Title
Interactions between Nutrition, Chromatin, and Gene Regulation in Yeasts

Author
Sadhu, Meru Jyoti

Publication Date
2012

Peer reviewed|Thesis/dissertation

Interactions between Nutrition, Chromatin, and Gene Regulation in Yeasts

by

Meru Jyoti Sadhu

A dissertation submitted in partial satisfaction of the
requirements of the degree of
Doctor of Philosophy
in
Molecular and Cell Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:

Professor Jasper Rine, chair
Professor Barbara Meyer
Professor Zac Cande
Professor Lisa Barcellos

Spring 2012

Interactions between Nutrition, Chromatin, and Gene Regulation in Yeasts

by Meru Jyoti Sadhu

Abstract

Interactions between Nutrition, Chromatin, and Gene Regulation in Yeasts

by

Meru Jyoti Sadhu

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Jasper Rine, Chair

The consumption of nutrients is one of the main ways organisms interact with their local environments. Especially considering the importance of nutrition in human disease, it is vital to understand the nature of this interaction. All the work described in this thesis was motivated by this goal, through experiments done on various yeast species. I explored whether deficiency of the vitamin folate affected a potentially heritable form of gene regulation, histone methylation. Also, I conducted experiments to understand the factors mediating the transcriptional regulation involved in the synthesis of sulfur-containing amino acids. Finally, I sought to characterize a highly-conserved gene predicted be involved in methionine synthesis.

The vitamin folate is required for proper methionine homeostasis in all organisms. Methionine is the precursor to s-adenosyl-methionine (SAM), which is used in myriad cellular methylation reactions, including all histone methylation reactions. Thus, I conducted experiments that showed that folate and methionine deficiency led to reduced methylation of lysine 4 of histone H3 (H3K4). This was not due to a general growth defect, as cells grown at low temperature did not show any effect on H3K4 methylation. The effect on H3K79 methylation was less pronounced. It was possible to exacerbate the effect on H3K79 methylation through the use of a hypomorphic allele, indicating the enzyme responsible for H3K79 methylation, Dot1, was less sensitive to changes in SAM concentration than the enzymes responsible for H3K4 methylation, Set1. Folate deficiency also caused changes in gene transcription that mirrored changes seen during complete loss of H3K4 methylation. Finally, methionine deficiency was also seen to affect H3K4 methylation in the fission yeast *Schizosaccharomyces pombe*, though folate deficiency did not.

Transcription of the *MET* regulon, encoding the proteins involved in the synthesis of the sulfur-containing amino acids, methionine and cysteine, is repressed by the presence of either methionine or cysteine in the environment. This is accomplished by ubiquitination of the transcription factor Met4, carried out by the SCF(Met30) E3 ligase. Previous work indicates cysteine is the repressive agent, and methionine downregulates

the *MET* regulon through its ability to be converted to cysteine. However, I found that a previously untested member of the *MET* regulon, *STR3*, was controlled by methionine availability, rather than cysteine. This fits the logic of the synthesis pathway, as Str3 is involved in synthesis of methionine from cysteine. Also, in order to understand how the activity of SCF(Met30) towards Met4 is controlled, I investigated whether there are post-translational modifications of Met30 that are differential between cells grown with and without sulfur-containing amino acids. Finally, I conducted a screen to find more components of the machinery controlling *MET* regulon repression. I found that loss of Cho2, which is involved in the methylation of phosphatidylethanolamine to produce phosphatidylcholine, leads to upregulation of the *MET* regulon, which is due to reduced cysteine synthesis in *cho2Δ* cells.

Methylenetetrahydrofolate reductase (MTHFR) produces the substrate methyltetrahydrofolate (MTHF), which is required for methionine synthesis. Genomic analysis indicates that many fungal species have two copies of MTHFR-encoding genes. In *S. cerevisiae* deletion of one of the MTHFR-encoding genes (*MET13)* causes methionine auxotrophy, whereas deletion of the other (*MET12)* does not. I found that the *MET13* ortholog was also essential for methionine prototrophy in *Saccharomyces bayanus*, indicating that *MET12* has not been able to support MTHF synthesis for a significant evolutionary period. To determine the critical differences between *MET12* and *MET13*, I tested a series of chimeric proteins composed of parts of Met12 and Met13 for the ability to support methionine prototrophy, and found that much of Met12 could be substituted into Met13 to produce functional chimeras. However, two small critical regions of Met12 crippled MTHFR function when substituted into Met13, both of which were in the catalytic domain.

Contents

Acknowledgements ii

Chapter 1: Introduction to chromatin and one-carbon metabolism 1
Figure 1.1: One-carbon metabolism in yeast 2

Chapter 2: Nutritional control of epigenetic processes in yeast 9
Figure 2.1: Interaction between folate, methionine, and cellular methylation 10
Results 11
Figure 2.2: Effect of environment on H3K4 methylation in *S. cerevisiae* 12
Figure 2.3: Effect of folate deficiency on the expression of *set1Δ-sensitive* genes 14
Figure 2.4: Effect of environment on H3K79 methylation in *S. cerevisiae* 16
Figure 2.5: Effect of environment on H3K4 methylation in *S. pombe* 17
Discussion 18
Materials and Methods 20

Chapter 3: Exploration of the complexity and logic of *S. cerevisiae* methionine regulation 23
Figure 3.1: Synthesis of sulfur-containing amino acids in *S. cerevisiae* 24
Results 25
Figure 3.2: Methionine starvation induction in different methionine auxotrophs 27
Figure 3.3: MET regulon induction in strains deficient for pathway intermediates 29
Figure 3.4: Effect of methionine availability on Met30 31
Figure 3.5: CHO2 mutations induce *MET3-GFP* 33
Discussion 34
Materials and Methods 37

Chapter 4: Characterization of an ancient duplication in fungal methylenetetrahydrofolate reductase 40
Results 41
Figure 4.1: Methionine prototrophy of *S. bayanus* deleted for *SbMET12* or *SbMET13* 42
Figure 4.2: Stability of Met12 in the absence of Met13 43
Figure 4.3: Mapping of functional domains of Met12 with chimeric proteins 45
Discussion 46
Materials and Methods 49

Table 1: Yeast strains used 51
Table 2: Oligonucleotides used 53
Table 3: Plasmids used 55
References 56

Acknowledgements

Jasper Rine, and his teachings and philosophies, have been instrumental in guiding me through the process of becoming a researcher. I leave graduate school with a goal not only to stay in academia and become a primary investigator, but to become a primary investigator in the image of Jasper, who manages to create a laboratory environment of independent, intelligent, and – most importantly – happy scientists. He has been my most important colleague in designing of my projects and experiments, as well as in the writing of this thesis.

My maturation as a researcher (as well as my continued sanity) also owes a great deal to the scientists I've had the pleasure of working with in my time at Berkeley. I hope I will always have colleagues as supportive, positive, and engaging as the ones in the Rine lab. Although I would like to write about each of my labmates at length, for the sake of brevity I'll simply name them here, and remember at least one happy moment I shared with each of them as I write their names: Josh Babiarz, Sarah Bissonnette, Yi Chao, Dago Dimster-Denk, Anne Dodson, Aisha Ellahi, Jen Gallagher, Qiaoning Guan, Jeff Halley, Laura Lombardi, Nick Marini, Jake Mayfield, Erin Osborne, Bilge Ozaydin, Dave Steakley, Lenny Teytelman, Debbie Thurtle, Oliver Zill, and Rachel Zunder.

One person I've worked very closely with is Jessica Pasqua, who started in the lab as an undergraduate. Jessica did all the bench work described in chapter four of this dissertation. At this point, she is almost certainly the world's leading expert on *MET12*. Over the course of the last two years, she has demonstrated a deep love for, and innate understanding of, science.

In addition to my mentorship of Jessica, I also had the opportunity to train two highly driven rotation students, Anjali Zimmer and Allison Craney, both of whom were very fun to work with. Anjali's work was essential for some of the experiments in chapter three.

My committee, composed of Barbara Meyer, Zac Cande, and Lisa Barcellos (as well as Jasper), has been very supportive during my graduate career. Zac Cande, as well as a former postdoc in his lab, Fei Li, collaborated with me for several years on some of the experiments in chapter two.

Back when I was an undifferentiated undergraduate, two professors were important in determining the path I was to take. Bruce Hay taught the Genetics course at Caltech that made me realize that I think "like a geneticist". And Scott Fraser, my undergraduate advisor, was a very involved mentor, and I highly appreciate the time and effort he spent.

My parents, Chanchal and Krishna Sadhu, obviously played a large role in making me who I am. Their support and encouragement has been essential my whole life. From a young age, they instilled a love of trying to understand the unknown. Though I never consciously acknowledged it, surely it is not a coincidence that I followed in their footsteps to pursue a doctorate in biology.

Practically speaking, graduate school has involved learning a great deal about biology, as well as a great deal about how to be an adult. I'm sure those lessons would have been a lot more challenging if not for the fact that I've had an exceptional roommate, Benjamin Lipshitz, the entire time I've been here.

And last, I can't imagine what my graduate school experience would have been like without my girlfriend Lyla Fadali. Through the periods of joy and frustration, epiphany and confusion, it has been enormously helpful to have someone to share with and confide in. I would also like to thank Lyla specifically for help writing this thesis – if this thesis is at all comprehensible, it is because of Lyla's advice and suggestions.

Chapter 1: Introduction to chromatin and one-carbon metabolism

The general theme of my graduate work has been the study of one-carbon metabolism, specifically the effects of one-carbon deficiencies on histone methylation, how feedback regulation in one-carbon metabolism might contribute to its homeostasis, and the role of duplication of genes involved in one-carbon metabolism.

One-carbon metabolism

One-carbon metabolism refers to the process of transferring methyl groups between donor and acceptor biological compounds (Suh, Herbig & Stover 2001). The two main "methyl donors" in one-carbon metabolism are folate molecules and s-adenosyl-methionine (SAM) (figure 1.1). Folate cofactors are used to perform a particular set of extremely important cellular methylation reactions. The synthesis of purines, thymidylate, and methionine all require folate cofactors for key methylation steps. Furthermore, methionine itself is a precursor to SAM. SAM, in contrast to folate, is used very widely as a methyl donor in DNA, RNA, lipid, small molecule and protein methylation reactions (Kagan, Clarke 1994). For instance, the synthesis of phosphatidylcholine, which comprises 40-45% of phospholipids in *Saccharomyces cerevisiae*, requires the consumption of three SAM molecules per molecule of phosphatidylcholine synthesized from phosphatidylethanolamine (Summers et al. 1988). The methyl group donated by a given molecule of SAM is, in fact, the same methyl group donated by folate to produce the methionine that was used to synthesize the SAM, thus linking the two components of one-carbon metabolism.

