Nutritional Control of Epigenetic Processes in Yeast and Human Cells

Meru J. Sadhu,^{*,†,1} Qiaoning Guan,^{*,†,2} Fei Li,^{*,3} Jade Sales-Lee,[†] Anthony T. Iavarone,[†]
Ming C. Hammond,^{*,†} W. Zacheus Cande,^{*} and Jasper Rine^{*,†,4}

^{*}Department of Molecular and Cell Biology, [†]Department of Chemistry, and [‡]California Institute for Quantitative Biosciences, University of California, Berkeley, California 94720-3220

ABSTRACT The vitamin folate is required for methionine homeostasis in all organisms. In addition to its role in protein synthesis, methionine is the precursor to S-adenosyl-methionine (SAM), which is used in myriad cellular methylation reactions, including all histone methylation reactions. Here, we demonstrate that folate and methionine deficiency led to reduced methylation of lysine 4 of histone H3 (H3K4) in *Saccharomyces cerevisiae*. The effect of nutritional deficiency on H3K79 methylation was less pronounced, but was exacerbated in *S. cerevisiae* carrying a hypomorphic allele of *Dot1*, the enzyme responsible for H3K79 methylation. This result suggested a hierarchy of epigenetic modifications in terms of their susceptibility to nutritional limitations. Folate deficiency caused changes in gene transcription that mirrored the effect of complete loss of H3K4 methylation. Histone methylation was also found to respond to nutritional deficiency in the fission yeast *Schizosaccharomyces pombe* and in human cells in culture.

METHYLATION of histone lysine residues plays specific, highly conserved roles in various aspects of eukaryotic gene regulation and chromosome biology. For example, methylation of lysine 4 of histone H3 (H3K4) is found at sites of active transcription in fungi (Bernstein *et al.* 2002), plants (Zhang *et al.* 2009), and animals (Gu and Fire 2010), whereas H3K9 methylation is found at repressed loci in the same broad range of organisms (Nakayama *et al.* 2001; Jackson *et al.* 2002; Peters *et al.* 2003). Furthermore, a single lysine can accept one, two, or three methyl groups, and these three different states of histone methylation can have different functions (Fingerman *et al.* 2005). The yeast *Saccharomyces cerevisiae* has widespread histone methylation at three lysine positions on histone H3: H3K4, H3K36 (Strahl *et al.* 2002), and H3K79 (van Leeuwen *et al.* 2002). Methylations

of lysines on histone H4 at H4K5, H4K8, H4K12, and H4K20 have also been reported (Edwards *et al.* 2011; Green *et al.* 2012). In *S. cerevisiae*, histone methyltransferase complexes are highly specific for a given lysine residue, with H3K4 methylation performed by the *Set1* methyltransferase, H3K36 methylation by the *Set2* methyltransferase, and H3K79 methylation by the *Dot1* methyltransferase.

The biochemical reaction is highly similar for these three methyltransferases—the methyl donor, S-adenosyl-methionine (SAM), is converted to S-adenosyl-homocysteine by the transfer of a single methyl group from SAM to the lysine acceptor (Figure 1). All known histone methyltransferases and DNA methyltransferases require SAM as the methyl donor (Cheng *et al.* 1993; Xiao *et al.* 2003; Sawada *et al.* 2004; Jia *et al.* 2007). Thus, chromatin methylation marks are, in principle, susceptible to nutritional limitation.

A potential source of such perturbations affecting SAM synthesis could come from nutritional deficiencies. SAM is synthesized from methionine and ATP in a reaction conserved across all domains of life (Thomas and Surdin-Kerjan 1991). Humans are dependent on diet for adequate methionine, classifying methionine as an essential amino acid (Townsend *et al.* 2004). Proper methionine homeostasis requires the vitamin folate, in the form of reduced folate cofactors, such as tetrahydrofolate and 5-methyltetrahydrofolate. The S-adenosyl-homocysteine resulting from a methyl transfer is broken

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¹Present address: Howard Hughes Medical Institute, Departments of Human Genetics and Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles, CA 90095.

²Present address: BGI@UC Davis, 2921 Stockton Blvd., Sacramento, CA 95817.

³Present address: Department of Biology, New York University, New York, NY 10003.

⁴Corresponding author: Department of Molecular and Cell Biology, and California Institute for Quantitative Biosciences, 374A Stanley Hall, UC Berkeley, Berkeley, CA 94720-3220. E-mail: jrine@berkeley.edu

Figure 1 Interaction between folate, methionine, and cellular methylation. Met2, not shown, is required for the synthesis of homocysteine. Note that although methionine synthesis requires cobalamin (vitamin B₁₂) in many organisms, methionine synthesis in *S. cerevisiae* is cobalamin independent. SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine; MTHFR, methylenetetrahydrofolate reductase.

down by S-adenosyl-homocysteine hydrolase to homocysteine, which is recycled back to methionine in a reaction using the reduced folate cofactor 5-methyltetrahydrofolate. This relationship between methionine homeostasis and folate is also conserved across biology (Suliman *et al.* 2005). Vitamin B₁₂ is required for methionine synthesis in many organisms, but not in *S. cerevisiae*. In organisms that synthesize methionine *de novo*, the final step in methionine biosynthesis is the same reaction between homocysteine and folate as used in methionine recycling (Pejchal and Ludwig 2004; Suliman *et al.* 2005). Thus, deficiencies in either methionine or folate could lead to lower intracellular SAM concentrations, which could theoretically affect histone methylation. In principle, if folate were limiting, cells may have the capacity to downregulate the activity of some methyltransferases to maintain SAM pools for more critical functions, such as membrane synthesis.

Folate deficiency is a particularly important topic of study, having been linked to several important diseases and common birth defects. Although the U.S. Food and Drug Administration has mandated folate fortification of grains in the United States since 1998, which has resulted in a substantial reduction in the frequency of some birth defects (Honein *et al.* 2001), most countries still do not mandate folate fortification of foods (Cordero *et al.* 2010). Historically, genetic variation among humans has not been considered in the establishment of nutritional guidelines. There is growing recognition, however, that natural genetic variation can considerably influence the level of a nutrient an individual requires. For example, methylene-tetrahydrofolate reductase

(MTHFR), the enzyme that produces the form of folate necessary to recycle homocysteine to methionine, has a common human variant (35% allele frequency in North America) that causes a significant reduction in the function of the MTHFR enzyme. Supplementation of cells with folate can remediate the function of this variant (Guenther *et al.* 1999; Yamada *et al.* 2001; Pejchal *et al.* 2006; Marini *et al.* 2008).

