine, and dimethylglycine (DMG), were measured (Figure 1F). It was found that the levels of these intermediates exhibited a graded response to changes in methionine concentration in the media. SAM and SAH were altered at concentrations below 25 μ M, concentrations well below those present in typical culture media. We next investigated the dose response of changes in histone methylation to changes in methionine availability (Figure 1G). Histone methylation responded differently to differing concentrations of methionine. H3K4me3 was affected at concentrations between 10 and 25 μ M, consistent with concentrations needed to deplete SAM and SAH in the methionine cycle. One possibility is that these changes were due to non-specific effects on cell proliferation. We therefore considered the effects of methionine restriction on cell proliferation over a course of 3 days (Figures 1H and S1C). At low concentrations of methionine, there were marked defects in cell morphology and cell proliferation but no activation of p53 when H3K4me3 was changing (Figures S1C and S1D), indicating that H3K4me3 dynamics occur before a cellular stress response. At 10 μ M, cells remained viable, with no gross alterations in morphology and only a modest decrease in cell proliferation that was fully recoverable at 25 μ M, confirming that the changes in histone methylation are not likely due to defects in cell proliferation. Together these findings demonstrate that methionine affects histone methylation and the dynamics of the methionine cycle.

Methionine Cycle and Histone Methylation Dynamics in Response to Methionine Restriction

Having observed alterations in the methionine cycle and concomitant changes in histone methylation, we sought to understand the dynamics of the process and its connection to cellular metabolism. We reasoned that the kinetics could provide further insights into how changes in metabolism alter histone methylation.

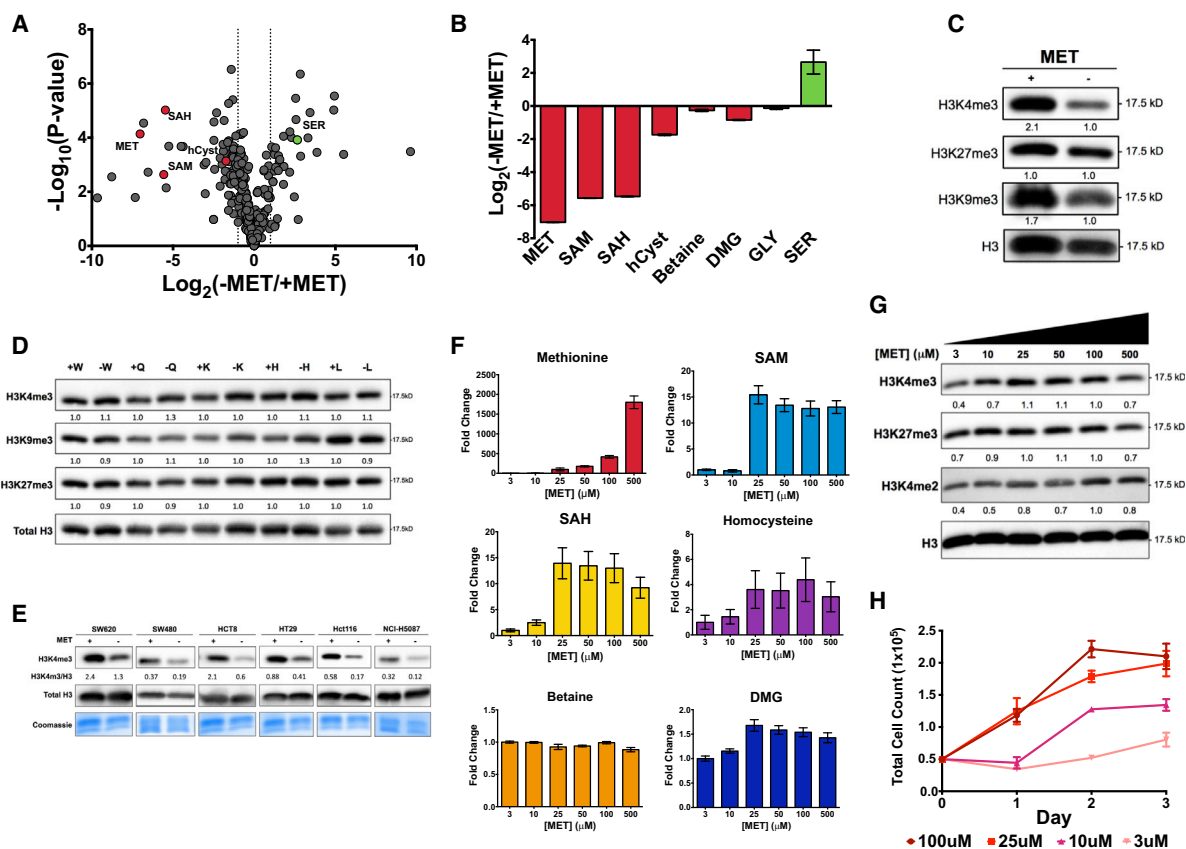


Figure 1. Methionine Metabolism Alters Histone Methylation Status

(A) Metabolomics profile of MR HCT116 cells. Log_2 fold change versus $-\log_{10}$ (p value) (Student's t test, two-tailed, $n = 3$).

(B) Effects of methionine restriction on one-carbon cycle metabolism.

(C) Effects of methionine restriction on histone methylation measured by immunoblotting. Band intensities are normalized to total H3 levels, and relative intensity was calculated compared with control cells.

(D) Effects of deprivation of other amino acids on histone methylation. Data are normalized to control media.

(E) Effects of methionine restriction on histone methylation across a panel of cell lines.

(F) Relative concentration of methionine cycle metabolites in cells cultured in different concentrations of methionine.

(G) Concentration-dependent effects of methionine restriction on histone methylation. Data are normalized to the 100 μM condition.

(H) Cell proliferation of HCT116 cells for differing levels of methionine.

All error bars are computed from SEM ($n = 3$), all quantitation is normalized to the total H3, and the fold change between experimental and control groups are reported.