Mammals cannot synthesize their own folate. Instead, mammals must obtain folate through their food, and thus folate is considered a vitamin. Folate deficiency in humans has been implicated in a range of birth defects. Mandatory supplementation of folate in grains in the United States led to a significant drop in the prevalence of the neural tube defects (NTDs), anencephaly and spina bifida, though both persist (Honein et al. 2001). Studies also suggest folate supplementation protects against cleft palate (Tolarova, Harris 1995). How folate deficiency causes these defects is not understood. Some studies have demonstrated greater risk of NTDs in mothers carrying a common hypomorphic variant in the enzyme methylenetetrahydrofolate reductase (MTHFR) (Botto, Yang 2000), which is necessary to produce the form of folate used to synthesize methionine. Thus, the focus in the field is on the possibility that methionine deficiency or a reduction in some important cellular methylation is the root cause of these defects.

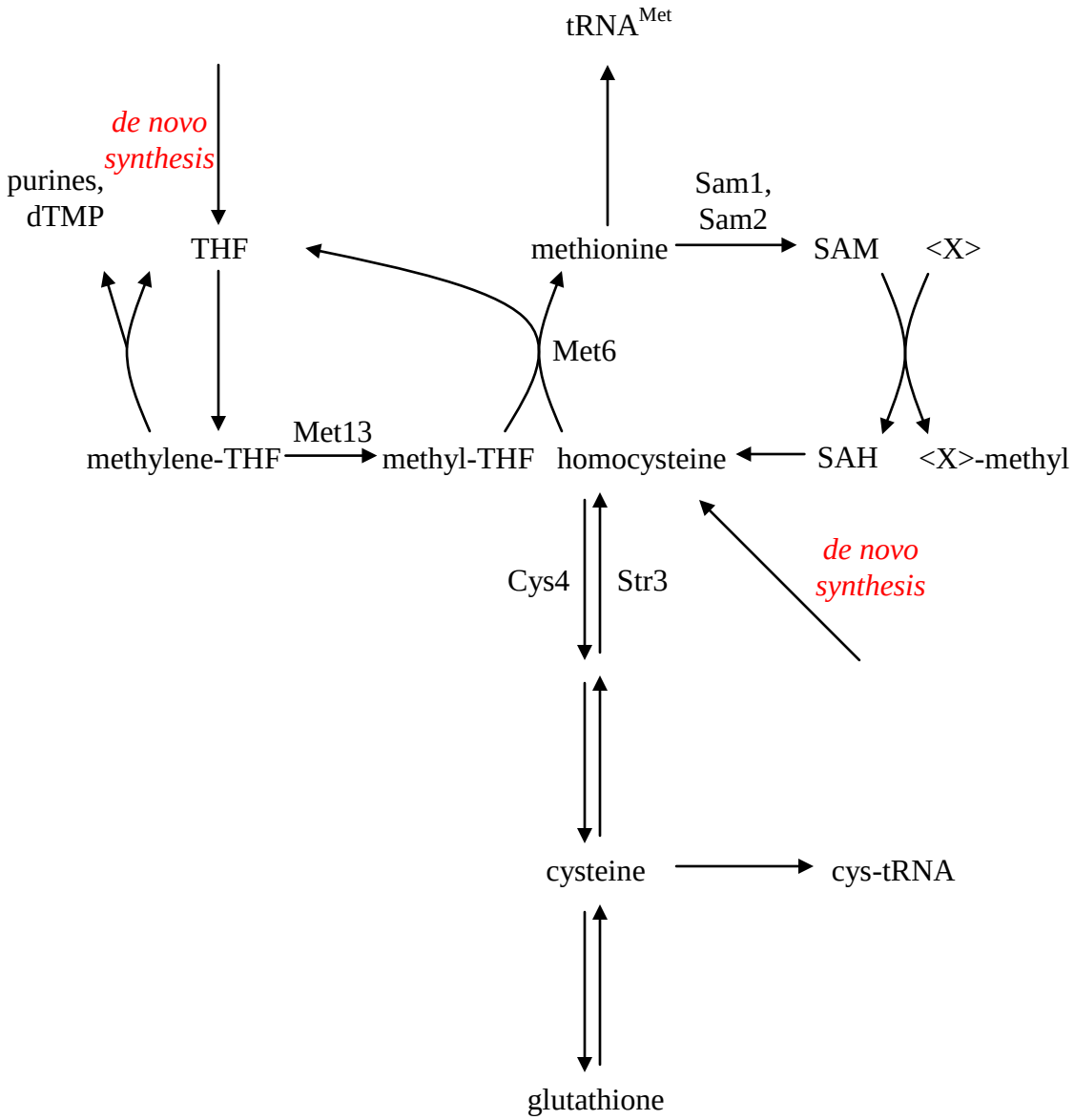


Figure 1.1: One-carbon metabolism in yeast. Select enzyme names are given. Both pathways marked "de novo synthesis" are not found in mammals.

Folate is used in *de novo* methionine synthesis in microorganisms, but in mammals methionine is an essential amino acid, meaning it is required in the diet (Cooper 1983). Still, folate is required for methionine homeostasis (figure 1.1). The consumption of SAM in methylation reactions produces s-adenosyl-homocysteine (SAH), which is hydrolyzed to homocysteine, the precursor of methionine. Homocysteine is then remethylated to methionine, in a reaction requiring folate. (This enzymatic reaction is conserved in microorganisms as the final step in *de novo* methionine synthesis.) Thus, a person with a methionine-rich, folate-poor diet could theoretically experience methionine stress due to the rapid consumption of methionine in the form of SAM without subsequent regeneration.

Microorganisms such as *S. cerevisiae* can synthesize folate and methionine. These synthesis pathways are well described, and thus it is possible to make mutant strains of yeast that mimic the nutritional requirements of humans by knocking out genes involved in the *de novo* synthesis pathways and supplementing the growth media with the proper nutrients (Bayly et al. 2001).

Methylation in chromatin

Because of the role of folate in SAM synthesis, folate deficiency could lead to a reduction in DNA or histone methylation. As described below, these methylation marks play important regulatory roles and thus could lead to developmental defects if disrupted. Additionally, patterns of DNA methylation and histone methylation have the potential to be inherited across generations, suggesting the possibility that folate deficiency could have effects even after the deficiency passed, either in the affected individual or in that individual's descendants. In chapter 2 I describe experiments that measured the effect of folate and methionine deficiency on histone methylation in *S. cerevisiae*, determined whether the observed effect was due to SAM limitation, and explored the phenotypic consequences of the changes in histone methylation.

Histone methylation occurs at multiple sites on multiple histone proteins. Methylation of different amino acids within the same histone can have very different effects on the associated genes. For instance, histone H3 methylations at positions lysine 4 (K4), K36, and K79 are found on actively transcribed genes, whereas H3 methylation on K9 and K27 is associated with silent chromatin (Kouzarides 2007). Furthermore, each lysine residue targeted for histone methylation can be methylated up to three times (referred to as mono-, di-, and trimethylation), and these different valencies of histone methylation can also have distinct roles (Fingerman et al. 2005). Apart from lysine methylation, there is methylation of histone arginine residues (Kirmizis et al. 2007).

The roles of distinct histone methylations are mostly defined by the protein factors binding the mark. For instance, in the fruit fly *Drosophila melanogaster* the silencing

complex PRC1 binds methylated H3K27, which is found in extended swaths at the silenced Hox genes (Ringrose, Paro 2004). H3K27 methylation has an essential role in silencing, as silencing is lost following mutation of the H3K27 methyltransferase, E(z) (Müller et al. 2002). The large size of silenced domains may facilitate their inheritance. For instance, during DNA replication unmethylated H3 histones are loaded amidst the nucleosomes carrying H3K27 methylation, which can recruit the complex containing E(z) to methylate neighboring unmethylated histones (Hansen et al. 2008). A similar mechanism was also proposed to underlie the inheritance of H3K9 methylation, another silencing mark (Bannister et al. 2001).

H3K4 methylation is found on nucleosomes associated with genes experiencing active transcription. The precise role of the methylation in transcription is unclear, but a global decrease in transcription was seen in *S. cerevisiae* strains lacking all H3K4 methylation due to deletion of the methyltransferase Set1 (Boa, Coert & Patterton 2003). Since Set1 is recruited to sites of active transcription by its association with PolII RNA polymerase, it is possible that H3K4 methylation plays some role in forming a positive feedback loop for transcriptional memory (Ng et al. 2003). There are also reports in *set1Δ* mutants of reduced meiotic double-strand break (DSB) formation, a key step in meiotic recombination (Borde et al. 2009).

Direct methylation of DNA comprises the other major class of chromatin methylation. DNA methylation is found in mammals predominantly on the two symmetric cytosine bases found in CG contexts (Jeltsch 2010) (referred to as "CpG methylation"). The most common sites of CpG methylation are transposons and other silenced regions of the genome. Its symmetric placement underlies a simple method for inheritance of the mark across cell divisions – after DNA is replicated each methylated CpG is paired with the newly synthesized strand's unmethylated CpG. This "hemi-methylated" DNA is bound by the DNA methyltransferase DNMT1, which then methylates the unmethylated cytosine (Goll, Bestor 2005).

Budding yeast have three widespread species of histone lysine methylation, all found on histone H3 – H3K4, H3K36, and H3K79. Each methyl mark is placed by a dedicated enzyme that can catalyze mono-, di-, and trimethylation. The first two are placed by the SET domain proteins Set1 (Roguev et al. 2001, Briggs et al. 2001) and Set2 (Strahl et al. 2002), respectively, whereas H3K79 methylation is placed by Dot1 (Ng et al. 2002), the only known non-SET domain histone methyltransferase. These methyl marks are conserved through to animals (Kouzarides 2007). The methyltransferases that place these marks are conserved as well, and their roles in gene regulation also seem to be related.

Curiously, budding yeast lack H3K9 methylation and H3K27 methylation, as well as DNA methylation. As mentioned above, all these marks are found on silenced chromatin. Phylogenetic analyses indicate that H3K9 methylation was lost in a unicellular ancestor of *S. cerevisiae* since the yeasts *S. pombe, Neurospora crassa,* and

Aspergillus fumigatus all carry homologs of Clr4 (Veerappan, Avramova & Moriyama 2008), and the *N. crassa* protein has been characterized as being an H3K9 methyltransferase (Tamaru, Selker 2001). Similarly, *N. crassa* and *A. fumigatus* each carry a homolog of the protein responsible for H3K27 methylation, E(z), and *N. crassa* has telomeric H3K27 methylation (Smith et al. 2008). DNA methylation was lost much more anciently, but is found in some mushroom species (Jeltsch 2010). (DNA methylation also appears in *N. crassa*, but the enzyme that catalyzes it is not related to canonical DNA methyltransferases, so an ancestor of *N. crassa* may have gained it independently.)

It is not clear why the *S. cerevisiae* lineage lost all these silencing-related chromatin modifications. One possibility is that in the reduction of the genome size of *S. cerevisiae* large amounts of heterochromatin were eliminated, obviating the need for silencing factors. Also, *S. cerevisiae* and related yeasts have Sir-protein-based silencing found at the silent mating type loci and telomeres (Rusche, Kirchmaier & Rine 2003); perhaps this novel silencing replaced silencing that used methylation. Of course, it is difficult to assign causality for any of these possibilities. Another difference in silencing mechanism in *S. cerevisiae* is the loss of an RNAi-like mechanism that silenced transposable elements, which was lost after the genesis of the beneficial killer virus (Drinnenberg, Fink & Bartel 2011).