A few studies have linked folate deficiency with changes in the bulk level of DNA methylation (e.g., Friso *et al.* 2002; Crider *et al.* 2011). However, whether folate or methionine deficiency specifically can affect histone methylation locally or globally is substantially understudied. The study described below demonstrated, in widely diverged yeast species and human cells, that folate and methionine limitation reduced histone methylation and revealed the consequences and potential universality of this relationship.

Materials and Methods

Strains, plasmids, and oligonucleotide sequences

Strains used are listed in Supporting Information, Table S1, plasmids used are listed in Table S2, and sequences of oligonucleotides used are listed in Table S3. JRY9339 was obtained from a tetrad dissection of the *fol3Δ/FOL3* heterozygote from the *Saccharomyces* essential heterozygote knockout collection (Open Biosystems YSC1057), on plates containing YPD + 50 μg/ml folic acid (FA). Diploids were sporulated on solid 1% potassium acetate plates supplemented with 200 μg/ml complete supplement mix (CSM). Deletion of *SET1* was accomplished with a modified lithium-acetate protocol (Becker and Lundblad 2001), and JRY9341 was made by tetrad dissection, as before, of a diploid formed by the mating of *fol3Δ* and *set1Δ* haploid strains. JRY9381 and JRY9382 were made by tetrad dissection of diploids formed by the mating of *fol3Δ* with *SET1-TAP* and *DOT1-TAP* from the TAP-tagged collection, respectively (Thermo Scientific YSC1178).

pJR2541 and pJR2542, carrying *DOT1* and *dot1-G401A* on a pRS315 plasmid backbone, respectively, were gifts from Daniel Gottschling (van Leeuwen *et al.* 2002). The plasmids were transformed by the modified lithium acetate protocol to make JRY9342 and JRY9343.

JRY9346 was made by deletion of *fol1* (SPBC1734.03) by electroporation in a stable *Schizosaccharomyces pombe* diploid, as described previously (Prentice 1992), which was then dissected to give haploids. (*fol1* in *S. pombe* is homologous to *FOL1* in *S. cerevisiae*, but was chosen instead of *S. pombe fol3* because it was more certain that *S. pombe fol1* lacked a paralog in the *S. pombe* genome.) Folate auxotrophy was confirmed by differential growth in media lacking or supplemented with folic acid. *S. pombe met6Δ* was a gift from Kaoru Takegawa (Fujita *et al.* 2006).

Yeast nutritional experiments

For folate deficiency experiments in *S. cerevisiae*, modified minimal medium (SD) (Amberg *et al.* 2005) was made from

YNB without vitamins and amino acids, supplemented with 180 µg/ml riboflavin, 30 µg/ml leucine, 45 µg/ml lysine, 20 µg/ml histidine, and 20 µg/ml uracil. Folate auxotrophic cells were grown overnight in liquid media supplemented with 50 µg/ml folinic acid and subsequently split into cultures supplemented with 10, 25, or 50 µg/ml folinic acid and grown for about six doublings to mid-log phase. For methionine deficiency experiments, SD was made as described above, but without leucine and with additional 10 µg/ml adenine. Cells were grown overnight, supplemented with 20 µg/ml methionine, and split into cultures supplemented with 3, 10, or 20 µg/ml methionine and grown for about eight doublings. To grow cells at different temperatures, SD + riboflavin, leucine, lysine, histidine, and uracil were used. Cells were grown overnight at 30°, then split between 30°, 21°, and 18° and grown for about six doublings. For all experiments, control prototrophic strains were grown without folinic acid or methionine.

For folate deficiency experiments in *S. pombe*, modified minimal medium was made as described above, but 1 mg/ml glutamic acid was used as the nitrogen source instead of ammonium sulfate, and 3 mg/ml potassium hydrogen phthalate and 5 mg/ml Na₂HPO₄ were added as supplements, in addition to uracil, adenine, histidine, and leucine added to 225 µg/ml. Folate auxotrophic cells were grown overnight in liquid medium supplemented with 50 µg/ml folinic acid and then split into cultures containing 4, 5, or 50 µg/ml folinic acid and grown for about six doublings. Methionine auxotrophic cells were grown overnight in liquid medium containing 80 µg/ml methionine and then split into cultures containing 30 or 80 µg/ml methionine and grown for about six doublings.

Human cell nutritional experiments

Freshly thawed human K562 cells were cultured in RPMI-1640 medium (from Gibco, contains 2.3 µM folate and 100 µM methionine) and supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (P/S) at 37° and 5% CO₂. Typical serum contains 8–35 nM folates and 20–30 µM methionine (Kimura *et al.* 2004). Cells were passaged at confluence of $<0.75 \times 10^6$ cells/ml throughout the experiments. To treat cells with desired level of folate and/or methionine, customized RPMI-1640 medium containing no folates or L-methionine (manufactured by UCSF Cell Culture Facility) was supplemented with both nutrients (Sigma) at defined concentrations. Dialyzed FBS (10%) was added to minimize the addition of folates from regular FBS to the customized medium. Dose-response and growth-rate experiments show that customized RPMI-1640 (supplemented with 1–100 nM folinic acid and/or 5–50 µM methionine in this study) supports cell growth at a rate comparable to that supported by conventional RPMI-1640 (data not shown).

The nutrition limitation experiment was done as follows: freshly thawed cells were cultured and passaged in conventional RPMI-1640 medium until the desired cell number for

each experiment was reached. Cells were washed twice in the customized RPMI-1640 medium without folate and L-methionine to remove residual levels of either nutrient, and then split into media with different concentrations of both nutrients. Forty-eight hours later, cells were collected by methods specific for the downstream analysis (bulk histone extraction or RNA extraction). All experiments were performed at a confluence of 0.3×10^6 to 0.6×10^6 cells/ml.