We considered the metabolic dynamics of methionine restriction across 12 time points (0, 5, 15, 30, 60, 90, 120, 240, 360, 540, 720, and 1,440 min), from very early time up to times leading to cell death (Figure 2A). At each of these time points, we profiled more than 300 metabolites spanning pathways across central carbon and secondary metabolism. A hierarchical clustering of the time course revealed coordinated dynamics across the metabolome in response to methionine restriction. Waves of coordinated changes that involve different metabolic pathways were observed. We used a recently developed algorithm for assessing the contribution to different pathways (i.e., pathway impact) defined by the Kyoto Encyclopedia of Genes and Genomes (Xia and Wishart, 2010) (Figure 2B). Pathway impact is computed by considering a weighted score of each metabolite, with greater weights being assigned to metabolites that are centrally located in a given metabolic pathway. At early times, a module related to cysteine and methionine metabolism

exhibited decreasing dynamics that were sustained across the time course. At intermediate times, alterations in metabolites belonging to phenylalanine, tryptophan, and lysine metabolism were observed. At later times, arginine and proline metabolism appeared altered together, suggesting that a complex compensatory amino acid response at later times occurs after the primary response to methionine restriction. Interestingly, pathways such as glycolysis, the tricarboxylic acid cycle, and anabolic metabolism associated with nucleotide and lipid metabolism appeared not to exhibit coordinated dynamics, suggesting that general effects on metabolism and cell proliferation do not appear altered throughout the kinetics. A further inspection of the module related to methionine cycle dynamics revealed that within minutes of methionine restriction, methionine was depleted within cells (Figure 2C). SAM levels exhibited a decay that occurred within 1 hr (Figure 2D). SAH levels exhibited more complex behavior involving a fast decrease, a recovery

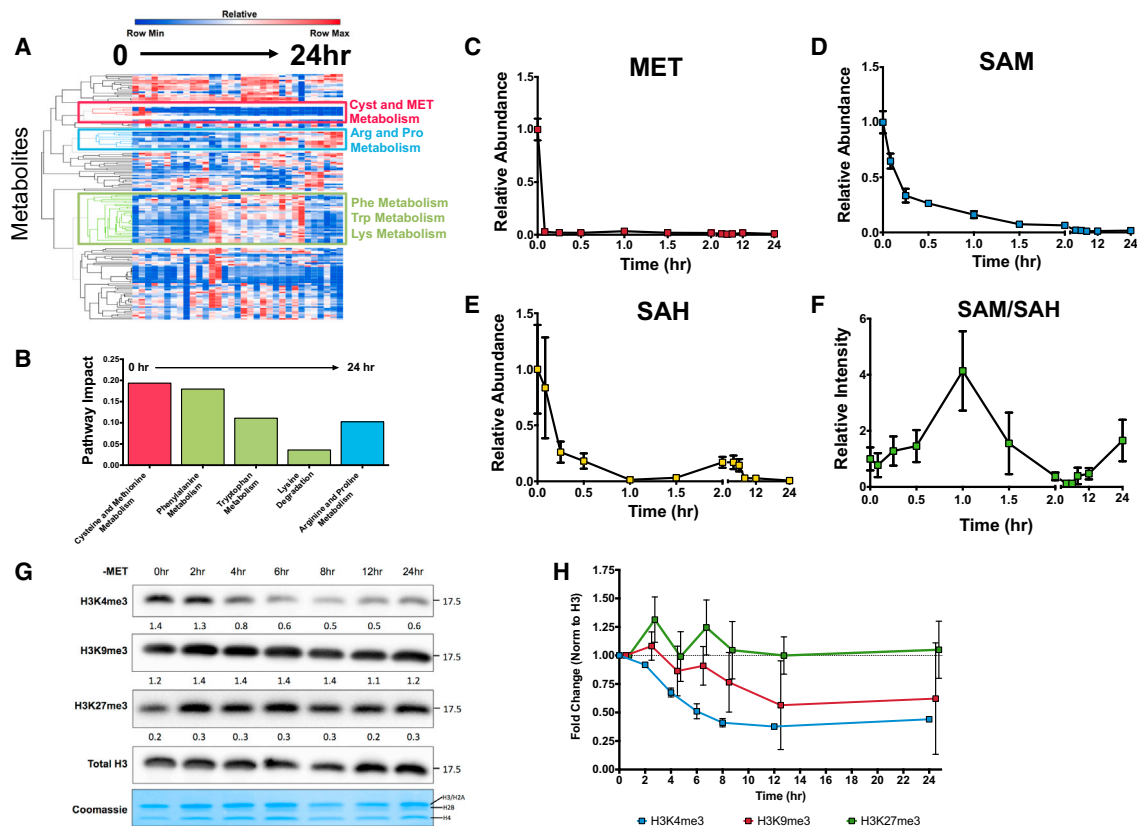


Figure 2. Histone Methylation Is Dynamically Regulated by Methionine Metabolism

(A) Global metabolic dynamics in response to methionine restriction.

(B) Pathway analysis of dynamics shown in (A).

(C) Dynamics of methionine.

(D) Dynamics of SAM.

(E) Dynamics of SAH.

(F) Dynamics of the SAM/SAH ratio.

(G) Dynamics of histone methylation.

(H) Quantitation of results in (G) and two other independent experiments.

Integrated intensities are normalized to total H3, and the fold change represents differences compared with 24 hr. All error bars are computed from SEM ($n = 3$).

at 2 hr, and a later decrease (Figure 2E). These dynamics are likely due to additional pathways, such as remethylation from one-carbon metabolism, methionine salvage, and flux into the transsulfuration pathway. These changes contributed to complicated dynamics of the SAM/SAH ratio that decreased at 2 hr (Figure 2F). In addition, 5-methyltetrahydrofolate did not contribute to the methionine cycle, as measured by ^{13}C serine, indicating that the folate cycle is largely uncoupled to the methionine cycle in these cells (Figure S2). We next monitored the dynamics of histone methylation to assess its interaction with metabolic dynamics. We considered the dynamics of the levels of H3K4/9/27 in response to methionine restriction (Figures 2G and 2H). Surprisingly, it was found that within 2–4 hr, a depletion of histone methylation was observed. These kinetics exhibited only a 1–2 hr lag between depletion of the methionine cycle and the subsequent changes in histone methylation. These dynamics are generally too short for a transcriptional or translational regulatory response. Together, these findings suggest that the dynamics of methionine metabolism result primarily in amino

acid compensatory responses and not general features that lead to disruption of cell proliferation. They also indicate that methionine dynamics can be coordinately sensed by histones to determine the levels of histone methylation.