Regulation of methionine synthesis

Over the course of my studies on how folate and methionine deficiency affect chromatin, I became interested in the regulatory response to SAM deficiency. After all, if folate deficiency or methionine deficiency leads to a decrease in SAM such that a particular cellular target becomes hypomethylated, this hypomethylation could be sensed and used as a signal to activate SAM synthesis. This hypothesis was especially compelling given the evidence that the presence or absence of protein methylation, in the form of histone methylation, can be read and acted upon by other proteins, as described above.

In addition its close relation to SAM synthesis, the synthesis of methionine is also intertwined with the synthesis of cysteine (figure 1.1). In *S. cerevisiae,* cysteine is synthesized solely from homocysteine (Cherest, Surdin-Kerjan 1992), which is also the direct precursor to methionine. The transcription of most genes involved in SAM, methionine, and cysteine synthesis is co-regulated by the activity of the leucine-zipper transcription factor Met4 (Thomas, Surdin-Kerjan 1997). Met4 has no DNA-binding activity and instead relies on the DNA-binding proteins Met31/Met32 (zinc-finger) or Cbf1 (basic helix-loop-helix) to be recruited to DNA. Met4 is only active under environmental conditions in which methionine and cysteine are limiting. This is due to the activity of the E3 ubiquitin ligase Met30, which rapidly ubiquitinates Met4 to

inactivate it under conditions of methionine or cysteine availability (Rouillon et al. 2000).

It is not known how Met30's activity is controlled by methionine and cysteine availability. For instance, it is not entirely clear what biomolecule is sensed that leads to repression of Met4 activity, or even if more than one biomolecule is sensed. Addition of either methionine or cysteine suffices to repress Met4's activity, but methionine and cysteine can be rapidly interconverted, so it is possible that only one of the two is actually a repressive agent. Additionally, it is possible that some other biomolecule that methionine or cysteine is converted to – such as $tRNA^{Met}$, $tRNA^{Cys}$, SAM, or the antioxidant glutathione – is the actual repressive agent. Experiments looking at mutants blocking the inter-conversion of methionine and cysteine indicate that cysteine deficiency is both necessary and sufficient for Met4 activity (Hansen, Johannesen 2000). However, this effect was only measured for the transcription of a small number of genes, and Met4 may not act equivalently at the promoters of all *MET* regulon members (Kuras et al. 2002).

The two DNA-binding proteins that recruit Met4 to chromatin have distinct DNA-binding motifs, whose distributions across the genome have been mapped (Lee et al. 2010). Most of the genes induced by starvation for methionine and cysteine have binding sites for both complexes. Interestingly, however, several induced genes encoding proteins responsible for steps in methionine or SAM synthesis after the branch point for cysteine synthesis (*STR3, MET6*, and *SAM1*) seem to have binding sites only for Met31/Met32. This dichotomy suggests the possibility that methionine and cysteine are both sensed to control the transcriptional response, with Met31/Met32's ability to recruit Met4 responding specifically to methionine. In this model, if the methionine/cysteine pool is too biased towards methionine, the cysteine deficiency will induce only those genes having Cbf1 binding sites, thus avoiding induction of methionine synthesis.

It is important to keep in mind that in addition to the regulation seen at the transcriptional level, there is also post-translational regulation of the components of one-carbon metabolism. MTHFR, which is dedicated to methionine synthesis, has a SAM-binding domain that downregulates MTHFR activity upon binding SAM (Roje et al. 2002). On the other hand, Cys4, which encodes cystathionine beta-synthase (CBS) and carries out the first dedicated step in cysteine synthesis (figure 1.1), has a SAM-binding domain that activates CBS activity upon SAM binding (Janošík et al. 2001). Thus, when SAM is plentiful methionine synthesis will be inactivated and homocysteine shunted to cysteine, and when SAM is deficient cysteine synthesis will be inactivated and homocysteine shunted to methionine, even in the absence of a transcriptional response.

In Chapter 3, I describe experiments to better understand how methionine and cysteine regulate transcription. I determined whether genes induced by methionine starvation are all induced by the same signal. I also investigated whether Met30's translation was reduced by the absence of methionine based upon my analysis of the

protein's primary sequence, inferred RNA structure and large-scale ribosome mapping datasets, and performed a screen to find more components of *MET* regulon control.

Function of duplicated MTHFR genes

One of the key enzymes in one-carbon metabolism is MTHFR, as it produces the form of folate that transfers a methyl group to homocysteine to form methionine, and eventually becomes the methyl group donated by SAM. Yeast have two genes that are predicted to encode MTHFR, named *MET12* and *MET13* (Raymond, Kastanos & Appling 1999). For some of my experiments concerning folate deficiency and histone methylation it was necessary to completely remove cellular MTHFR activity, which led to my interest in the functions of these two genes.

Deletion studies of *MET12* and *MET13* indicate that Met13 is solely responsible for MTHFR function in the cell – *met13Δ* strains are methionine auxotrophs, indicating that the presence of Met12 is insufficient to produce enough methionine for survival (Raymond, Kastanos & Appling 1999). Furthermore, overexpression of *MET12* in *met13Δ* cells still does not compensate for the methionine auxotrophy. Indeed, no enzymatic activity is detectable for Met12. Perhaps not surprisingly, then, *met12Δ* strains are not methionine auxotrophs, nor do they have any other noticeable phenotypes.

However, various observations inspired us to take a closer look at *MET12.* First, the duplication that created *MET12* is extremely ancient – *S. pombe*, one of the yeasts most distantly related to *S. cerevisiae,* also has two genes that encode for MTHFR (Naula et al. 2002). Indeed, nearly all completely sequenced yeast species carry two copies of genes encoding MTHFR, and phylogenetic analysis indicates they all stem from a single duplication event (Sienko et al. 2007). The fact that two MTHFR-encoding genes have been maintained for so long in the *S. cerevisiae* genome is strong evidence that *MET12* has a functional role, given the highly compact nature of the genome. For instance, a recent *S. cerevisiae* ancestor experienced a whole-genome duplication, from approximately 5,000 genes to 10,000, after which the genome underwent rapid gene loss, and currently contains approximately 5,500 genes (Scannell, Butler & Wolfe 2007). Yet, a single copy each of *MET13* and *MET12* has been retained in *S. cerevisiae* and in all other post-duplication species studied.

Furthermore, the deletion phenotypes for *MET12* and *MET13* homologs in other yeasts paint a more complicated picture for *MET12* function. In *Aspergillus nidulans* (Sienko et al. 2007) and *S. pombe* deletion of either MTHFR homolog causes methionine auxotrophy. This is a slightly surprising result – if Met12 and Met13 are functionally interchangeable MTHFRs, then normally one expects neither single deletion to cause methionine auxotrophy. Rather, one would expect half of the normal MTHFR activity to suffice, as seen in diploid *S. cerevisiae* hemizygous for *MET13*. Instead, these results

indicate that in *A. nidulans* and *S. pombe*, neither protein is capable of shouldering the complete MTHFR requirement.

Finally, in *S. cerevisiae* Met12 and Met13 form a heterodimer (Brooks et al. 2010). In non-fungal species MTHFR proteins function as homodimers (Matthews et al. 1984). At the very least, this result demonstrates *MET12* encodes a protein that behaves structurally like an MTHFR. This result would also seem to rule out one possibility suggested by the previous observation, namely, that *MET12* and *MET13* encode MTHFRs that reside in separate cellular compartments, such as the mitochondria and cytoplasm, as that would preclude their binding each other. Intriguingly, MTHFR homodimers from the bacteria *Thermus thermophilus* have only one subunit bound to the FAD cofactor, yet the overall activity of such a dimer was higher than the activity of dimers where FAD was forced to incorporate into both subunits (Igari et al. 2011). Met12 may be dedicated to playing a similar role to the enzymatically inactive *T. thermophilus* subunit.

In chapter 4, I describe experiments I conducted with an undergraduate, Jessica Pasqua, to investigate the role of *MET12*. We extended the phylogenetic analysis of *MET12* function to *Saccharomyces bayanus*, a species more closely related to *S. cerevisiae* than *A. nidulans* is, to determine if the observed difference between Met12 function in *S. cerevisiae* and *A. nidulans* was due to very recent changes in the *S. cerevisiae* lineage. To see if Met12 was a functional MTHFR that required Met13 binding for activity, we measured the abundance of Met12 protein in the absence of Met13. We also mapped the critical regions of Met12 that precluded its ability to function as the sole cellular MTHFR.

Chapter 2: Nutritional control of epigenetic processes in yeast

Introduction

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, 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, Gafken & Gottschling 2002). Methylations of lysines in histone H4 have also been recently reported (Green et al. 2012, Edwards, Dang & Berger 2011). Because unique lysine methylations have such different roles, it is not surprising that histone methyltransferase complexes are highly specific for a given lysine residue. In yeast H3K4 methylation is 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 (SAH) by the transfer of a single methyl group to the lysine acceptor (figure 2.1). All described histone methyltransferases and DNA methyltransferases require SAM as the methyl donor (Xiao et al. 2003, Sawada et al. 2004, Cheng et al. 1993, Jia et al. 2007). Thus, chromatin methylation marks are in principle susceptible to conditions that result in perturbations of the cellular SAM pool.

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 branches of life (Thomas, Surdin-Kerjan 1991). Humans are dependent on diet for methionine, classifying methionine as an essential amino acid (Townsend, Tew & Tapiero 2004). Proper methionine homeostasis requires the vitamin folate, in the form of reduced folate cofactors. The SAH produced by a methyl transfer is returned to the methionine pool by being broken down by SAH 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. In organisms that synthesize methionine *de novo,* the final step in methionine biosynthesis is the same reaction between homocysteine and folate as seen in methionine recycling (Suliman et al. 2005, Pejchal, Ludwig 2004). Thus, deficiencies in either methionine or folate could lead to lower intracellular SAM concentrations, which could in principle affect histone methylation.