Protein extract preparation and quantitative immunoblotting

Yeast whole-cell protein extracts were precipitated using 20% (wt/vol) trichloroacetic acid and solubilized in SDS loading buffer. Human histone proteins were isolated from cells by acid extraction as described in Shechter *et al.* (2007) and <http://www.abcam.com> (Histone Extraction Protocol).

Histones were electrophoretically separated on 15% (29:1 acrylamide/bis from BioRad) SDS/PAGE gels and transferred to Amersham Hybond-ECL Nitrocellulose Membrane (GE Healthcare). Immunoblotting was done with standard procedures and blots were imaged using the LiCOR Odyssey imager. Antibodies used in the immunoblots were anti-H3K4me2 (Abcam Ab7766), anti-H3K4me3 (Abcam Ab8580), anti-H3K79me2 (Abcam Ab3594), and anti-H3K79me3 (Abcam Ab2886). Either anti-H4 (Abcam Ab17036) or anti-Pgk1 (Invitrogen A6457) was used as a loading control. All histone methylation values were normalized to loading control values for the same sample. To combine values between experiments all values were normalized to the value obtained for a prototroph on the same blot.

RNA extraction, cDNA preparation, and RT-qPCR

RNA was purified from yeast cells using the hot acid/phenol and chloroform extraction and from human cells using Trizol reagents. Residual DNA was removed by DNase treatment (Roche 04716728001), after which RNA was purified again by use of a Qiagen RNeasy kit. cDNAs were prepared with an Invitrogen Superscript III kit and were quantified with a Stratagene MX3000 quantitative PCR system. All primer-set amplification values in yeast were normalized to *ACT1* amplification values and in human cells were normalized to *ACTB* amplification values.

Chromatin immunoprecipitation

S. cerevisiae cells were cross-linked with 1% formaldehyde for 20 min and then quenched with glycine at a final concentration of 300 mM. Cells were washed twice with tris-buffered saline and lysed in FA lysis buffer by mechanical lysis (Lombardi *et al.* 2011). Isolated chromatin was sonicated to fragments of average length 400–600 bp. Immunoprecipitation was by incubating overnight at 4° with 25 µl protein A Sepharose (GE Healthcare), 3 µl antibody (Ab8580 for H3K4 trimethylation and Ab1791 for H3, both from Abcam), and the sonicated chromatin from 16 OD₆₀₀ units of cells (for H3K4 trimethylation) or 8 OD₆₀₀ units of cells (for H3). Resin washing, IP elution, and DNA cross-link reversal were performed as previously described (Aparicio

et al. 2005). Quantification of DNA was done by qPCR as described above. Values are expressed as the H3K4 trimethylation enrichment at loci of interest relative to *HMRA1* (a negative control locus), normalized to H3 enrichment at the same locus relative to *HMRA1*.

For K562 cells, chromatin immunoprecipitation (ChIP) assays were carried out as described in Lee *et al.* (2006) and <http://www.abcam.com> (X-ChIP protocol). 2×10^7 cells were incubated in growth medium containing 1% formaldehyde for 10 min and quenched for 5 min with 125 mM glycine. Nuclear lysates were sonicated such that the average fragment lengths were around 500 bp. A fraction of the fragmented chromatin was reserved as input control, and aliquots of the remainder were incubated at 4° overnight with 5–10 µg of Ab8580 (H3K4 trimethylation, Abcam). Protein–DNA complexes were captured on protein A Dynabeads (Invitrogen), washed, and eluted in 1% SDS TE buffer at 65°, followed by the reversal of formaldehyde cross-links at 65° for a minimum of 6 hr. Between two and four preparations of chromatin were examined with each antibody. Values are expressed as the H3K4 trimethylation enrichment at the *HBG1* gene relative to the input value at *HBG1*, normalized to the H3K4 trimethylation enrichment at the *HBE1* gene relative to the input value at *HBE1* (a negative control locus).

Metabolite extraction and detection

Metabolites were extracted by treatment with perchloric acid (Warner *et al.* 1976). Frozen cell pellets were resuspended in 500 µl cold water and then treated with 500 µl 20% perchloric acid. Cell debris was pelleted for 10 min at $10,000 \times g$ and then the supernatants were adjusted to pH 6 using a solution of 2 M KOH/0.02 M K_2HPO_4 , after which the precipitated salt debris was pelleted and removed. Metabolites were quantified by liquid chromatography-mass spectrometry analysis (LC-MS), as described previously (Mayfield *et al.* 2012).

For detection of SAM by the Pi SAM-I riboswitch aptamer, extracts were diluted 1:50, and 1 µl was applied to 9 µl radiolabeled RNA in TBMK buffer (90 mM Tris, 89 mM boric acid, 10 mM $MgCl_2$, 10 mM KCl), as described previously (Karns *et al.* 2013). Before mixing with lysate, the RNA had been renatured at 70° for 3 min and then allowed to return to room temperature for 10 min. After a 5-min equilibration, samples were quenched with 50% glycerol and run on a 10% native PAGE gel. Band density was determined with ImageQuant software. Percentage of maximal binding was calculated with the equation $(x-y)/(z-y)$, in which x was the bound RNA fraction for a given sample, y was the bound RNA fraction for 0 mM SAM, and z was the bound RNA fraction for 1 mM SAM (which represents maximal binding).

Results

Folate deficiency compromised histone methylation

Folate is an important biological cofactor in all domains of life (Suh *et al.* 2001). Many organisms, including *S. cerevisiae*, are capable of synthesizing folate and its reduced cofactor

derivatives (Bayly *et al.* 2001) and are thus capable of normal growth even when there is a complete lack of folate in their environment. Therefore, to study the consequences of folate deficiency in yeast, we compromised folate synthesis by deleting *FOL3*, which encodes dihydrofolate synthetase. Growth of *fol3Δ* cells is completely dependent on folate supplementation in the form of FA (Bayly *et al.* 2001).