Methionine Metabolism and Histone Methylation Dynamics Are Reversible

Thus far we have shown that methionine metabolism can induce alterations in histone methylation levels and that this effect is dynamic and occurs on timescales consistent with that of a direct sensing mechanism. Another necessary requirement of such a signal transduction mechanism is that the dynamics be reversible. We therefore questioned whether methionine and histone methylation can be recovered after methionine restriction. Cells were subjected to methionine restriction for 24 hr (between day 1 and day 2), at which point we allowed the cells to be cultured for an additional 24 hr in media containing the full concentration of methionine (between day 2 and day 3) (Figure 3A). We carried out metabolomics at each time point, as considered previously

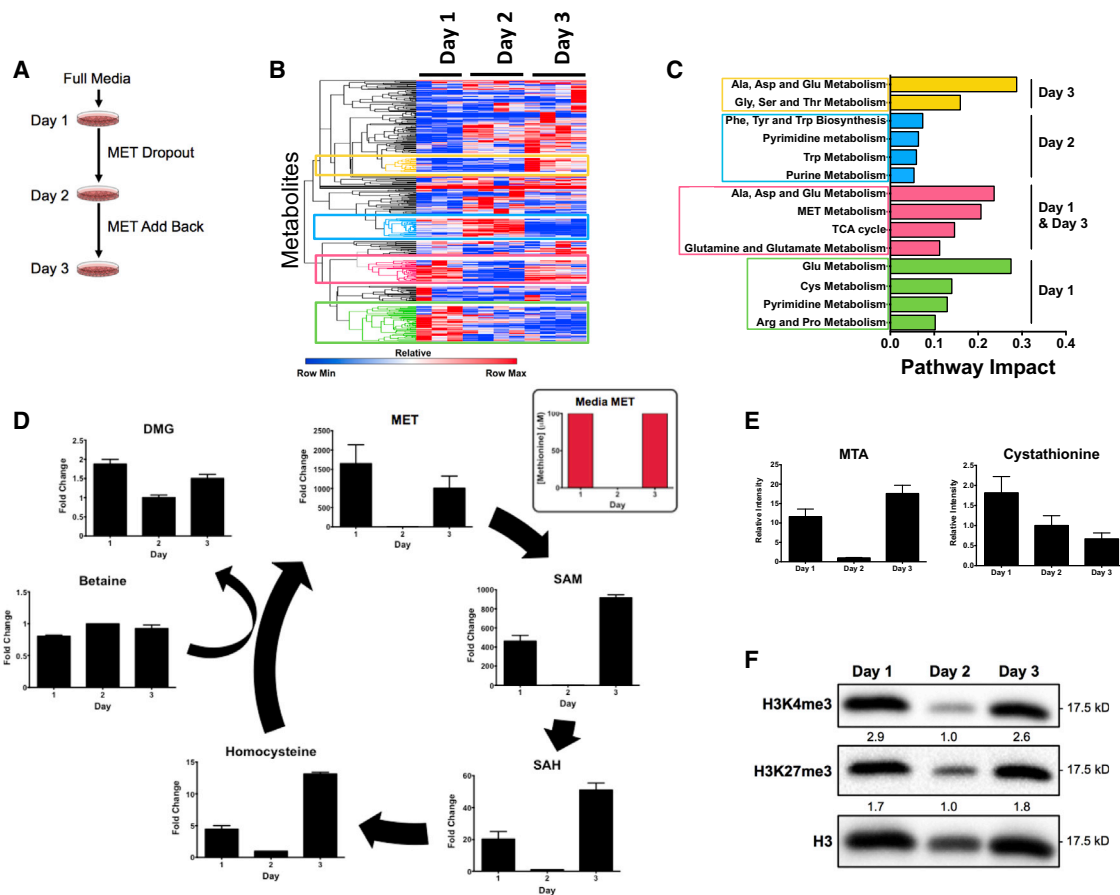


Figure 3. Histone Methylation and Methionine Cycle Dynamics Are Reversible

- (A) Experimental setup of methionine restriction and recovery.
 (B) Effects of methionine restriction and recovery on global metabolism.
 (C) Pathway analysis of methionine recovery.
 (D) Effect of methionine restriction and recovery on methionine cycle metabolism.
 (E) Effect of methionine restriction and recovery on the methionine salvage and transsulfuration pathways.
 (F) Response of histone methylation from methionine restriction and recovery.

Integrated intensities are normalized to total H3, and fold change was calculated. All error bars are computed from the SEM ($n = 3$).

(Figure 3B). Hierarchical clustering of each profile revealed marked changes across the metabolic network at days 1, 2, and 3. The gross measured differences in metabolism from day 1 and day 3 revealed that overall metabolism is not reversible under these conditions. An assessment of the pathways involved indicated that several amino acid metabolism pathways were not recoverable at 3 days (Figure 3C). Of the pathways recovered after reincorporation of methionine into the culture media, cysteine and methionine metabolism was one of three pathways shown to be reversible. This effect is illustrated in Figure 3D, in which the methionine cycle is plotted, and for each metabolite in the cycle, some recovery of its concentration and the concentration of the metabolites in its salvage pathway (Figure 3E) was observed. To investigate whether these dynamics correlated with histone methylation, we measured histone methylation levels (Figure 3F) and found that methylation levels exhibited a concomitant recovery back to levels observed in the original culture conditions. Together these findings indicate that the methionine cycle is reversible even when the remainder of global metabolism is

altered. Furthermore, these results show that the recovery of the levels of metabolites in the methionine cycle is sufficient to restore the levels of histone methylation.

Methionine Restriction Decreases H3K4me3 Signature Peaks and Alters Gene Expression

Although results from immunoblotting identified global alterations in histone methylation after methionine restriction, it remained unclear whether methionine metabolism affected specific marks on the genome to mediate gene expression. We therefore sought to determine the precise location H3K4me3 on the genome using chromatin immunoprecipitation with sequencing (ChIP-seq) and the consequences on gene expression using RNA sequencing (RNA-seq). The specificity of the H3K4me3 antibody was first confirmed with modified histone peptide array, and sensitivity was tested using serial titrations of H3K4me3 peptides, as suggested in the Encyclopedia of DNA Elements guidelines (Bailey et al., 2013) (Figures S4A and S4B). Cells were subjected to methionine restriction ($-MET$) or normal methionine ($+MET$) conditions