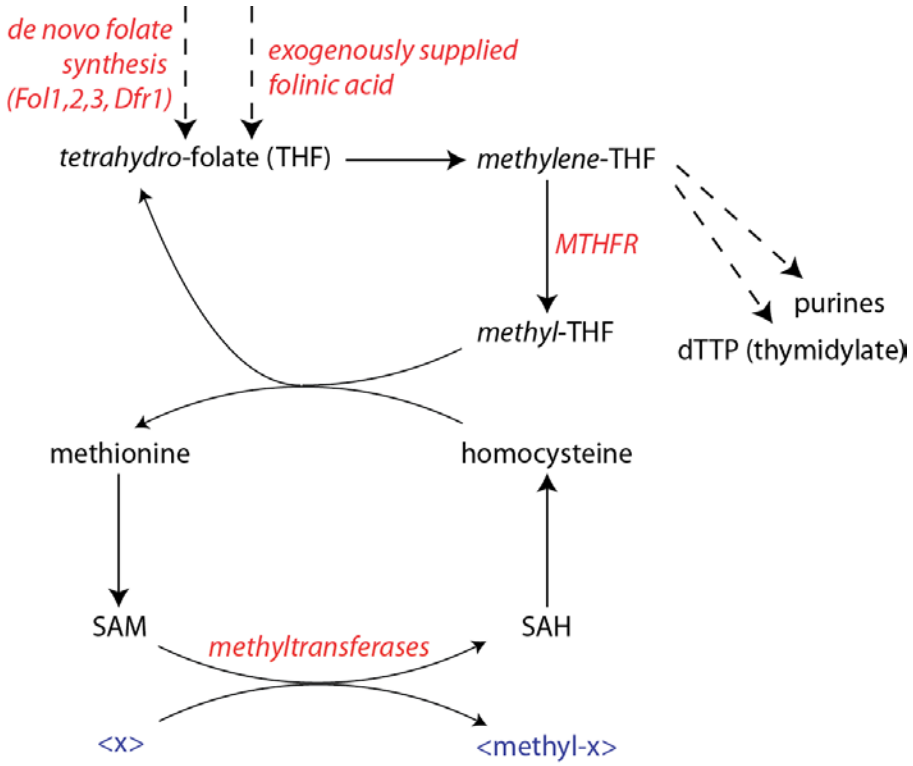


Figure 2.1: Interaction between folate, methionine, and cellular methylation. Met2, not shown, is required for the synthesis of homocysteine. Abbreviations: SAM, s-adenosyl-methionine; SAH, s-adenosyl-homocysteine; MTHFR, methylenetetrahydrofolate reductase.

Folate deficiency is a particularly important topic of study, having been linked to several important diseases and common birth defects. Although the United States 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 is not

considered in the establishment of nutritional guidelines. There is growing recognition, however, that natural genetic variation can play a large role in determining how much of a nutrient is enough. For example, methylenetetrahydrofolate 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 (Kim 2005). Supplementation with folate can remediate the function of this variant (Marini et al. 2008).

A few studies have shown a link between folate deficiency and changes in the bulk level of DNA methylation (Friso et al. 2002, Crider et al. 2011). However, to the best of our knowledge, no studies have examined whether folate or methionine deficiency can affect histone methylation locally or globally. The study described below demonstrated that in yeast folate and methionine limitation reduced histone methylation, and revealed the consequences and potential universality of this relationship.

Results

Folate deficiency compromised histone methylation

Folate is an important biological cofactor in all kingdoms of life (Suh, Herbig & Stover 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, strains were compromised for folate synthesis by deleting *FOL3,* which encodes dihydrofolate synthetase. Growth of *fol3Δ* cells is completely dependent on folate supplementation in the form of folinic acid (FA).

As determined by quantitative immunoblotting on whole cell extracts, genome-wide H3K4 methylation levels were reduced in cultures grown in 10 ug/mL FA relative to cultures grown in 50 ug/mL FA – H3K4 dimethylation was reduced about 25%, and trimethylation was reduced about 45%, both of which were statistically significant (figure 2.2a). This reduction implied that in cells experiencing folate deficiency, the nuclear concentration of SAM was lower than normal, and in particular may have been below Set1's K_M. Even in cells grown at 50 ug/mL FA the H3K4 methylation levels was 25-30% below that of folate prototrophs, suggesting that even those cultures experienced some degree of folate starvation. Cultures grown with 25 ug/mL FA lost about 30% of H3K4 trimethylation, which was statistically significant; these cultures did not have a statistically significant change in H3K4 dimethylation.

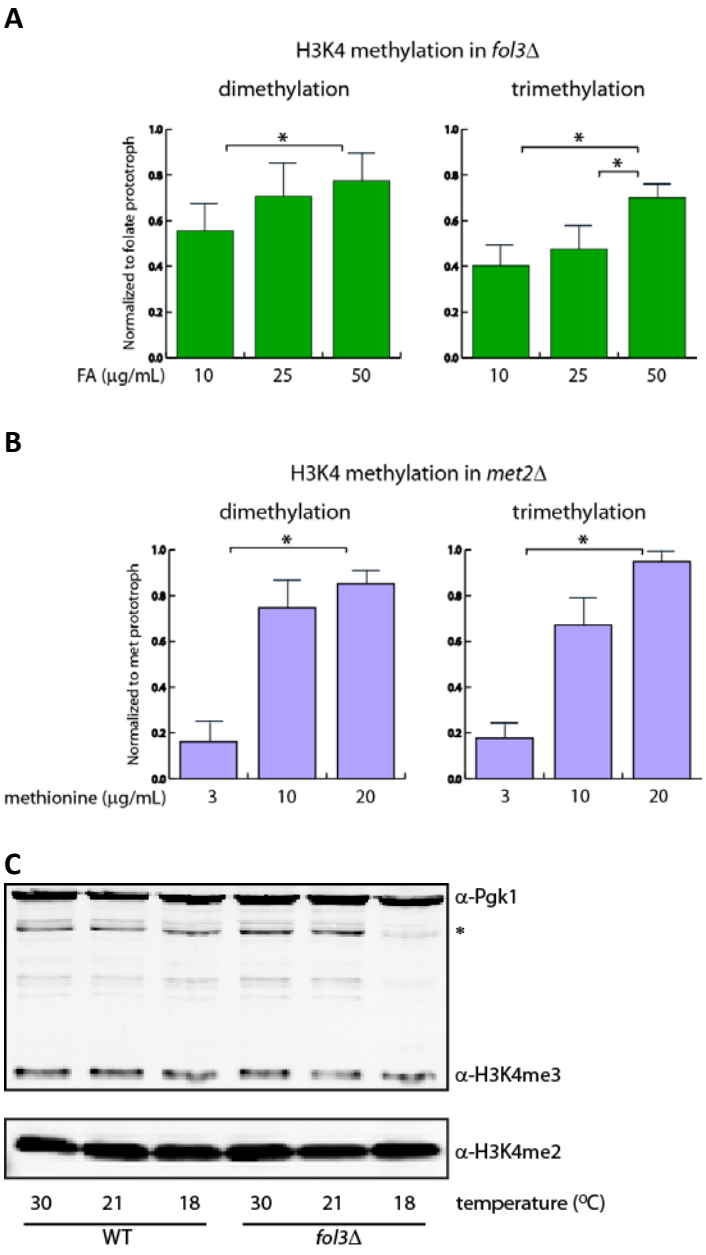


Figure 2.2: Effect of environment on H3K4 methylation in *S. cerevisiae*. **(A)** H3K4 di- and tri-methylation in *fol3Δ* cells grown at different concentrations of FA. All values were normalized to the value of a loading control from the same sample, and then normalized internally to the value for a folate prototroph on the same blot. n=7 for dimethylation, n=8 for trimethylation. Error bars show standard error. *$p < 0.05$, Student's T-test. **(B)** H3K4 di- and tri-methylation in *met2Δ* cells grown at different concentrations of methionine. Normalization was as in (a), n=3. **(C)** H3K4 di- and tri-methylation in wild type and *fol3Δ* cells grown at different temperatures. Pgk1 band density served as a loading control. The asterisk denotes a non-specific band. Quantification (not shown) of band density did not indicate any differences in histone methylation abundance.

If the effect of folate deficiency on histone methylation were due to a decrease in intracellular methionine levels, leading in turn to a decrease in nuclear SAM levels, directly limiting methionine in a methionine auxotroph should also reduce histone methylation. As with folate limitation, growth of *met2Δ* in 3 ug/mL methionine, conditions under which the cells could still divide, led to reduced H3K4 di- and trimethylation (figure 2.2b). Thus, nutritional deficiency, of either folate or methionine, could affect chromatin modifications.

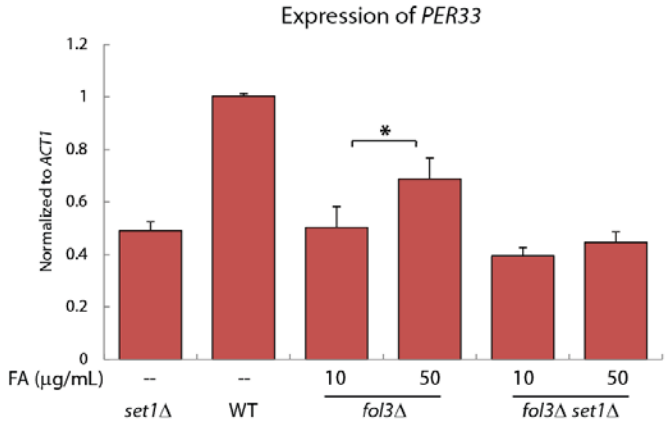
Both folate deficiency and methionine deficiency decreased yeast growth rate. To determine whether slower growth had an effect on histone methylation, cultures were grown at temperatures that slowed growth as much or more than the folate or methionine limitation conditions did, and histone methylation levels in these cultures were then assayed (figure 2.2c). Growth rate variation had no observable effect of on either H3K4 di- or trimethylation levels. Thus, the effect of nutrient limitation on histone modification was likely direct.

Effects of folate limitation at specific loci

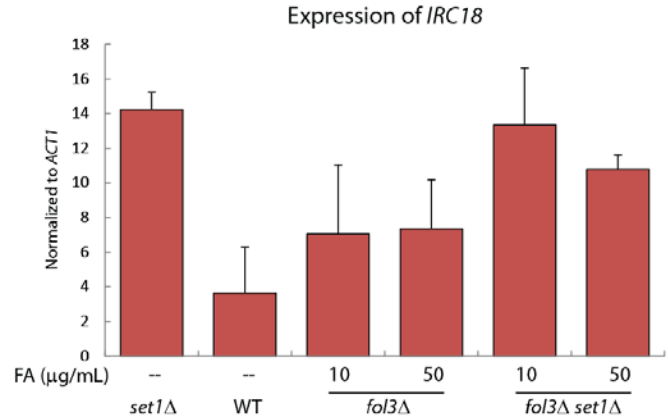
To determine if the effect of folate deficiency on histone methylation had consequences for gene regulation, genes whose expression was affected by the *set1Δ* mutation were tested for altered expression in folate deficient cells. A microarray analysis (Venkatasubrahmanyam et al. 2007) was used to select candidate genes whose expression was decreased in a *set1Δ* strain. Several candidates were tested, of which *PER33* was the only gene whose expression statistically significantly decreased in *set1Δ* (data not shown). Folate deficiency likewise reduced *PER33* expression (figure 2.3a). 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. If folate deficiency and absence of Set1 activity had independent effects on *PER33* expression, folate deficiency would reduce *PER33* expression in *set1Δ* cells. In contrast, if folate deficiency acted on *PER33* by abrogating Set1 activity, folate deficiency would not change *PER33* expression in *set1Δ*. In *set1Δ* cells folate deficiency had no significant effect on *PER33* expression beyond that of the *set1Δ* mutation itself. Therefore folate deficiency changed *PER33* expression through loss of H3K4 methylation.