As determined by quantitative immunoblotting on whole-cell extracts, genome-wide H3K4 methylation levels were reduced in cultures grown in 10 µg/ml folinic acid relative to cultures grown in 50 µg/ml folinic acid—H3K4 dimethylation was reduced on average by 25%, and trimethylation was reduced ~45%. Both reductions were statistically significant (Figure 2A). In principle, these reductions in H3 methylation in cells experiencing folate deficiency could reflect a reduction in SAM levels, but alternate explanations were also possible (see below). Even in cells grown at 50 µg/ml folinic acid, the H3K4 methylation levels were typically 25–30% below that of folate prototrophs, suggesting that cells grown at 50 µg/ml folinic acid also experienced some degree of folate limitation. Cultures grown with 25 µg/ml folinic acid lost ~30% of H3K4 trimethylation, which was also statistically significant. In contrast, H3K4 dimethylation was not significantly affected in cultures grown with 25 µg/ml folinic acid.

If the effect of folate deficiency on histone methylation was due to a decrease in intracellular methionine levels, directly limiting methionine in a methionine auxotroph should also reduce histone methylation. As with folate limitation, growth of a *met2Δ S. cerevisiae* strain in 3 µg/ml methionine, conditions under which the cells divided slowly, led to reduced H3K4 di- and trimethylation (Figure 2B). Thus, nutritional deficiency of either folate or methionine affected chromatin modifications. The effect of exogenously provided methionine on histone methylation in folate-deficient cultures was also tested. Immediately after adding methionine to a final concentration of 15 µg/ml to *fol3Δ* cells, H3K4 di- and trimethylation increased (Figure 3A), although at later time points it declined somewhat, for reasons not yet understood.

Both folate deficiency and methionine deficiency decreased yeast growth rate. To determine whether slower growth itself caused reduced histone methylation, cultures were grown in nutrient-replete defined media at temperatures that slowed growth as much as, or more than, the folate or methionine limitation conditions did. Whereas folate auxotrophs had a doubling time 5% longer when grown in folate-deficient media compared to folate-replete media, growth at room temperature increased doubling time by ~50%, and growth at 18° increased doubling time by three-fold. The cultures reached the same overall density as in the nutrient limitation experiments. Growth-rate variation had no observable effect on either H3K4 di- or trimethylation levels (Figure 3B). The effect of folate deficiency on the abundance of the *Set1* methyltransferase itself was also tested; neither *SET1* mRNA, as measured by quantitative PCR, nor TAP-tagged *Set1* protein levels were significantly affected by

Figure 2 Effect of exogenous folinic acid and methionine on H3K4 methylation in *S. cerevisiae*. (A) H3K4 di- and trimethylation in *fol3Δ* cells grown at different concentrations of folinic acid. Here and elsewhere, folinic acid is abbreviated FA. Here and for Figures 5 and 7, for each replicate, histone methylation values were normalized to the value of a loading control from the same sample, either histone H4 or Pgk1, and then normalized to the value for a folate prototroph on the same blot. $n = 7$ for dimethylation; $n = 8$ for trimethylation. Error bars, standard error. (*) $P < 0.05$, Student's t -test. Representative blots are shown below. (B) H3K4 di- and trimethylation in *met2Δ* cells grown at different concentrations of methionine. Normalization was as in A; $n = 3$.

folate deficiency (Figure 3C). Thus, the effect of nutrient limitation on histone H3K4 methylation was not merely an indirect consequence of altered growth rate or due to quantitative changes in the methyltransferase.

Effects of folate limitation on gene expression

To determine if the effect of folate deficiency on histone methylation had consequences for gene regulation, genes whose expression was affected by the *set1Δ* mutation were tested for altered expression in folate deficient cells. A microarray analysis (Venkatasubrahmanyam *et al.* 2007) was used to select *PER33* as a candidate gene whose expression was decreased in a *set1Δ* strain. Quantitative RT-PCR analysis confirmed that *PER33* expression decreased significantly in *set1Δ* cells. Folate deficiency likewise reduced *PER33* expres-

sion (Figure 4A). To determine whether the effect of folate deficiency on expression was due to a change in *Set1* activity or to a different, unrelated mechanism, *PER33* expression was also tested in *set1Δ* cells in folate-limited conditions. In *set1Δ* cells folate deficiency had no significant additional effect on *PER33*. Therefore, folate deficiency changed *PER33* expression through loss of H3K4 methylation.

Although *Set1* is commonly considered to contribute to transcriptional activation (Boa *et al.* 2003), some genes, such as *BNA2*, have increased expression in *set1Δ* strains. *BNA2* expression was tested in cells experiencing folate deficiency, to see if the increased expression observed in *set1Δ* was recapitulated. *fol3Δ* cells grown at low folate had increased expression of *BNA2* (Figure 4B). However, in *set1Δ* cells, folate deficiency appeared to have a minor effect on

Figure 3 Directness of effect of nutrition on H3K4 methylation. (A) H3K4 di- and trimethylation in folate-deficient *fol3Δ* cells after adding 15 μg/ml methionine. Quantification of band density normalized to quantification of Pgk1 band density is shown below each blot, after normalization to wild-type values. $n = 2$, with a representative blot shown. (B) H3K4 di- and trimethylation in wild-type and *fol3Δ* cells grown at different temperatures. Growth rate is shown as doubling time, calculated over the whole growth of the culture. Pgk1 band density served as a loading control. Quantification of band density normalized to quantification of Pgk1 band density is shown below each blot, after normalization to the wild-type value at 30°. (C) *SET1* mRNA levels (left) and Set1-TAP protein levels (right) in folate-deficient, folate-replete, and wild-type cultures. RNA was measured by quantitative PCR and protein was measured by quantitative Western blotting. *SET1* mRNA values were normalized to *ACT1* mRNA from the same sample and then to wild type. Set1-TAP values were normalized to Pgk1 from the same sample as a loading control and then to wild type. $n = 3$.

BNA2 expression, although not statistically significant. Conceivably folate deficiency had other small effects on *BNA2* expression, apart from through H3K4 methylation by *Set1*.