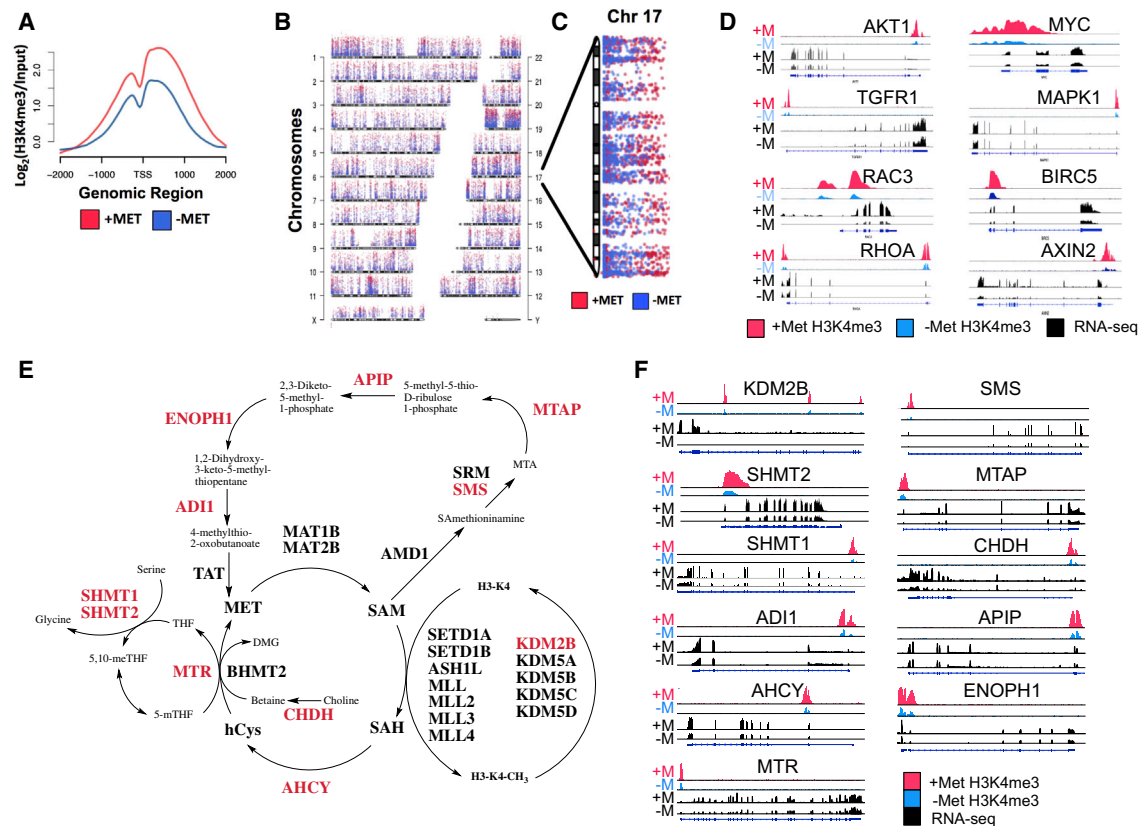


Figure 4. Methionine Restriction Decreases H3K4me3 and Alters Gene Expression

- (A) Change in H3K4me3 distribution around the TSS after 24 hr methionine restriction.
 (B) Genome-wide distribution of H3K4me3 peaks in +MET (red) and –MET (blue) conditions.
 (C) Enlargement of chromosome 17.
 (D) Changes in H3K4me3 ChIP-seq signal with corresponding changes in gene expression from RNA-seq for colon cancer genes.
 (E) Enzymes with decreased H3K4me3 and gene expression essential in one-carbon metabolism and related pathways are highlighted in red.
 (F) H3K4me3 ChIP-seq and RNA-seq signals for enzymes in (E) before and after methionine restriction.

for 24 hr, and crosslinked chromatin was collected for H3K4me3 ChIP-seq analysis, with total RNA collected for RNA-seq in parallel. After peak calling ([Experimental Procedures](#)), replicate samples for the +MET and –MET conditions clustered together, suggesting the genome-wide profile of H3K4me3 marks are similar between replicates but vastly different between the conditions ([Figure S4C](#)). Next, we investigated the change in H3K4me3 peaks at the transcription start site (TSS) of genes, because H3K4me3 at gene promoters has been associated with gene transcription. Overall, we found that the H3K4me3 peaks around the TSS are bimodal and decrease, on average, after methionine restriction ([Figure 4A](#)). In addition, H3K4me3 peaks decreased in breadth after methionine restriction ([Figure S4D](#)), but no significant change in H3K4me3 binding site distribution around the TSS was observed ([Figure S4E](#)).

Next, peak counts were plotted for each chromosome, showing that H3K4me3 peak distribution across the genome is relatively consistent ([Figure 4B](#)). Closer inspection of individual chromosomes revealed that particular genomic regions are differentially marked by H3K4me3 ([Figure 4C](#)), and further differential binding analysis indicated significantly depleted regions of H3K4me3 ([Figure S4F](#)).

Because promoter H3K4me3 is a signature for active transcription, we further investigated how decreased H3K4me3 at promoters affects gene transcription. To this end, we performed RNA-seq ([Figure S4G](#)) under MR conditions in HCT116 and compared these results with our ChIP-seq analysis. Interestingly, we found that colorectal cancer (CRC)-associated genes were enriched for loss of H3K4me3 at promoters, with resulting decreased expression ($p = 0.02$, Fisher's exact test). Notably, the cancer-associated genes *AKT1*, *MYC*, and *MAPK*, among others, were responsive to decreases in promoter H3K4me3 ([Figure 4D](#)). In addition, CRC genes that had significant decreases in gene expression also showed significant decreases in H3K4me3 over the promoter region when compared with genome-wide loss of H3K4me3 ([Figure S4H](#)). Overall, these results suggest that nutrient status has a dramatic effect on the chromatin state and gene regulation in cells with likely effects on pathophysiological outcomes.

To further evaluate specific physiological consequences of this mechanism, we identified a network of enzymes involved in one-carbon metabolism that show decreased H3K4me3 with concomitant decreases in expression ([Figure 4E](#)). This suggests a feedback mechanism that decreases consumption of