Although Set1 is commonly considered to be a transcriptional activator (Boa, Coert & Patterton 2003), some genes, such as *IRC18* and *BNA2,* have increased expression in *set1Δ* strains*. IRC18* expression was tested in cells experiencing folate deficiency, to see if the increased expression observed in *set1Δ* was recapitulated. Relative to *FOL3* cells, *fol3Δ* mutants had a higher expression level of *IRC18* regardless of the level of folate

A



B



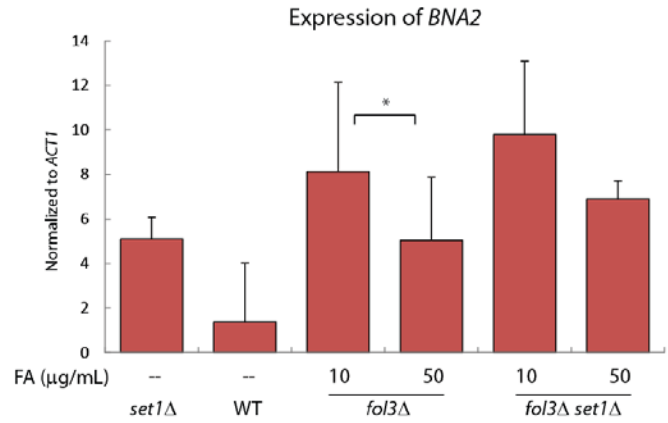


Figure 2.3: Effect of folate deficiency on the expression of *set1Δ*-sensitive genes. **(A)** *PER33* expression, as determined by rt-qPCR. All cDNA values were normalized to *ACT1* cDNA values. Several other comparisons gave showed statistically significant differences, but were not demarcated, to avoid visual clutter. n=4. **(B)** *IRC18* expression. Normalization was as in (a), n=2.

supplementation. Thus, it was possible that the reduction in H3K4 methylation seen in *fol3Δ* grown at high folate relative to *FOL3* was sufficient to cause increased *IRC18* expression. Indeed, *IRC18* expression in *set1Δ* cultures was unaffected by *FOL3* genotype (figure 2.3b), indicating that *IRC18* expression was sensitive to the reduction in Set1 activity seen in *fol3Δ* relative to *FOL3*. *BNA2* expression was also found to be higher in cultures experiencing folate deficiency. In *set1Δ* cells folate deficiency also appeared to affect *BNA2* expression, though the effect was not statistically significant. So it is possible that there are additional effects of folate deficiency on *BNA2* expression, apart from the effect mediated by changes in Set1 activity.

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 places 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. Folate deficiency caused about a 15% decrease in H3K79 trimethylation ($p<0.05$), and had no statistically significant effect on H3K79 dimethylation (figure 2.4a).

The more modest effect of folate limitation on H3K79 methylation compared to the effect on H3K4 methylation suggested that Dot1's K_M might be lower than Set1's. If Dot1's K_M for SAM were close to the concentration of SAM in the nucleus of a folate-deficient cell, the effect on its activity would be minimal. One prediction of this model is that a mutant hypomorphic Dot1 with a higher K_M would be more strongly affected by a decrease in SAM availability. This possibility was tested with a *dot1-G401A* mutant allele. Mutation of G401 to alanine leads to partial methylation loss, with H3K79 trimethylation essentially absent and dimethylation reduced by 60% (van Leeuwen, Gafken & Gottschling 2002). The crystal structure of Dot1 predicts that Glycine-401 resides close to the Dot1's SAM-binding region (Sawada et al. 2004). Thus, a *dot1G401A fol3Δ* strain was tested for effects of folate deficiency on H3K79 methylation (figure 2.4b). When this strain was grown in low-folate medium, H3K79 dimethylation was reduced by 45% relative to high-folate medium, which was statistically significant. Thus, H3K79 methylation was more susceptible to folate deficiency in a strain whose Dot1 enzyme was partially crippled. This supports the hypothesis that H3K79 methylation was more resistant to folate deficiency than H3K4 methylation because wild-type Dot1 had a lower K_M than Set1 under conditions of SAM stress.

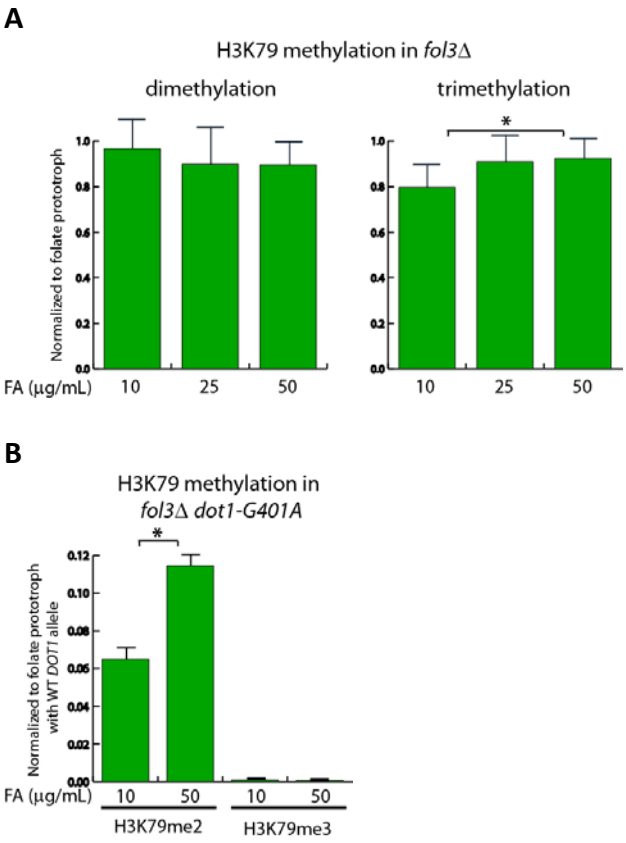


Figure 2.4: Effect of environment on H3K79 methylation in *S.cerevisiae*. All normalizations are as in figure 2.2. **(A)** H3K79 di- and tri-methylation in *fol3Δ* cells grown at different concentrations of FA. n=3 for dimethylation, n=4 for trimethylation. **(B)** H3K79 di- and tri-methylation in *fol3Δ dot1-G401A* cells grown at different concentrations of FA. Normalization was to folate prototroph with wild-type *DOT1*. n=3.

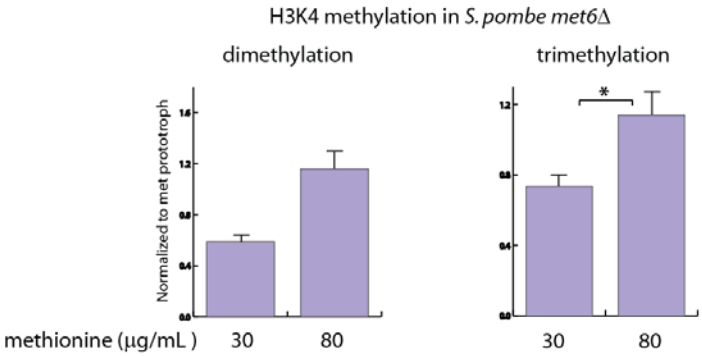
Histone methylation-nutrition interaction in S. pombe

The fission yeast *Schizzosaccharomyces pombe* is among the most distant relatives of *S. cerevisiae* among yeast species – one estimate places their divergence as having occured 330-420 million years ago (Sipiczki 2000), whereas another places it as having occurred 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 was 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, Gafken & Gottschling 2002), yet has not been described in *S. pombe*. H3K4 methylation is present in both. The H3K4 methyltransferase in *S. pombe* is an ortholog of *S. cerevisiae's* Set1

(Noma, Grewal 2002) (and goes by the same name), and the localization pattern of H3K4 methylation in gene bodies is also conserved. Set1 in *S. pombe* also requires SAM as a cofactor. Folate and methionine appear to play the same essential roles in SAM synthesis, as all the genes predicted to encode for proteins involved 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 use 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 a 35% decrease in H3K4 trimethylation, which was statistically significant (figure 2.5). Though histone methylation did appear to be decreased in folate-deficient cultures, the effect was not statistically significant.

A



B

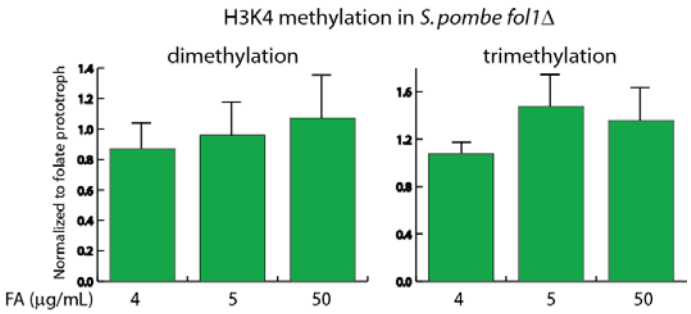


Figure 2.5: Effect of environment on H3K4 methylation in *S. pombe*. All normalizations are as in figure 2.2, n=3 for all graphs. **(A)** H3K4 di- and tri-methylation in *S. pombe met6Δ* cells grown at different concentrations of methionine. **(B)** H3K4 di- and tri-methylation in *S. pombe fol1Δ* cells grown at different concentrations of FA.

The effect of methionine deficiency on histone methylation levels implied that sufficiently low methionine availability in *S. pombe* decreased the SAM concentration past the point where Set1's activity was affected. The lack of an effect of low folate could indicate that unlike in *S. cerevisiae*, folate deprivation did not lower methionine levels enough to cause a significant impact on SAM concentrations. This could happen if the K_M of methionine synthase for 5-methyltetrahydrofolate were lower in *S. pombe* than in *S. cerevisiae.* Another possibility is that methionine concentration and SAM concentration were substantially affected, but the large increase in doubling time seen in *S. pombe* at low folate (data not shown) allowed sufficient time for H3K4 methylation, even though the methylation may have been less efficient.

Discussion

Nutritional deficiency affects histone methylation

The studies described above 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 reduce histone methylation, in two distantly related species of yeast. Furthermore, in *S. cerevisiae*, the changes in histone methylation resulting from folate deficiency caused changes in gene expression that appeared to be direct effects.

The effect of nutritional limitation was more pronounced on H3K4 methylation than H3K79 methylation, suggesting that Set1's K_M for SAM may be higher than Dot1's. Indeed, levels of H3K79 methylation placed by a hypomorphic mutant form of Dot1 were more labile in response to nutritional changes. Relatedly, cultures grown with an intermediate concentration of folate had a statistically 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 statistically significant degree. One possibility is that Set1's K_M for SAM was higher in the trimethylation reaction than it was for the dimethylation reaction. Alternatively, perhaps H3K4 methylation is placed distributively, rather than processively, meaning that Set1 dissociates with H3 after a methylation event. So, genome-wide H3K4 dimethylation would occur overall earlier in the cell cycle than H3K4 trimethylation. In this model, in cells grown with an intermediate folate concentration cell division proceeded after the

appropriate levels of H3K4 dimethylation were achieved genome-wide, but prior to H3K4 trimethylation being completed. However, given the precise pattern of the three H3K4 methylation marks within gene bodies, with trimethylation predominantly found at 5' regions, followed by di- and mono-methylation (Liu et al. 2005), Set1 seems unlikely to act distributively.