ChIP was used to determine the status of H3K4 methylation directly at *PER33* and *BNA2*. The local H3K4 trimethylation at *PER33* was lower in folate auxotrophic cultures grown at high folate than in wild-type cultures (Figure 4C), which is consistent with the lower *PER33* expression observed. The local H3K4 trimethylation did not further decrease in folate auxotrophic cultures grown at low folate, which suggests that the further decrease in expression is mediated by changes in H3K4 methylation at loci other than *PER33* itself. H3K4 trimethylation at *BNA2* in folate auxotrophic cultures grown at high folate was higher than that in wild-type cultures (Figure 4D), consistent with the higher expression observed (Pokholok *et al.* 2005). This result suggested that the change in expression was either mediated through a decrease in H3K4 methylation elsewhere in the genome such as at a repressor of *BNA2* or in a manner independent of *Set1*. Moreover, this result hinted that the effect of folate limitation on histone methylation was not due to a simple direct coupling of folate and SAM levels.

H3K79 methylation was more resistant to folate limitation than H3K4 methylation

To see if the effect of folate limitation on H3K4 methylation applied to other histone methylation reactions, H3K79 methylation was evaluated under conditions of nutritional stress. The enzyme that catalyzes H3K79 methylation, *Dot1*, is a member of the only known non-SET-domain histone methyltransferase family (Sawada *et al.* 2004). Thus, assaying both H3K4 and H3K79 methylation covers a considerable evolutionary spread in histone methyltransferase structure. In wild-type cells, folate deficiency caused a slight, although statistically significant, decrease in H3K79 trimethylation ($P < 0.05$) and had no statistically significant effect on H3K79 dimethylation (Figure 5A). There was no effect of folate deficiency on TAP-tagged *Dot1* protein levels (data not shown).

A simple explanation for the modest effect of folate limitation on H3K79 methylation compared to the effect on H3K4 methylation would be that *Dot1*'s K_m for SAM is lower than *Set1*'s. If so, the effect of folate deficiency would be less pronounced as measured by H3K79 methylation than when measured by H3K4 methylation. One prediction of this

Figure 4 Effect of folate deficiency on the expression of *SET1*-responsive genes. (A) *PER33* expression, as determined by RT-PCR. All cDNA values were internally normalized to *ACT1* cDNA values from the same cDNA preparation. Within an experiment, the expression values are normalized to wild type. $n = 4$. Here and elsewhere, (*) $P < 0.05$, Student's t -test. Error bars, standard error. (B) *BNA2* expression. Normalization was as in A; $n = 3$. (C) ChIP analysis of H3K4 trimethylation at *PER33*, as determined by qPCR. Values are expressed as the H3K4 trimethylation enrichment at *PER33* relative to *HMRA1* (a negative control locus), normalized to H3 enrichment at *PER33* relative to *HMRA1*. $n = 2$. (D) ChIP analysis of H3K4 trimethylation at *BNA2*, as determined by qPCR. Normalization was as in C; $n = 2$.

model was that a hypomorphic mutant *Dot1* enzyme might be more strongly affected by a decrease in folate deficiency than wild-type *Dot1*. The *dot1-G401A* mutant allele used for this analysis causes a nearly complete loss of H3K79 trimethylation and a 60% reduction in dimethylation (van Leeuwen *et al.* 2002). Indeed, in a *dot1-G401A fol3Δ* strain grown in low-folate medium, H3K79 dimethylation was significantly reduced, on average by 45% relative to high-folate medium (Figure 5B). Thus, H3K79 methylation was more susceptible to folate deficiency in a strain with a partially defective *Dot1* enzyme. The crystal structure of *Dot1* predicts that glycine-401 resides close to the *Dot1* SAM-binding region (Sawada *et al.* 2004) and thus could plausibly affect its K_m for SAM. Regardless of the mechanism, this result established the existence of a hierarchy of vulnerabilities among histone methyltransferases for limiting folate levels.

S-Adenosyl-methionine detection in cell extracts

The results described above were consistent with a hypothesis that cells in low-folate medium experienced a shortage of SAM. To test this notion, SAM levels were measured in cell extracts by two independent and orthogonal methods. By LC-MS, SAM was 55% lower in cultures grown in low folate than high folate, a difference that was statistically significant (Figure 6A). However, surprisingly, SAM levels measured from wild-type cells were similar to the levels seen in the *fol3Δ* cultures grown in low folate and were also significantly lower than the level of SAM measured from

fol3Δ cultures grown in high folate. To address the possibility that the efficiency of SAM extraction was different between genotypes, the levels of several other metabolites were determined in the same extracts, as a control for extraction efficiencies. However, levels of lysine, arginine, tryptophan, and *S*-adenosyl-homocysteine did not vary between genotypes in the same way as SAM levels varied (Figure 6B). Thus, differences in metabolite extraction efficiency among the strains were unlikely to account for the differences in SAM levels.

As an independent test of the SAM levels as detected by LC-MS, a second method was used to determine SAM levels in the cellular extracts. Riboswitches are a class of RNA molecules that contain an aptamer domain that changes conformation upon binding metabolites or ligands (Roth and Breaker 2009). Several naturally occurring SAM-binding riboswitches have been found. The mobility of a riboswitch aptamer on a native PAGE slab gel changes upon SAM binding, and the ratio of the bound and unbound species can be used to measure SAM concentration, for riboswitch aptamers with slow interconversion rates between the bound and unbound state (Karns *et al.* 2013), such as the SAM-I riboswitch aptamer from *Polaribacter irgensii*. When incubated with extracts from *fol3Δ* cells grown with high folate, more of the SAM-I riboswitch aptamer was bound to SAM than when incubated with extracts of *fol3Δ* cells grown with low folate or with extracts of wild-type cells (Figure 6, C and D). This result was consistent with that

Figure 5 Effect of exogenous folinic acid on H3K79 methylation in *S. cerevisiae*. All normalizations are as in Figure 2. (A) H3K79 di- and trimethylation in *fol3Δ* cells grown at different concentrations of folinic acid. $n = 3$ for dimethylation; $n = 4$ for trimethylation. Error bars, standard error. The difference between H3K79 trimethylation between 50 $\mu\text{g/ml}$ folinic acid and 10 $\mu\text{g/ml}$ folinic acid was marginally significant at $P < 0.05$, Student's t -test. (B) H3K79 di- and tri-methylation in *fol3Δ dot1-G401A* cells grown at different concentrations of folinic acid. Normalization was to the level of methylation in a folate prototroph with wild-type *DOT1*. $n = 3$. (*) $P < 0.05$, Student's t -test.

seen for SAM detection by LC-MS. Thus, SAM quantification by two independent methods indicated that SAM levels were comparable in wild-type cells and in folate auxotrophic cultures grown in low-folate medium and higher in folate auxotrophs grown in high-folate medium.