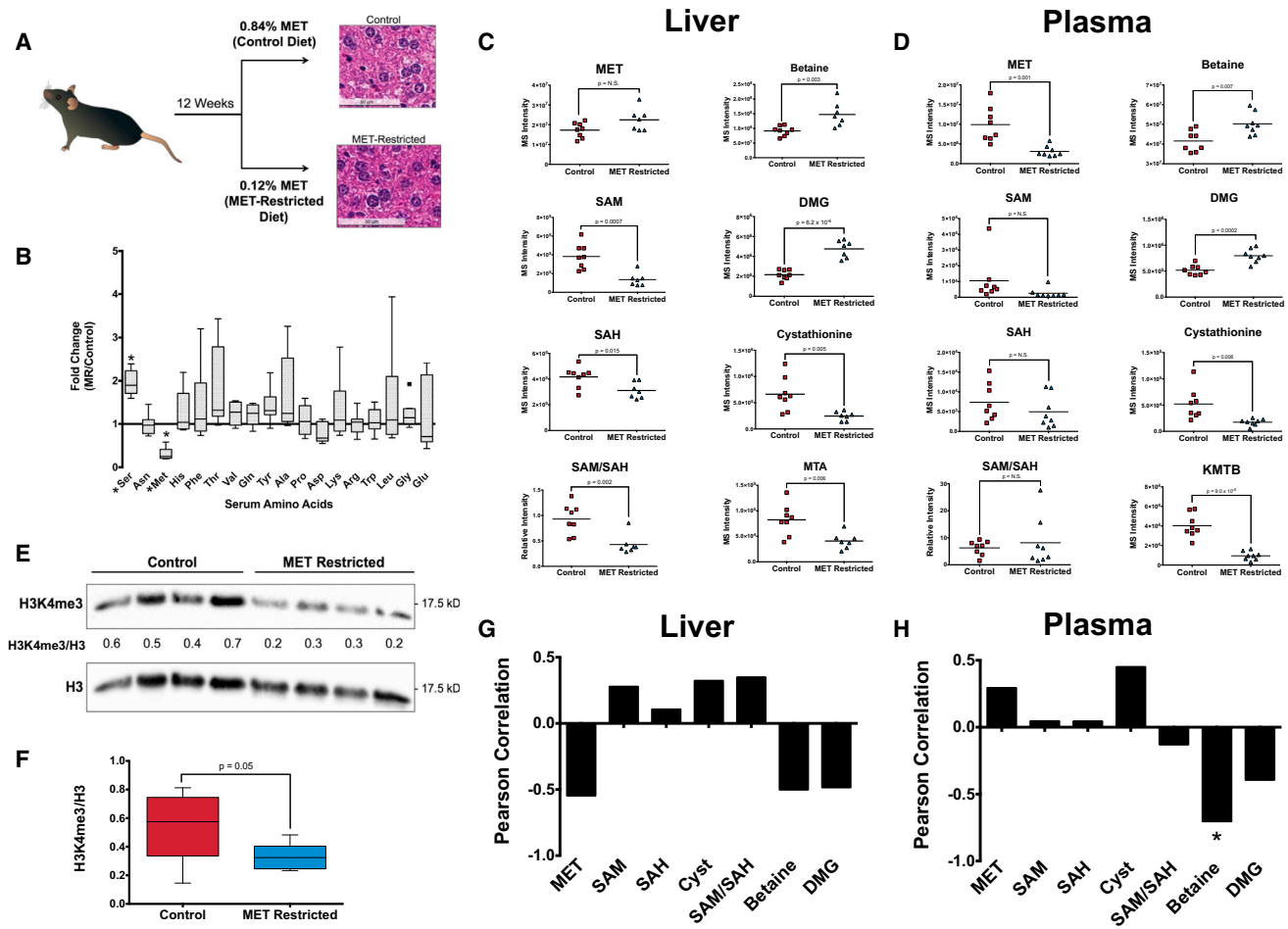


Figure 5. Alterations in Methionine Metabolism Can Be Sustained by Diet In Vivo

(A) Methionine restriction in mice with representative histology in MR and control mice. Images are at 40 \times with hematoxylin and eosin staining. (B) Fold change of serum amino acids in –MET compared with +MET, measured by LC-MS (* $p < 0.001$). (C) Methionine cycle metabolites in liver of MR mice. (D) Methionine cycle metabolites in plasma of MR mice. (E) Histone methylation in liver of MR mice. Integrated intensities were normalized to total H3. (F) Quantitation of results histone methylation from $n = 8$ mice in each cohort. p value was obtained from a Student's t test with equal variance. (G and H) Correlation of histone methylation and methionine cycle metabolites in liver and plasma.

SAM by alternative pathways and an attempt to maintain H3K4me3 by regulation of *JARID1B* (*KDM5B*), H3K4me3 demethylase that is deregulated in cancer (Yamane et al., 2007). This finding is indicative of a signal transduction mechanism by which alterations in H3K4me3 directly feed back to one-carbon metabolism and methylation-related enzymes to maintain physiological homeostasis.

Methionine Cycle Alterations Can Be Sustained by Diet In Vivo

We next questioned whether changes in methionine can occur through and are maintained in response to alterations in nutrient availability in a longer term physiological setting. We considered an analysis of the effects of methionine restriction in vivo at a concentration that has been shown to have health-promoting effects (Ables et al., 2012). Seven-week-old C57Bl6 mice were randomized into two groups, and one arm was fed a standard

diet consisting of 0.84% (w/w) methionine and the other a MR diet consisting of more than seven times less methionine (0.12% w/w) (Figure 5A). We first confirmed that the MR diet specifically decreased serum methionine levels compared with the levels of all other amino acids (Figure 5B). At 12 weeks, mice were sacrificed, and no gross phenotypic alterations were observed as measured by liver histology and as previously described (Ables et al., 2012) (Figure 5A). An examination of the metabolite profile in the fasting serum and liver by carrying out unsupervised hierarchical clustering revealed global alterations in metabolism in the liver and plasma (Figure S5A), with a pathway analysis revealing that cysteine and methionine metabolism was predominantly altered in plasma (Figure S5B). Other amino acid metabolism pathways to a lesser extent exhibited alterations. Inspection of metabolism in the liver exhibited sustained alterations in cysteine and methionine, along with alterations in taurine metabolism and other amino acid metabolism

pathways (Figure S5C). Globally, an analysis of the quantile-quantile plot showed skewness toward more changes observed in the liver but with more significant changes occurring in the serum (Figure S5D). These differences were further exemplified by comparing correlations of metabolite levels between liver and plasma (Figure S5E), with metabolites related to beta oxidation such as acylcarnitines, amino acid metabolism, and one-carbon metabolism found to correlate with the extent of methionine levels in the plasma (Figure S5F).

Together these findings indicate that the regions of the metabolic network that are altered in response to sustained methionine restriction *in vivo* are comparable with those aspects of the metabolic network that are dynamic in response to acute methionine restriction in cells. Furthermore, the metabolic state of fasting liver is dramatically different in the animals that were maintained on different diets for the long term.

Methionine Restriction *In Vivo* Sustains Altered Methionine Cycle and Histone Methylation

Having demonstrated that cysteine and methionine metabolism exhibit sustainable alterations in mice, we then asked whether these changes led to differences in the methionine cycle. In the liver (Figure 5C), methionine concentrations exhibited no significant changes, suggesting that compensatory fluxes involving its consumption or intake are occurring. These changes in fluxes resulted in decreases in SAM, SAH, the SAM/SAH ratio, cystathionine, and methylthioadenosine and increases in the methyl donors DMG and betaine. However, in plasma (Figure 5D), methionine levels were maintained at lower levels. In addition, cystathionine and 2-keto-4-methylthiobutyrate, a component of the methionine salvage pathway, were also significantly decreased. Conversely, betaine and DMG each exhibited increases suggestive of their possibility as plasma biomarkers. The methionine cycle metabolites SAM and SAH did not show changes in plasma. This observation was expected because cells are not thought to secrete these metabolites at appreciable concentrations (Agrimi et al., 2004). We next asked whether these sustained changes in the methionine cycle were sufficient to induce changes in histone methylation. We observed decreases in H3K4me3 in the MR liver (Figure 5E) that we quantified (Figure 5F), indicating that the alterations in SAM and SAH levels observed in the liver are sufficient to induce global changes in histone methylation. We found that SAM, the SAM/SAH ratio, and cystathionine positively correlated with H3K4me3 levels, and methionine, betaine, and DMG negatively correlated with H3K4me3 levels (Figures 5G and S5G). In plasma, the levels of methionine and cystathionine were positively correlated, and betaine and DMG were negatively correlated (Figures 5H and S5H). Together these findings provide evidence that levels of SAM and SAM/SAH ratio are predictive of histone methylation levels, that plasma metabolites in methionine cycle metabolism are predictive of histone methylation in the liver, and that these levels can be directly modulated by diet in mice.