Folate, methionine, and SAM have essential functions in cells unrelated to histone methylation – for instance, folate is necessary for synthesis of purines (Rébora, Laloo & Daignan-Fornier 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 functions before affecting histone methylation, the cell would likely die of the nutritional deficiency without histone methylation levels being perturbed. Thus, the results in this study suggest that eukaryotic cells may have evolved a metabolic triage system in which the less essential metabolic functions of folate, methionine, and SAM are more vulnerable than essential processes under conditions of nutritional stress.

Connections to observations in other species

To the best of our knowledge, this is the first report of a link between histone methylation and nutritional modulation of SAM availability. However, previous studies have demonstrated a link between DNA methylation and SAM availability. 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, 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, Selker 2001). So, if the effect of SAM availability on histone methylation observed here also holds in *Neurospora,* a reduction in DNA methylation could be due to effects of SAM insufficiency on the DNA methyltransferase, or the combined effects on the DNA and histone methyltransferases .

Genes in *Drosophila melanogaster* can become subject to silencing based on their genomic context, and elucidation of the mechanism of these silencing events was important in understanding the role of repressive histone methylation marks. For instance, the histone methyltransferase *E(z),* which places H3K27 methylation (Czermin et al. 2002) and is one of the founding members of the SET-domain family of methyltransferases (Tschiersch et al. 1994), was originally described by gain-of-function mutations that enhanced the silencing of w^{is} by the *zeste* allele z^{1} (Kalisch, Rasmuson 1974). Interestingly, a loss-of-function mutation in SAM synthase, termed *Su(z)5*, was found to suppress w^{is} silencing (Larsson, Rasmuson-Lestander 1994). At the time, *E(z)*'s function as a histone methyltransferase was unknown, so the possibility that a chromatin methylation mark was affected in *Su(z)5* was not considered. However,

given our current state of knowledge, and especially in light of the results described here, one parsimonious explanation for the involvement of SAM synthase in w^{is} silencing is that *E(z)* is sensitive to changes in SAM availability, and thus mutants with impaired SAM synthase function have silencing defects due to reduced H3K27 methylation.

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 (Papp, Müller 2006, Cao et al. 2002). 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. If histone methylation perturbation were causing NTDs, the common A222V allele of MTHFR might be a risk factor for NTDs. 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 for proper methionine homeostasis. 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 A222V variant seem to be at higher risk for neural tube defects (Botto, Yang 2000).

Antifolates such as methotrexate are commonly used in cancer treatment (Alkins et al. 1996). The proposed mechanism of action is to inhibit 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 (Esteller 2007).

Materials and Methods

Strains, plasmids, and oligonucleotide sequences

Strains used are listed in table 1, and sequences of oligonucleotides used are listed in table 2. JRY9339 was obtained from a tetrad dissection of the *fol3Δ/FOL3* heterozygote from the essential heterozygote knockout collection (Open Biosystems YSC1057), on plates containing YPD + 50 µg/mL folinic acid (FA). Diploids were

sporulated on solid 1% potassium acetate plates supplemented with 200 µg/mL CSM (complete supplement mix). Deletion of *SET1* was accomplished with a modified lithium acetate protocol (Becker, Lundblad 2001), and JRY9341 was made by tetrad dissection, as before, of a diploid formed by the mating of *fol3Δ* and *set1Δ* haploid strains.

pFvL54, carrying *dot1-G401A* on a pRS315 plasmid backbone, was a gift from Daniel Gottschling (van Leeuwen, Gafken & Gottschling 2002). It was transformed by the modified lithium acetate protocol to make JRY9342 and JRY9343. The *dot1Δ* strain was taken from the MATa knockout collection (Open Biosystems YSC1053).

JRY9346 was made by deletion of *fol1Δ* by electroporation in a stable *S. pombe* diploid, as described previously (Prentice 1992), which was then dissected to give haploids. Folate auxotrophy was confirmed by differential growth in media lacking or supplemented with FA. *S. pombe met6Δ* was a gift from Kaoru Takegawa (Fujita et al. 2006).

Media

For folate deficiency experiments in *S. cerevisiae*, modified minimal media (SD) (Amberg, Burke & Strathern 2005) was made from YNB without vitamins and amino acids, supplemented with 180pg/mL riboflavin, 30µg/mL leucine, 45µg/mL lysine, 20µg/mL histidine, and 20µg/mL uracil. Folate auxotrophic cells were grown overnight in liquid media supplemented with 50 µg/mL folinic acid (FA), and subsequently split into cultures supplemented with 10, 25, or 50 µg/mL FA and grown for about 6 doublings. 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 8 doublings. To grow cells at different temperatures, SD + riboflavin, leucine, lysine, histidine, and uracil was used. Cells were grown overnight at 30^{o}C, then split between 30^{o}C, 21^{o}C, and 18^{o}C and grown for about 6 doublings. For all experiments, control prototrophic strains were grown without FA or methionine.

For folate deficiency experiments in *S. pombe*, modified minimal media was made as described above, but 1 mg/mL glutamic acid was used as nitrogen source instead of ammonium sulfate, and 3 mg/mL potassium hydrogen phthalate and 5 mg/mL Na_2HPO_4 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 media supplemented with 50 µg/mL FA, and then split into cultures containing 4, 5, or 50 µg/mL FA and grown for about 6 doublings. Methionine auxotrophic cells were grown overnight in liquid media containing 80 µg/mL methionine, and then split into cultures containing 30 or 80 µg/mL methionine and grown for about 6 doublings.

Whole cell extract preparation, immunoblotting

Protein extracts were precipitated using 20% trichloroacetic acid and solubilized in SDS loading buffer. 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). Anti-H4 (Abcam Ab17036) or Anti-Pgk1 (Invitrogen A6457) was used as loading controls. All histone methylation values were normalized to loading control values for the same sample, and to combine values between experiments all values are normalized to the value obtained for a prototroph on the same blot.

RNA extraction, cDNA preparation, RT-qPCR

RNA was purified using hot acid phenol and chloroform. 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 were normalized to *ACT1* amplification values.

Chapter 3: Exploration of the complexity and logic of *S. cerevisiae* methionine regulation

Introduction

The budding yeast *Saccharomyces cerevisiae* is capable of synthesizing the sulfur-containing amino acids methionine and cysteine, and is also capable of synthesizing each from the other (figure 3.1) (Thomas, Surdin-Kerjan 1997). The amino acid homocysteine is the precursor to both methionine and cysteine. It is synthesized *de novo* by covalent attachment of hydrogen sulfide, produced by the sulfur assimilation pathway, to o-acetyl-homoserine, produced by the homoserine synthesis pathway. In addition, both methionine and cysteine can be converted back to homocysteine. The methionine-to-homocysteine conversion requires the hydrolysis of s-adenosyl-methionine (SAM) to s-adenosyl-homocysteine (SAH), which accompanies all cellular methylation reactions, including methylation of proteins, lipids, and nucleic acids.

The transcription of the *MET* regulon – the genes encoding proteins involved in the synthesis of sulfur-containing amino acids – rapidly shifts from low basal transcription to high active transcription if methionine and cysteine are absent from the environment. This induction is mediated by the transcription factor Met4. Met4 does not bind DNA. Instead, Met4 is recruited to specific sites in the regulatory region of genes by the DNA-binding protein Cbf1, which uses a basic-helix-loop-helix domain, or one of Met31 and Met32, which are highly similar zinc-finger domain proteins that share a binding site. The binding sites of these DNA-binding proteins have been mapped genome-wide in *S. cerevisiae* (Lee et al. 2010). Many *MET* regulon genes have binding sites for both proteins in their promoters, though several have only one or the other. Genes encoding proteins involved in the uptake of sulfur and its assimilation into the amino acid backbone are more likely to have only the Cbf1 binding site in their promoters, whereas genes encoding proteins involved in the uptake of methionine and synthesis of methionine from cysteine are more likely to have only the Met31/Met32 binding site in their promoters. Thus, there is a potential for alternative regulation even within the *MET* regulon.

Met4 is inactivated by ubiquitination when sulfur-containing amino acids are present in the environment (Rouillon et al. 2000), as are Met31, Met32, and Cbf1 (Ouni, Flick & Kaiser 2010). Ubiquitination can affect Met4 in one of two ways. Depending on the nutritional state, Met4 is polyubiquitinated and degraded, or it is oligoubiquitinated and remains stable with altered activity (Kuras et al. 2002). The ubiquitination on Met4 is placed by the ubiquitin ligase complex SCF^{Met30}, in which the F-box protein responsible

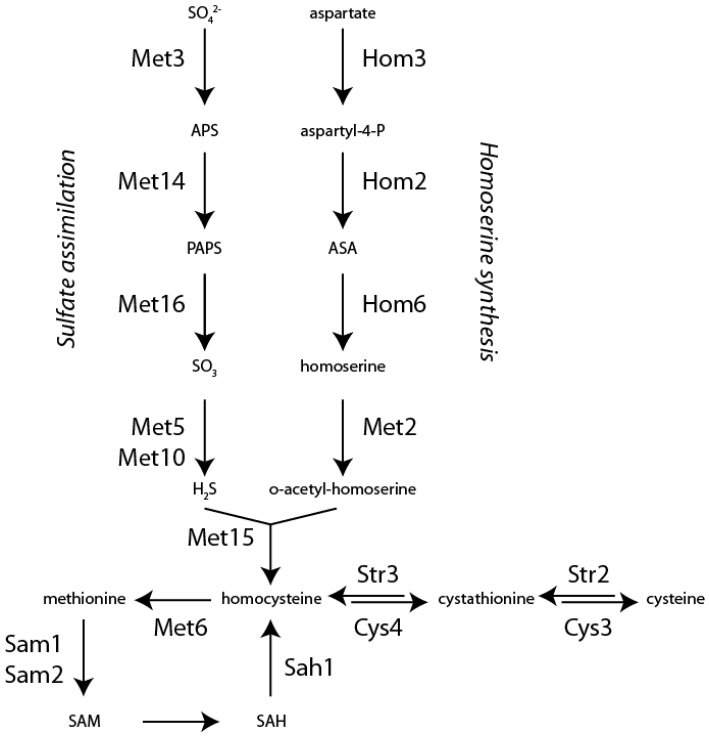


Figure 3.1: Synthesis of sulfur-containing amino acids in *S. cerevisiae.* Met13, not depicted, is required for the synthesis of methyltetrahydrofolate, a substrate used by Met6 in the synthesis of methionine from homocysteine. The conversion of SAM to SAH occurs through myriad cellular methylation reactions. Abbreviations used: APS, adenylyl sulfate; PAPS, phosphoadenosine phosphosulfate; ASA, aspartate semialdehyde; SAM, s-adenosyl-methionine; SAH, s-adenosyl-homocysteine

for interacting with Met4 is Met30. How these ubiquitination events are confined to conditions of sulfur-containing amino acid availability is unknown.