Histone methylation-nutrition interaction in *S. pombe*

The fission yeast *S. pombe* is among the most distant relatives of *S. cerevisiae* among yeast species—one estimate places their divergence as having occurred 330–420 million years ago (Sipiczki 2000), and another places it at more than a billion years ago (Heckman *et al.* 2001). To evaluate whether the relationship between nutrients and chromatin modification was conserved over a large timescale of evolutionary history, and hence would be expected to be a widespread principle among eukaryotes, these analyses were extended to *S. pombe*. The pattern of histone methylation differs between the two species—H3K9 methylation is present in *S. pombe* (Nakayama *et al.* 2001), yet missing in the *S. cerevisiae* lineage (Klose *et al.* 2007), whereas H3K79 methylation is present in *S. cerevisiae* (van Leeuwen *et al.* 2002), but has not been detected in *S. pombe*. H3K4 methylation is present in both species. The H3K4 methyltransferase in *S. pombe* is an ortholog of *S. cerevisiae*'s *Set1* (Noma and Grewal 2002), and the localization pattern of H3K4 meth-

ylation in gene bodies is also conserved. Set1 in *S. pombe* also requires SAM as a cofactor (Roguev *et al.* 2003). Folate and methionine appear to play the same essential roles in SAM synthesis, as all the genes predicted to encode for proteins involved in SAM synthesis from folate and methionine have orthologs in the *S. pombe* genome. Thus, every component required for the interaction observed between H3K4 methylation and nutritional status in *S. cerevisiae* is also present in *S. pombe*.

As with *S. cerevisiae*, *S. pombe* can synthesize folate and methionine, so it was necessary to create auxotrophic strains of *S. pombe*. *S. pombe fol1Δ* and *met6Δ* were tested for histone methylation levels in cultures grown with differing folate and methionine concentrations, respectively, as described above. Methionine deficiency caused H3K4 trimethylation to decrease on average 35%, which was statistically significant (Figure 7B). Although histone methylation appeared to be decreased in folate-deficient cultures, the effect was not sufficiently robust to support statistical significance (Figure 7A).

The effect of methionine deficiency on histone methylation levels established the generality of nutrient limitation on chromatin modification. At present, the lack of a significant effect of low folate on H3K4 methylation is not understood.

Figure 6 Measurements of SAM concentration in cell extracts from *fol3Δ* cells grown at varying folate concentrations. (A) SAM detection by LC-MS. $n = 3$. (*) $P < 0.05$, Student's t -test. Error bars, standard error. (B) Detection of arginine, lysine, tryptophan, and S-adenosyl-homocysteine (SAH) in the same cellular extracts as in A, by LC-MS. (C) Binding of the *Pi* SAM-I riboswitch aptamer to either purified SAM or SAM from cellular extracts. $n = 3$ for extracts; a representative gel is shown. (D) Quantification of the fraction of RNA bound to SAM from the experiment as shown in C (graph includes all three replicates); values are expressed as the percentage of aptamer RNA in the bound fraction (lower band) compared to the percentage of aptamer RNA in the bound fraction in the 1 mM SAM control lane, after the background signal from the 0 mM SAM control lane had been subtracted from both values.

Histone methylation–nutrition interaction in human cells

Humans are auxotrophic for both folate and methionine. (Although humans can synthesize methionine from homocysteine, humans lack genes required for *de novo* homocysteine synthesis.) In addition to the methionine taken from diet, 5-methyltetrahydrofolate is still required for proper methionine homeostasis in humans, through the remethylation of homocysteine produced by methylation reactions. Moreover, many histone methylations are conserved from yeast to humans; for instance, H3K4 methylation in humans is placed by human SET-domain proteins and is enriched at transcriptionally active loci (Ruthenburg *et al.* 2007), just as in yeast species. In addition, humans have well-described histone methylations on lysines K9, K27, K36, and K79 of histone H3, and lysine K20 of histone H4 (Barski *et al.* 2007; Mikkelsen *et al.* 2007), all of which are shared with *S.cerevisiae*, *S. pombe*, or other fungal species (Smith *et al.* 2008). H3K9 methylation is enriched in heterochromatin (Barski *et al.* 2007), making it functionally distinct from H3K4 methylation. To determine whether the effects observed in *S. cerevisiae* and *S. pombe* pertained to human cells, histone methylation levels in human K562 cells were tested at a range of folate and methionine concentrations that are within the range of concentrations in human serum (Friso *et al.* 2002; Kimura *et al.* 2004).

Relative to K562 cells grown in medium with high folate and high methionine, cells grown with low levels of both nutrients averaged a 23% reduction in H3K4 trimethylation

(Figure 8A). Trimethylation of H3K9 was also reduced in K562 cells grown in low folate and methionine medium, to a similar degree (Figure 8B). Growth in medium with sufficient methionine but low folate did not significantly reduce H3K4 trimethylation or H3K9 trimethylation levels.

To determine if the effect of nutritional limitation on chromatin modification caused changes in gene expression, we examined the human *HBG1* locus, encoding γ -globin, because it is expressed in K562 cells and because its histone methylation state is well characterized (Kim *et al.* 2007). We determined its expression level by quantitative RT-PCR on mRNA from cells grown in these media. The expression of *HBG1* was reduced by 46% in cultures grown in low folate and methionine relative to cultures grown with high folate and methionine (Figure 8C), which is consistent with effects through loss of H3K4 methylation. Enrichment of H3K4 trimethylation at the *HBG1* locus was decreased 43% in cultures grown in low folate and methionine relative to cultures grown with high folate and methionine, which was statistically significant (Figure 8D).