Humans Exhibit Variability in Methionine Levels

We next questioned whether variability in methionine metabolism exists in humans and how it can be regulated. Standard clinical parameters and a record of dietary intake over 4 days were considered to reflect variations in habitual diet, as is stan-

dard practice in clinical nutrition (Levine et al., 2014) across a cohort of healthy human subjects. Fasting serum was collected and subjected to a metabolomics analysis (Figures 6A and S6A). We performed an unsupervised hierarchical clustering of the nutrient intake for each subject and found sets of defined modules that were able to classify the subjects into discrete dietary behaviors, such as groups that were high in fruits and vegetables or carbohydrates (Figure 6B). We next measured the concentration of methionine along with a panel of amino acids in these subjects (Figure 6C). Strikingly, the concentration of methionine exhibited substantial variation, with values ranging from 3–30 μM and methionine exhibiting the largest variation (Figure 6D). This variation in concentration is on the same order as that needed to induce changes in histone methylation in cells and in mice. In addition, methionine in the serum correlates with N,N,N-trimethyllysine and N-methylglycine (sarcosine), both of which are methylated by the transfer of methyl groups from SAM, suggesting that methionine levels in the serum are indicative of cellular methylation status (Figure 6E), with clear patterns of food consumption that corresponded to both high and low methionine intake (Figures 6F and S6B). Vegetable-based nutrients such as fiber correlated with low methionine levels, and age, body weight, and fat intake correlated with high methionine levels. Surprisingly, protein intake exhibited no correlation with methionine. An analysis of the metabolites and pathways that correlated with methionine levels (Figures 6G and S6C) revealed pathways related to ketogenesis and amino metabolism. Taken together, these findings demonstrate that the variability in methionine concentration in humans is on the same scale as that needed to induce alterations in histone methylation and that these differences correlate with changes in diet and health status.

A Computational Model Identifies Factors that Contribute to Methionine Variability

Finally, having identified associations of methionine with diet and other factors, we built a computational model to identify the direct influences on methionine concentration. We considered a mixed-effects model that aimed to identify causal features in high-dimensional data and thus identify factors that give rise to the variation in methionine levels (Figure 7A). We considered dietary intake variables and clinical variables such as age, gender, and body composition measured by dual-energy X-ray absorptiometry (DEXA). To reduce the dimensionality of the data matrix, we filtered the variables according to their correlations with methionine, carried out a principal-components analysis on the DEXA variables (Figure S7A), and checked the resulting 12 variables for collinearity (Figure S7B). We then carried out a regression using least-squares minimization with maximum likelihood estimation (Supplemental Information) and obtained a model with good fit ($p < 10^{-4}$, F test) to the experimental methionine concentrations (Figure 7B).

An analysis of the coefficients (Figure 7C) revealed several contributions to methionine variation, including age, body composition, gender (with maleness contributing to a positive influence), and diet, including variables known to be associated with methionine, including zinc and tocoferol. Using established guidelines (Experimental Procedures), it was found that the diet variables could be related to major sources of food intake