Much work has been done to identify which metabolites are sensed to control the *MET* regulon. In the absence of Cys3 or Cys4, which are required to synthesize cysteine, all tested members of the *MET* regulon are highly transcribed when grown with abundant methionine but no cysteine (Hansen, Johannesen 2000, Menant et al. 2006). Thus, it appears that cysteine deficiency, or the deficiency of some compound synthesized from cysteine, is sufficient to stimulate *MET* regulon transcription, independent of methionine availability. To address the possibility that deficiency in methionine, or a compound synthesized from methionine, could also stimulate *MET*

regulon transcription, *MET14* and *MET15* were tested for transcriptional stimulation in cells grown with abundant cysteine but no methionine (Hansen, Johannesen 2000). In the absence of Str2 or Str3, which catalyze the conversion of cysteine to methionine, cells grown with no methionine but abundant cysteine induced neither *MET14* nor *MET15*. Thus, at least for the genes tested, deficiency in cysteine or a derivative of cysteine is both necessary and sufficient for transcriptional induction.

How cysteine, or a derivative of cysteine, controls the *MET* regulon is unknown. One possibility is that cysteine inhibits an enzyme involved in sulfur-containing amino acid synthesis, and the product of this enzyme directly activates the *MET* regulon transcriptional machinery. The *LEU* regulon is controlled in this manner by leucine. Leucine inhibits the enzymatic action of the orthologous proteins Leu4 and Leu9, which produce the leucine precursor α-isopropylmalate (αIPM) (Chang et al. 1984). The transcription factor Leu3 binds αIPM in order to upregulate *LEU* regulon transcription (Sze et al. 1992). Thus, when leucine is present, αIPM is not synthesized and the regulon is not transcribed, but when leucine is absent, Leu4 and Leu9 are able to produce αIPM, which binds Leu3 to stimulate *LEU* regulon transcription.

In this study, analysis of whether methionine or cysteine controls the expression of genes in the *MET* regulon was expanded to include the expression of *STR3*. *STR3* was selected because it codes for a protein involved in the synthesis of methionine from cysteine, whereas all genes whose expression was previously tested are involved in the synthesis of both amino acids. In addition, end-product inhibition of upstream steps in methionine synthesis was investigated for a potential role in the transcriptional regulation of the *MET* regulon. Met30 protein was also examined to determine if any posttranscriptional modification of Met30 restricted its activity to conditions of sulfur-containing amino acid depletion. Finally, a genetic screen was performed to expand the number of genes known to affect *MET* regulon transcription.

Results

Determinants of STR3 *induction*

Previous studies of the expression of the *MET* regulon found that all genes tested are induced when cysteine is specifically lacking in the environment, but not when methionine is lacking (Hansen, Johannesen 2000, Menant et al. 2006). These results lead to the conclusion that only cysteine levels control *MET* regulon induction. However, it remains undetermined whether this also applies to the expression of genes involved in the synthesis of methionine from cysteine. This pathway requires the activity of two enzymes, Str2 and Str3 (figure 3.1). *STR2*'s expression does not change significantly when cells are starved simultaneously for both sulfur-containing amino

acids (Lee et al. 2010). However, *STR3*'s expression is highly induced in cells undergoing simultaneous starvation for methionine and cysteine. This induction requires Met4 – deletion of either *MET4* or concurrent deletion of both *MET31* and *MET32* abolishes *STR3* induction. Furthermore, the promoter of *STR3* is bound by Met31 and Met32. It would be counterintuitive if *STR3* transcription were induced specifically by cysteine starvation and not by methionine starvation, since its enzymatic action would exacerbate cysteine deficiency.

To test whether methionine deficiency alone can induce *STR3*, it was necessary to create conditions of methionine scarcity coupled to cysteine availability, which was complicated by the ability of *S. cerevisiae* cells to synthesize methionine from cysteine. Furthermore, since *STR3* itself codes for a protein involved in the synthesis of methionine from cysteine, it was prudent to avoid the use of strains harboring deletions of members of this pathway, which was the method used in the study that assessed the effect of methionine deficiency on *MET14* and *MET15* induction (Hansen, Johannesen 2000). Thus, a strategy was employed in which cells were genetically engineered to be unable to synthesize either both of the sulfur-containing amino acids or just methionine. Met15 is responsible for the synthesis of homocysteine, the precursor to both methionine and cysteine, whereas Met13 is required for the synthesis of methionine from homocysteine, and has no role in cysteine synthesis. *met15Δ, met13Δ,* and a strain prototrophic for both methionine and cysteine were tested for *MET* regulon induction after being moved to medium without any sulfur-containing amino acids. The *met15Δ* cells were expected to experience starvation for both methionine and cysteine, whereas the *met13Δ* cells were expected to experience only methionine starvation. It was possible that the prototrophic strain would also induce members of the *MET* regulon, as it too would need to switch from transporting the sulfur-containing amino acids from the medium to synthesizing them, though it would not experience full starvation. Thus the point of comparison was whether the amino acid starvations in the mutant strains would lead to higher levels of induction than that seen in the prototrophic strain. If methionine starvation, but not cysteine starvation, induced *STR3* transcription, then *met13Δ* and *met15Δ* cells would have equivalent, large transcriptional inductions. Alternatively, if only cysteine starvation induced *STR* transcription, the *met15Δ* cells would have a large transcriptional induction while the *met13Δ* cells would induce only as much as the prototrophic strain. Finally, if starvation for either amino acid were independently able to induce *STR3* transcription, then both strains would show a large induction, though *met15Δ* cells would be expected to induce *STR3* more than *met13Δ* cells.

First, the expression of *MET3* was measured by rt-qPCR after 60 minutes growth without sulfur-containing amino acids (figure 3.2a). As expected from previous studies, *MET3* expression was induced significantly in the *met15Δ* strain, while *met13Δ* and the prototrophic strain induced *MET3* to a lower level. This result supported the notion that cysteine starvation is solely responsible for *MET3* induction, and validated the approach used here. In contrast to *MET3*, the expression of *STR3* was strongly induced in both

met13Δ and *met15Δ*, implying that methionine starvation induced its expression (figure 3.2b). Moreover, the induction was similar in magnitude between the strains, which would be expected if only methionine limitation induced *STR3* expression, and cysteine limitation played no role. Thus, different members of the *MET* regulon responded differently to starvation for the sulfur-containing amino acids. Moreover, these data provided evidence for a role of methionine, or possibly a metabolite related to methionine, in controlling the transcription of members of the *MET* regulon.

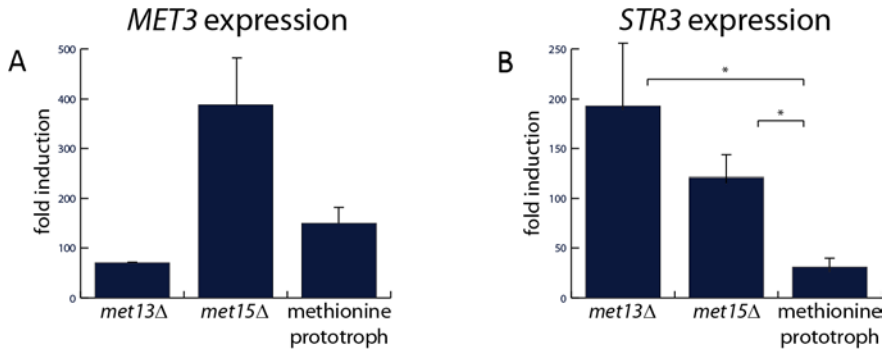


Figure 3.2: Induction of *MET3* and *STR3* by methionine starvation in different methionine auxotrophs. Expression was compared between uninduced cells and cells 60 minutes after transfer to media without sulfur-containing amino acids. **(A)** *MET3* expression, as determined by rt-qPCR. Data was normalized to *ACT1*, as well as to the expression of *MET3* in the methionine prototrophic strain in media containing methionine. Error bars show standard error. n=3. **(B)** *STR3* expression. Data was normalized as in (a). *p < 0.05, Student's T-test. n=3.

Regulation of the MET *regulon by intermediate abundance*

It is possible that control of *MET* regulon transcription by sulfur-containing amino acids involves blocking the synthesis of a pathway intermediate that positively regulates the regulon. This mode of regulation is seen for the *LEU* regulon, where leucine inhibits the enzymatic action of the enzymes that synthesize the leucine precursor α-isopropylmalate (αIPM) (Chang et al. 1984). αIPM, when present, binds the transcription factor Leu3 to upregulate *LEU* regulon transcription. Thus, leucine controls the *LEU* regulon indirectly, by controlling the abundance of the direct positive regulator of Leu3, αIPM (Sze et al. 1992). One feature of the *LEU* regulon is that the transcription of *LEU4, LEU9,* and all other genes encoding proteins upstream of αIPM synthesis is not drastically affected by leucine levels, in contrast to the transcription of genes encoding proteins downstream of αIPM synthesis (Chin et al. 2008). This observation may generally apply to this mode of regulation, as the enzymes that produce the sensed intermediate need to already be present to make the intermediate when the end product repression is lifted. Thus, if the transcription of any gene involved in methionine synthesis were relatively unaffected by methionine starvation, it could be a

clue as to the identity of an intermediate in methionine synthesis that is sensed as part of the regulation.

Methionine synthesis involves two branches; the sulfur assimilation pathway takes sulfur from the environment and produces hydrogen sulfide, while the homoserine synthesis pathway produces o-acetyl-homoserine. The two are combined to make homocysteine, the immediate precursor to methionine. Examination of a microarray data set measuring the expression of all *S. cerevisiae* genes after 80 minutes of methionine starvation (Lee et al. 2010) revealed expression increases greater than 10-fold for all genes encoding proteins involved in sulfur assimilation, whereas the expression of all genes involved in homoserine biosynthesis changed by less than 5-fold, and most less than 2.5-fold (figure 3.3a). Thus, it was possible that the presence of an intermediate in homoserine synthesis was a signal for the need to upregulate the rest of the *MET* regulon.