Discussion

Nutritional deficiency affects histone methylation

The studies described here established an evolutionarily conserved link between the nutritional status of a eukaryotic cell and methylation at two different sites on histone H3, at least one of which has demonstrated epigenetic potential (Ng *et al.* 2003). Either folate or methionine limitation could

Figure 7 Effect of exogenous folinic acid and methionine on H3K4 methylation in *S. pombe*. All normalizations are as in Figure 2; $n = 3$ for all graphs. (*) $P < 0.05$, Student's t -test. Error bars, standard error. (A) H3K4 di- and trimethylation in *S. pombe fol1Δ* cells grown at different concentrations of folinic acid. (B) H3K4 di- and tri-methylation in *S. pombe met6Δ* cells grown at different concentrations of methionine.

reduce histone methylation in two distantly related species of yeast, as well as in human cells in culture. Furthermore, the changes in histone methylation resulting from folate deficiency caused changes in gene expression that appeared to be direct effects of the change in histone methylation.

The effect of nutritional limitation was more pronounced on H3K4 methylation than on H3K79 methylation, suggesting a hierarchy of sensitivities to nutritional limitations. Indeed, levels of H3K79 methylation placed by a hypomorphic mutant form of *Dot1* were more susceptible to nutritional changes. Relatedly, yeast cultures grown with an intermediate concentration of folate had a significantly reduced level of H3K4 trimethylation, but not dimethylation. In contrast, at the lowest concentration of folate, both trimethylation and dimethylation were lowered to a significant degree.

If folate levels were directly coupled to SAM levels, these observations could easily be understood if the K_m of the *Set1* and *Dot1* methyltransferases differed, perhaps with nuanced differential impacts on dimethylation vs. trimethylation. However, to our surprise, SAM levels, as measured by two completely different methods, were virtually identical between a folate auxotroph grown in low-folate medium and a folate prototroph.

Folate, methionine, and SAM have essential functions in cells unrelated to histone methylation—for instance, folate is necessary for synthesis of purines (Rébora *et al.* 2005), methionine is crucial for all protein synthesis, and methylation via SAM is required for rRNA maturation (Tollervey *et al.* 1991). In principle, if a nutritional deficiency were to compromise one of these other functions before affecting histone methylation, the cell would likely die of the nutritional de-

ficiency without histone methylation levels being perturbed. Thus, the results in this study implied the existence of a metabolic triage mechanism in which the less essential metabolic functions of folate, methionine, and SAM in histone methylation were compromised so that SAM levels could be maintained for essential functions, similar to the triage mechanism proposed by Ames (2006). This triage mechanism was not mediated through transcriptional or translational control of the histone methyltransferases, but could have involved direct regulation of methyltransferase activity, cellular compartmentalization of the metabolites, or less direct mechanisms.

Generality of the link between one-carbon metabolism and histone methylation

To the best of our knowledge, this is among the first reports of a link between histone methylation and nutritional status. However, previous studies have demonstrated a link between DNA methylation and nutritional status. In *Neurospora crassa*, a fungal species that has DNA methylation, temperature-sensitive methionine auxotrophs have reduced DNA methylation, and this effect can be reversed by methionine supplementation (Roberts and Selker 1995). It is worthwhile to note that in *Neurospora* H3K9 methylation is necessary for DNA methylation, as H3K9 methylation recruits the DNA methyltransferase DIM-2 to DNA (Tamaru and Selker 2001). Thus, if the effect of nutritional limitation on histone methylation observed here also holds in *Neurospora*, a reduction in DNA methylation could be due to effects on the DNA methyltransferase or the combined effects on the DNA and histone methyltransferases.

Figure 8 Effect of exogenous folinic acid and methionine on histone methylation in human K562 cells. (A) H3K4 trimethylation in cells grown in defined medium containing different levels of folinic acid and methionine. Values were normalized to values for H4 from the same sample and then normalized to the nutrient-rich culture from the same experiment. $n = 6$. (*) $P < 0.05$, Student's t -test. Error bars, standard error. (B) H3K9 trimethylation in cells grown in defined medium containing different levels of folinic acid and methionine. Normalization was as in A. (C) Expression of *HBG1* in cells grown in defined medium containing different levels of folinic acid and methionine. Values were normalized to *ACTB* from the same sample. $n = 3$. (D) Enrichment of H3K4 trimethylation at *HBG1* relative to enrichment at *HBE1* from the same sample (both values first normalized to input). $n = 3$.

The results of this study uncovered the unexpected compensatory mechanism for maintaining SAM levels in the face of nutritional limitations, which was not evident in previous studies. Genes in *Drosophila melanogaster* can be silenced due to their genomic context. The histone methyltransferase *E(z)*, which catalyzes H3K27 methylation (Czermin *et al.* 2002), is one of the founding members of the SET-domain family of methyltransferases (Tschiersch *et al.* 1994). *E(z)* was originally described by gain-of-function mutations that enhanced the silencing of *w^{is}* by the *zeste* allele *z¹* (Kalisch and Rasmuson 1974). Interestingly, a loss-of-function mutation in SAM synthetase, termed *Su(z)5*, suppresses *w^{is}* silencing (Larsson and Rasmuson-Lestander 1994). In light of the results described here, one parsimonious explanation for the involvement of SAM synthetase in *w^{is}* silencing is that *E(z)* is sensitive to changes in SAM availability, and thus mutants with impaired SAM synthetase function have silencing defects due to reduced H3K27 methylation. Similarly, depletion of SAM synthetase by RNAi in *Caenorhabditis elegans* reduces histone methylation genome wide (Towbin *et al.* 2012), and inactivation of *S*-adenosyl-homocysteine hydrolyase in *Arabidopsis thaliana* disrupts gene silencing through effects on chromatin methylation (Baubec *et al.* 2010). Because the experimental conditions in these experiments targeted SAM levels directly, it

is not clear whether the compensatory mechanism revealed in this study operates in *Drosophila* and *C. elegans*.