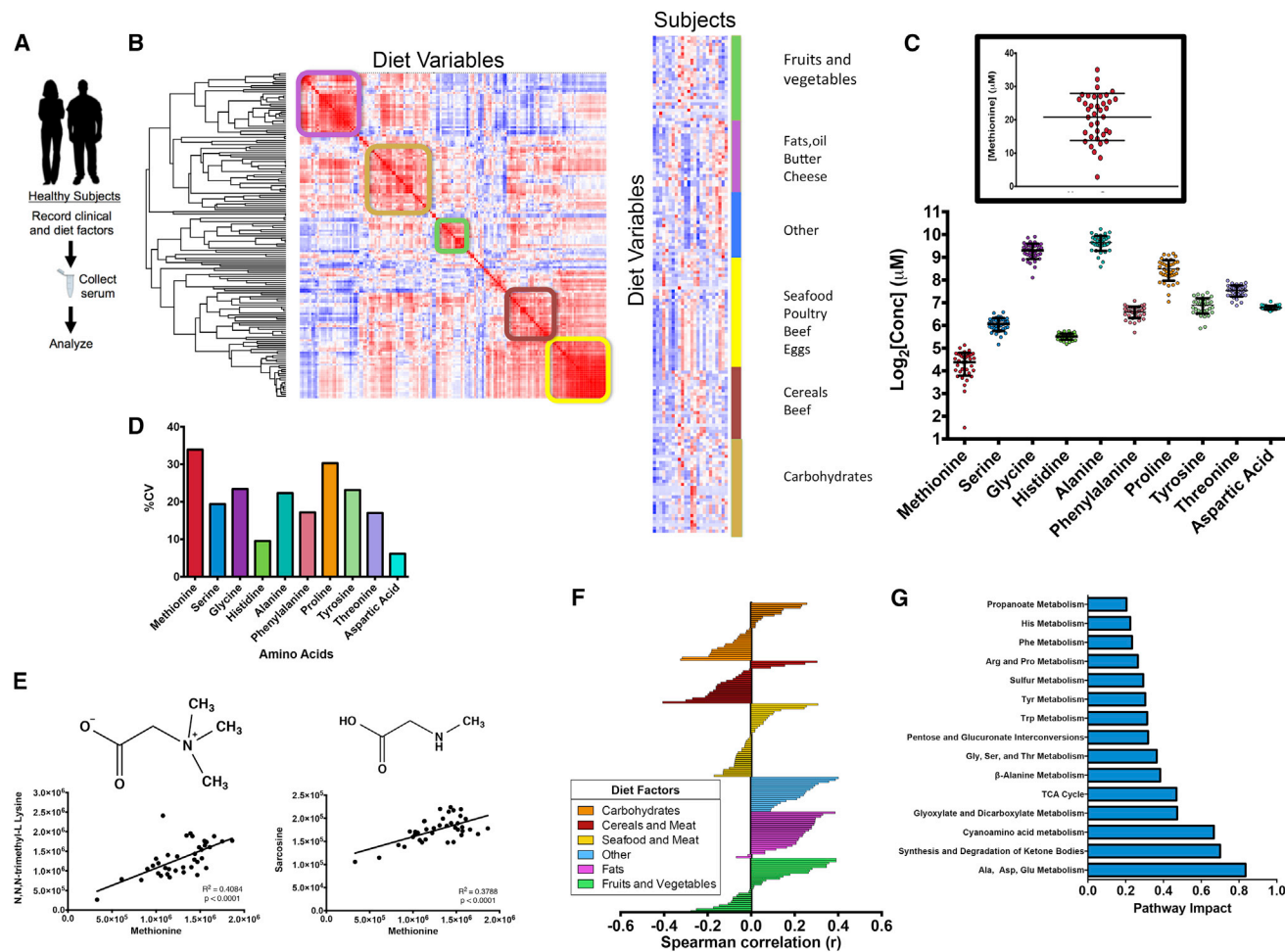


Figure 6. Methionine and Metabolic Variation in Human Subjects

- (A) Measurement of serum methionine and clinical and dietary variables in human subjects.
 (B) Left: Hierarchical clustering of the distance matrix of diet variables. Right: k-Means clustering of subjects and diet variables (n = 24).
 (C) Absolute concentrations of amino acids in fasting serum in 38 human subjects.
 (D) Coefficients of variation of amino acids in fasting serum.
 (E) Correlation of methionine in the serum with methylated serum metabolites, N,N,N- trimethyllysine, and sarcosine.
 (F) Correlation of methionine concentrations with dietary variables obtained from habitual diet records.
 (G) Pathways enriched with metabolites that correlate ($|r| > 0.2$) with serum methionine concentration.

(Figure 7D) with, for example, fats, seafood, and meat contributing to higher concentrations of methionine. Finally, we performed a variance partitioning calculation (Figure 7E) and found that about 30% of the variation was explained by diet, about 30% by clinical variables including gender and age, and the remaining unaccounted variance likely due to genetic factors. These results indicate that methionine concentrations observed in humans can be decomposed into several factors, including diet.

DISCUSSION

These findings together provide evidence for a dynamic regulatory mechanism whereby the status of the methionine component of one-carbon metabolism is sensed by histones to determine the levels of methylation on critical residues that mediate cellular epigenetic status. We first demonstrated that methionine deprivation and subsequent depletion of SAM and SAH induce

changes in histone methylation. Furthermore, we tested and confirmed that these dynamics occurred in a manner consistent with a signal transduction mechanism. Necessarily, we found that changes in histone methylation occurred at quantitatively relevant concentrations of methionine. The kinetics of turnover of histone methylation in response to SAM and SAH availability were found to occur within hours and thus are much shorter than times corresponding to changes in cell-cycle progression or global stress responses that also affect histone methylation. Importantly, these dynamics were found to be reversible, as the depletion of histone methylation could be recovered upon restoration of methionine availability even when the remainder of cellular metabolism was not recovered. Each of these properties is consistent with a mechanism whereby the status of key histone marks such as H3K4me3 that mediate chromatin structure, gene expression, and cell identity directly sense the metabolic status of one-carbon metabolism.

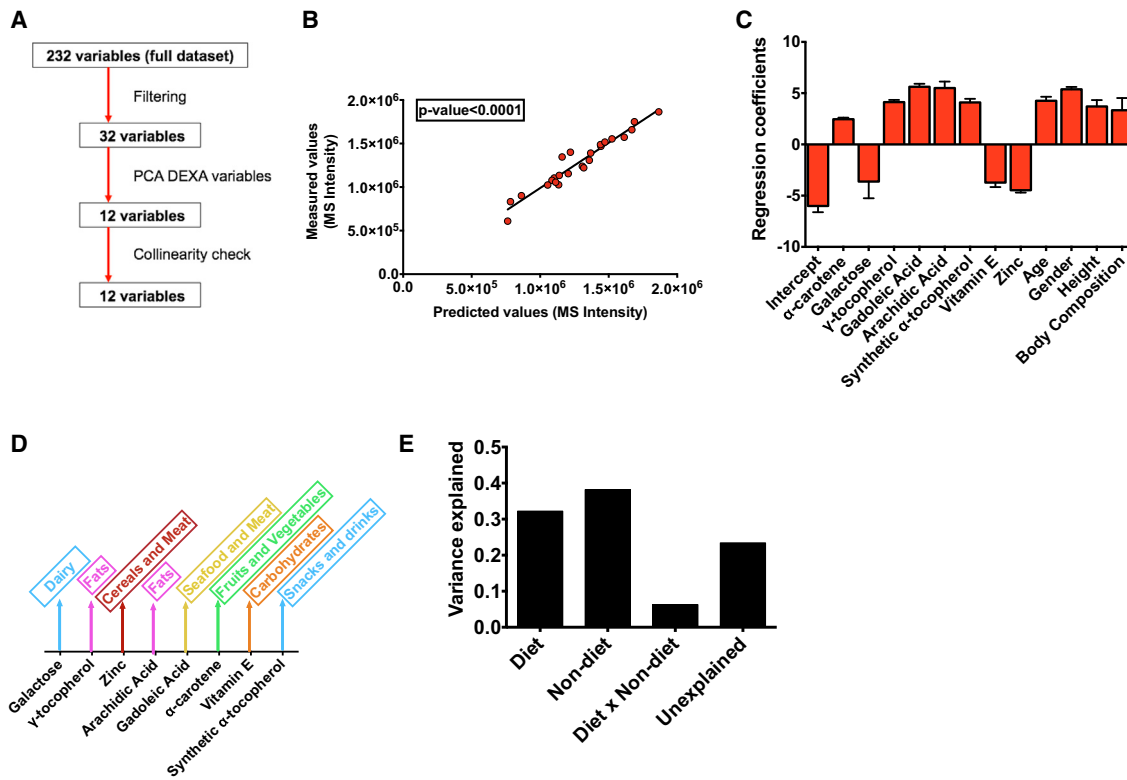


Figure 7. A Computational Model Identifies Determinants of Methionine Variability

(A) Overview of variable selection for the computational model. (B) Predicted versus measured methionine levels in human subjects. (C) Regression coefficients. Error bars are obtained from maximum likelihood estimates. (D) Schematic of dietary factors that contribute to each modeled variable. (E) Results from variance partitioning.

In vivo, the dynamic response to methionine restriction further provided support of such a mechanism, because alterations in SAM and SAH could be sustained in liver, and these changes were sufficient to induce changes in histone methylation. Interestingly, although this dietary intervention maintained reduced methionine levels in the serum, the levels in the liver were not altered. This suggested compensatory adaptations in methionine fluxes occurred, likely through other remethylation pathways, for example, from folate metabolism. These metabolic adaptations likely serve to maintain other essential processes involved in methionine metabolism, possibly providing a basis for why methionine restriction can be sustained in healthy animals even when changes in histone methylation are observed. Notably, methylation status and metabolism were measured in fasting conditions, in which methionine restriction was shown to alter histone methylation. This fasted condition, we expect, sets a lower bound on the extent to which the methionine cycle can be depleted, with feeding contributing to increases in methionine levels. Given this observation, it is tempting to speculate that feeding or a diurnal rhythm might regulate this effect, perhaps raising methionine levels to recover depletions in histone methylation.

In humans, the variation of methionine in healthy individuals was found to be largest across a panel of serum amino acids. Although these measurements are by no means exhaustive of

human population dynamics, concentrations in many individuals were found to be far lower than the concentration required to induce changes in methylation levels. Computational modeling of diet and clinical variables found that about 30% of the variation could be due to fundamental clinical variables such as age, body composition, and gender, and about 30% was modulated by diet. In the future, the results from these models could further be integrated with genomics data to specifically define the genetic contributions, as have been identified to associate with serum metabolite levels (Suhre et al., 2011). Nevertheless, it is tempting to speculate that basic dietary factors such as vegetable and fat intake could mediate human epigenetics through modulation of methionine metabolism.

One-Carbon Metabolism as a Metabolic Signal Transduction Mechanism

Cellular decision making occurs through signal transduction that uses post-translation modifications. For example, protein kinases establish networks of protein phosphorylation with multiple layers of nonlinear regulation that control almost all aspects of physiology. Despite the ubiquitous importance of histone and other macromolecular methylation, relatively little is known about how methylation is regulated. In contrast to protein kinases, whereby the substrate, ATP, is in vast excess, the

concentrations of SAM and SAH are on the order of the kinetic parameters that determine enzyme activity. Thus, much of histone and other methylation could be regulated in large part by the status of methionine metabolism and one-carbon cycle flux, consistent with relatively small numbers of methyltransferases compared with the more than 500 protein kinases that exist. This type of regulation by virtue of its biochemistry would have fundamentally different control properties and information capacity than that of protein kinase-mediated signaling. This regulation is evident in the feedback control observed in genes in one-carbon metabolism, whose expression and H3K4me3 signature is modulated in response to decreases in SAM and SAH levels, likely as a specific mechanism to maintain homeostasis in one-carbon metabolism. In particular, enzymes important in regenerating methionine by using SAM are some of the most downregulated genes with loss of H3K4me3 at their promoters.

Methionine Metabolism in Pathophysiology through Its Modulation of Chromatin State

Numerous studies have documented a contribution of methionine metabolism and its restriction to beneficial metabolic health, including the extension of mammalian lifespan, the acquisition of resistance to diet-induced obesity, and the therapeutic efficacy of ketogenic diets (Kraus et al., 2014; Malloy et al., 2006; Orentreich et al., 1993; Pissios et al., 2013). In each of these cases, the biological mechanisms contributing to these outcomes are largely unknown. Although alterations in methylation status likely do not account for all of these effects, it is possible if not likely that the alterations in gene expression of key genes are important for the relevant physiological effect in each of these cases. Indeed, chronic diseases, including obesity, diabetes, and cancer, often stem from the inability of the organism to adapt to the demands of its nutritional load (Hotamisligil, 2010; Laplante and Sabatini, 2012). These adaptations require signal transduction mechanisms that integrate nutritional status to achieve the desired physiological demand. There are numerous signal transduction pathways that sense nutritional status, and it is likely that the sensing of one-carbon metabolism by histones is one of these mechanisms.

Interestingly, this mechanism affected gene regulation of several cancer-promoting genes. Many cancers are vulnerable to disruptions in one-carbon metabolism (Locasale, 2013), and our results demonstrate the necessity of methionine metabolism for the maintenance of histone methylation and the consequences for expression of oncogenic programs. Thus it is tempting to speculate that some of the anti-tumor effects of targeting one-carbon metabolism may occur through changes in cancer epigenetics, and further studies could more deeply elaborate these mechanisms.

EXPERIMENTAL PROCEDURES

Methionine Restriction in Cell Culture

Briefly, CRC cells were grown in RPMI supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin. For methionine restriction experiments, media was changed to either conditional media lacking methionine (–MET) or media with methionine (+MET) for indicated times. Detailed protocol and media recipes are provided in the [Supplemental Information](#).

Mouse Feeding and Tissue and Plasma Analysis

Mouse feeding and sample collection were performed as previously described (Ables et al., 2012). Briefly, C57BL/6J mice were randomly assigned to either an isocaloric, 0.85% (w/w) methionine (control) or a 0.12% (w/w) methionine (MR) diet and sacrificed after 12 weeks. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Orentreich Foundation for the Advancement of Science (Permit Number 0511MB). Detailed methods are described in the [Supplemental Information](#).

Metabolite Extraction and LC-HRMS Analysis

The procedures for cultured cells, serum, and tissues were described in previous studies (Liu et al., 2014a, 2014b). Additional methods for liquid chromatography mass spectrometry (LC-MS) analysis are provided in the [Supplemental Information](#).

RNA-Seq

Total RNA from +MET and –MET conditions was polyA selected, and libraries were prepared according to a standard protocol provided by Illumina and sequenced on the Illumina HiSeq 2500 Rapid Run sequencer.

ChIP-Seq

Hct116 cells from +MET and –MET conditions were crosslinked, and each immunoprecipitation was performed as previously described (Ercan et al., 2007; Landt et al., 2012) using 1.5×10^6 cells. Libraries were made and pooled according to Illumina instructions, then sequenced on an Illumina HiSeq 2500 Rapid Run sequencer.

Human Studies

Serum samples, 4-day diet records, and body composition results (via DEXA scan) on 24 de-identified healthy older adults were provided by Marcas Baman at the University of Alabama at Birmingham (UAB). As part of the UAB Institutional Review Board-approved parent project, all 24 subjects agreed to have their samples and data used for future research.

Statistical Analysis

Error bars represent SEM. Significance figures are from Student's *t* tests unless otherwise noted.

Computational Modeling

In brief, a set of 12 predictor variables were obtained after a variable selection process that incorporates methionine correlations, a dimensional reduction of the DEXA data using principal-components analysis, and a collinearity assessment using the variance inflation factor. We next generated a mixed-effects linear model including the set of predictor variables, a random-effect term, and noise. Model selection was carried out using exhaustive sampling and optimization of the Akaike information criterion. Variance contributions for each variable and random effect were summed to define the total contribution of variance for each variable and unexplained factor. The full details of the modeling are contained in the [Supplemental Information](#).

ACCESSION NUMBERS

The accession number for the ChIP-seq and RNA-seq data sets reported in this paper is GEO: GSE72131.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2015.08.024>.

AUTHOR CONTRIBUTIONS

S.J.M. and J.W.L. designed the study. S.J.M., S.N.N., D.G., M.M., L.H., X.L., P.G.P., D.M., and G.A., performed experiments and contributed to data

analysis. A.E.T.-M., M.M.B., and D.G. carried out the diet record and serum collection. S.N.N. led the feeding studies. S.N.N., D.M., and G.A. carried out the feeding studies and harvested tissues in mice. S.J.M. and J.W.L. wrote the paper.

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