Human disease

Folate deficiency in humans is a strong risk factor for neural tube defects (NTDs) (Wald *et al.* 1991), a common form of birth defect. Histone methylation plays important roles in development. For instance, H3K27 methylation is essential for proper Hox gene patterning, conserved from flies to mammals (Cao *et al.* 2002; Papp and Müller 2006). How folate deficiency causes NTDs is an area of active research, and one possibility suggested by this work is that folate deficiency causes defects in histone methylation that disrupt development. To date, the evidence concerning links between maternal MTHFR genotype and risk for NTDs is mixed; however, among some ethnicities children born to mothers with the common A222V variant seem to be at higher risk for neural tube defects (Botto and Yang 2000). MTHFR synthesizes the form of folate used in methionine synthesis, and therefore the reduced enzyme function of the A222V allele could create a stronger requirement for folate in proper methionine homeostasis. If histone methylation perturbation were the cause of NTDs, the common A222V allele of MTHFR might cause altered histone methylation. A more comprehensive evaluation of genetic variants of all

genes affecting folate metabolism has provided a stronger link to NTDs than previously reported and is also consistent with a mechanism rooted in chromatin modification (Marini *et al.* 2011).

Antifolates such as methotrexate are commonly used in cancer treatment (Alkins *et al.* 1996). Their proposed mechanism of action is inhibition of DNA synthesis in cancer cells, as folate has roles in the synthesis of purines and dTTP (Takezawa *et al.* 2011). Given the results of this study, it may be important to consider the effects of antifolate treatment on histone methylation. Perhaps the effectiveness of antifolates in cancer chemotherapy is due to a combined ability to inhibit DNA synthesis and to restrict the epigenetic options available to promote the evolution of the tumor.

Importantly, as histone methylation patterns have the potential to be inherited epigenetically across cellular generations (Ng *et al.* 2003; Hansen *et al.* 2008), consequences of nutritional deficiency could continue to manifest even after the nutritional deficiency has passed. The coat color of mice with the *Agouti viable yellow* allele is correlated with the DNA methylation and histone modifications (Morgan *et al.* 1999, Dolinoy *et al.* 2010) at a transposon at the *Agouti* locus. Elegant studies have demonstrated a nutritional effect on the coat color of these mice (Morgan *et al.* 1999), which persists even in progeny fed normal diets (Wolff *et al.* 1998, Cropley *et al.* 2006). Persistent effects of nutritional deficiency have also been noted in humans, as the health of individuals gestated during famine is affected in various ways later in life, with these individuals also showing DNA methylation pattern changes at various genetic loci involved in disease (Tobi *et al.* 2009).

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Nutritional Control of Epigenetic Processes in Yeast and Human Cells

**Meru J. Sadhu, Qiaoning Guan, Fei Li, Jade Sales-Lee, Anthony T. Iavarone,
Ming C. Hammond, W. Zacheus Cande, and Jasper Rine**

Table S1 Yeast strains used in this study

Name	Species	Genotype	Source
JRY6331	<i>S. cerevisiae</i>	<i>MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i> (BY4742)	Jef Boeke
JRY9339	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i>	this study
JRY0527	<i>S. cerevisiae</i>	<i>MATα met2 ade2-101 his3-200 lys2-801 ura3-52</i>	Mark Johnston
JRY9340	<i>S. cerevisiae</i>	<i>set1Δ::HIS3MX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i>	this study
JRY9341	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX set1Δ::HIS3MX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i>	this study
JRY8879	<i>S. cerevisiae</i>	<i>sir1Δ::TRP1 hmr-a1::K.I.URA3 MATα ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1</i> (W303)	Osborne, 2009
JRY9342	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX sir1Δ::TRP1 dot1Δ::HIS3MX hmr-a1::K.I.URA3 MATα ade2-1 lys2 leu2-3,112 his3-11 trp1-1 ura3-1</i>	this study
JRY9343	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX sir1Δ::TRP1 dot1Δ::HIS3MX hmr-a1::K.I.URA3 MATα ade2-1 lys2 leu2-3,112 his3-11 trp1-1 ura3-1</i> [pJR2541]	this study
JRY9344	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX sir1Δ::TRP1 dot1Δ::HIS3MX hmr-a1::K.I.URA3 MATα ade2-1 lys2 leu2-3,112 his3-11 trp1-1 ura3-1</i> [pJR2542]	this study
JRY9345	<i>S. pombe</i>	<i>h- ura4-D18 leu1-32 his3-301 ade6-M210</i>	Zac Cande
JRY9346	<i>S. pombe</i>	<i>fol1::KanMX h- ura4-D18 leu1-32 his3-301 ade6-</i>	this study
JRY9380	<i>S. pombe</i>	<i>met6::loxP h- ura4-M190T leu1-32</i>	Fujita, 2006
JRY9381	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX SET1-TAP::his3MX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i>	this study
JRY9382	<i>S. cerevisiae</i>	<i>fol3Δ::KanMX DOT1-TAP::his3MX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1</i>	this study

Table S2 Plasmids used in this study

plasmid	backbone	yeast selection	insert	source
pJR2541	pRS315	LEU2	DOT1	van Leeuwen, 2002
pJR2542	pRS315	LEU2	dot1(G401A)	van Leeuwen, 2002

Table S3 Oligonucleotides used in this study*RT-qPCR*

<i>ACT1</i>	GGCATCATACCTTCTACAACGAATTG GTTTTGTCCTTGACTCTTCCGGTAG
<i>BNA2</i>	GACGTTGCATTTCATGCTG CCCTGCACTGCTTAAGAAG
<i>PER33</i>	TTCCAGGAATCGTCCCATG CGATGGCATATGTAACGGAT
<i>HBG1</i>	TGGCAAGAAGGTGCTGACTTC GCAAAGGTGCCCTTGAGATC
<i>ACTB</i>	AAGATCAAGATCATTGCTCCTC ATACTCCTGCTTGCTGATCC

ChIP qPCR

<i>HMRA1</i>	CACGGGCATTTTGTAGAAGAGG TTGAGAACAAGAGCAAGAC
<i>BNA2</i>	AACCGGACCACAAGTACTAC TCCTCTTGCAAAAGAGAGGG
<i>PER33</i>	TTCCAGGAATCGTCCCATG CGATGGCATATGTAACGGAT
<i>HBG1</i>	TGGCAAGAAGGTGCTGACTTC GCAAAGGTGCCCTTGAGATC
<i>HBE1</i>	CACAACTTAGTGCCATCCATCAC CCCTGTTCTCCATGGTACTTAAAG