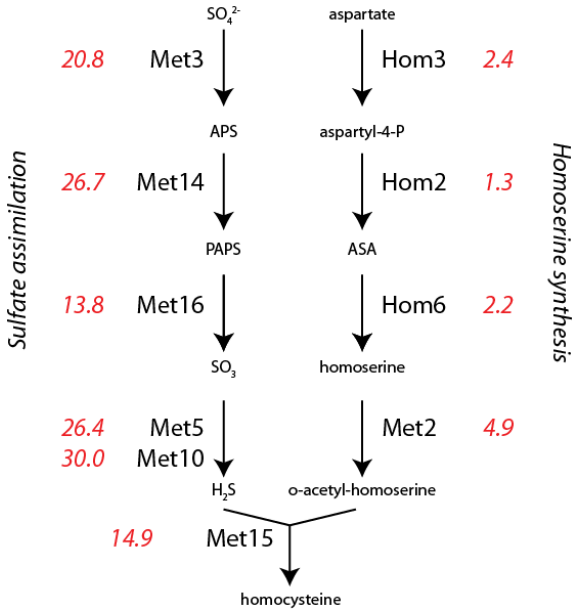
A prediction of this model was that deletion of *HOM3*, the gene that encodes the protein that performs the first step in homoserine synthesis (Rafalski, Falco 1988), would reduce the induction of the *MET* regulon during methionine starvation, since the hypothetical intermediate whose presence stimulates the *MET* regulon would be absent. The expression of *MET3* after 60 minutes growth without methionine was significantly lower in *hom3Δ* cells than *met15Δ* cells (figure 3.3b). *MET3* was still substantially induced in *hom3Δ* cells, indicating that at least one inducing signal of the *MET* regulon functioned independently of intermediates of the homoserine biosynthesis pathway. Still, this result suggested inhibition of the homoserine biosynthetic pathway played a role in controlling *MET* regulon transcription.

An alternate possibility for the difference between *hom3Δ* and *met15Δ* cells was that the media was contaminated with low levels of intermediates in the homoserine synthesis pathway, which in *hom3Δ* cells can be synthesized into methionine and cysteine to repress the *MET* regulon. *met15Δ* cells, on the other hand, would be unable to synthesize methionine and cysteine from these intermediates. To address this possibility, the expression of *MET3* in doubly mutant *hom3Δ met15Δ* cells was compared to *MET3* expression in the single mutants. If the effect in *hom3Δ* cells were due to absence of an intermediate, then the double mutant would be affected in the same way. On the other hand, if the effect were due to synthesis of methionine from contaminating intermediates, then the double mutant would act the same way as the *met15Δ* cells. Doubly mutant *hom3Δ met15Δ* cells induced *MET3* to the same level as *hom3Δ* cells (figure 3.3b), implying the effect in *hom3Δ* was indeed due to absence of an intermediate of the homoserine synthesis pathway.

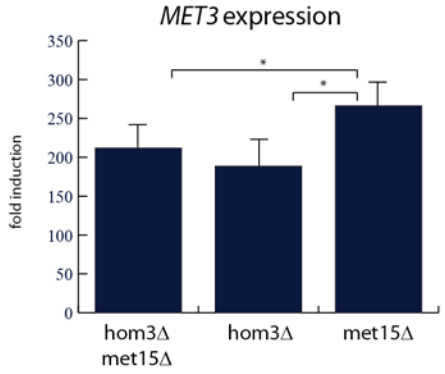
To determine which end product, methionine or cysteine, was likely targeting the homoserine biosynthesis pathway, the expression of *STR3* was also tested, as its expression was induced specifically by methionine deficiency. In contrast to the result for *MET3, STR3* expression was not statistically different between *hom3Δ* and *met15Δ*

cells (figure 3.3c). Thus, it appeared that cysteine deficiency induced the *MET* regulon through interaction with the homoserine biosynthesis pathway, whereas methionine deficiency did not.

A



B



C

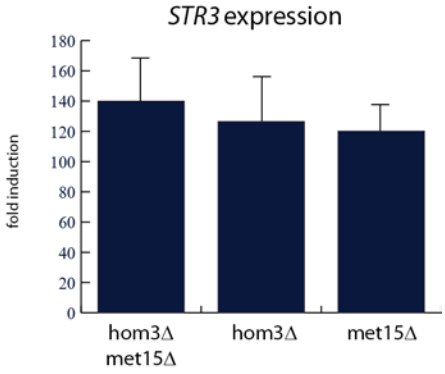


Figure 3.3: *MET* regulon induction in strains deficient for pathway intermediates. **(A)** Gene induction after 80 minutes starvation for sulfur-containing amino acid synthesis (Lee et al. 2010). Values are shown as fold-induction, in red. **(B)** *MET3* induction. Values are normalized to *ACT1* expression, as well as to the expression of *MET3* in the *met15Δ* strain grown in media containing methionine. *$p < 0.05$, Student's T-test. n=5. **(C)** *STR3* induction. Data was normalized as in (a). n=5.

Effects of sulfur-containing amino acid availability on Met30 protein

Ultimately, these metabolites must differentially affect Met30, Met4, or perhaps an unknown interacting partner of these proteins. For instance, translation of the *MET30* transcript could be affected by methionine abundance. The N-terminus of Met30 was found to be rich with methionine residues, containing 8 methionines in the first 70 amino acids, making it among the most methionine-dense N-termini in the *S. cerevisiae* proteome (figure 3.4a). In cells grown without methionine, it is possible that translation could stall on these methionine codons, or that a different methionine codon than the first one in the ORF could be chosen as the initiator. Moreover, a predicted RNA hairpin with a stem of 15 base pairs was found to overlap the last of these methionine codons (figure 3.4b). The structure of this RNA hairpin was preserved in all the *sensu stricto* species in spite of multiple mutations in the RNA sequence. RNA hairpins have been shown to affect translation (Chartrand et al. 2002, Reineke et al. 2008), and the relative positioning with the methionine codons suggests translation of the *MET30* transcript might be especially difficult in conditions lacking methionine.

An epitope-tagged Met30 which provided full Met30 function was used to determine if the abundance or size of Met30 was affected by the availability of sulfur-containing amino acids. Use of a C-terminal tag, under the native *MET30* promoter, allowed observation of whether the 5' UTR and translational initiation process modulated translation. *met15Δ* cells carrying epitope-tagged *MET30* on a plasmid under its native promoter were grown with methionine and then shifted to media without methionine and cysteine, after which proteins were extracted and detected by immunoblot (figure 3.4c). If translation of the *MET30* transcript were blocked by methionine starvation, Met30 abundance would decrease in cells grown without methionine, but contrary to that expectation, Met30 abundance increased. *MET30* mRNA transcription is known to be induced by growth without methionine and cysteine (Rouillon et al. 2000), as *MET30* is a Met4 target. Indeed, *MET30* transcription was induced to a similar degree to the protein level increase (figure 3.4d), indicating that the translational efficiency of Met30 is not affected by starvation for methionine and cysteine.

Interestingly, in addition to the reduced abundance of Met30 in the presence of methionine, there was a shift in the position of the Met30 band for the culture grown without methionine compared to the culture grown with methionine. This differential mobility could reflect a post-translational modification, cleavage, or use of an alternate start codon. Thus, although Met30 protein abundance did not decrease in methionine-starved conditions, other forms of direct protein regulation may control its activity.

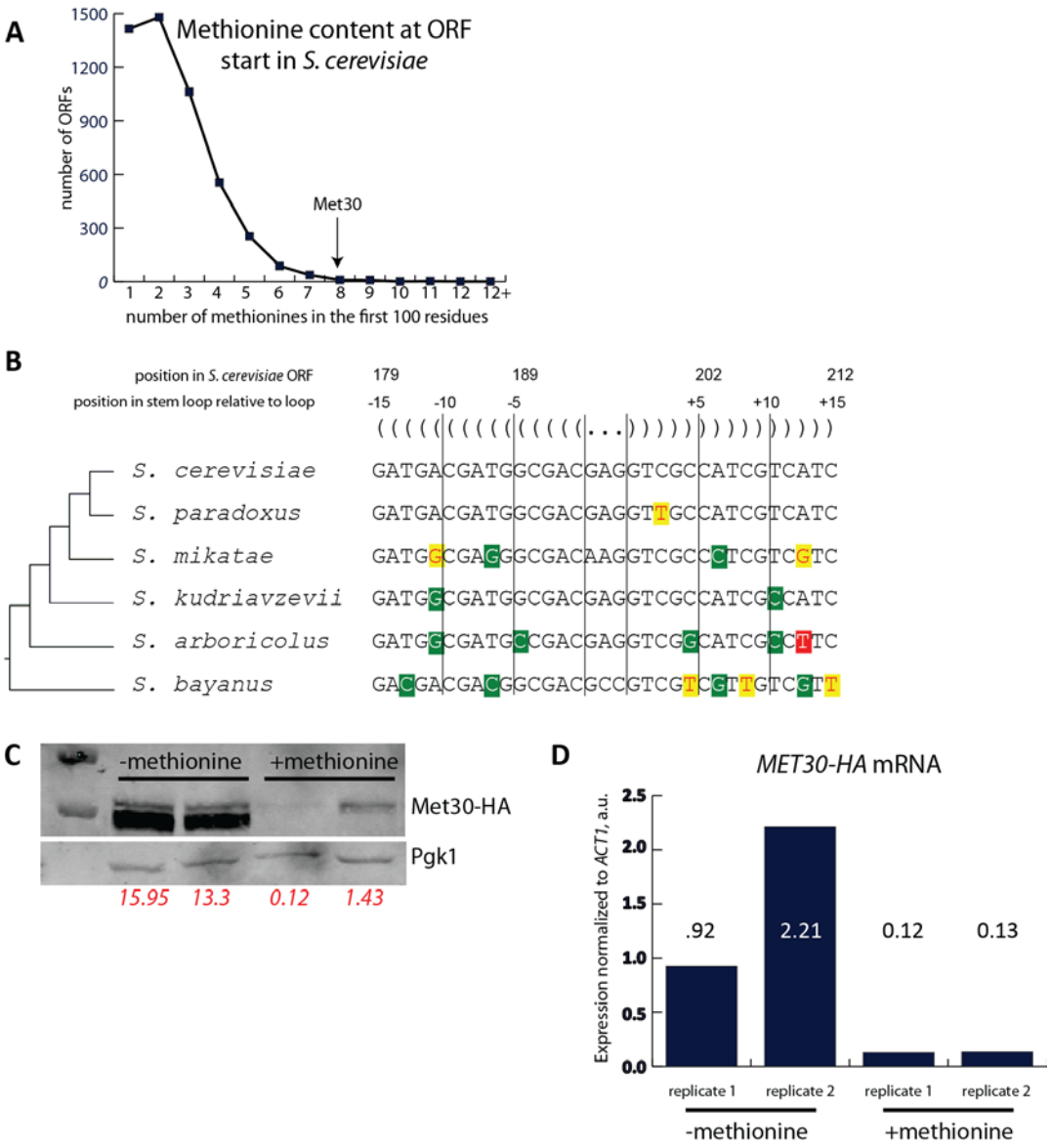


Figure 3.4: Effect of methionine availability on Met30 **(A)** Histogram of methionine content of the first 100 amino acids for all ORFs in *S. cerevisiae*. For ORFs shorter than 100 amino acids, the total number of methionines was counted. Met30 is marked. **(B)** A predicted RNA hairpin in *MET30*. DNA sequence is shown, with bracket notation for the *S. cerevisiae* RNA shown above. Aligned DNA sequence for the other species of the *sensu stricto* clade are shown below, with mutations highlighted. Green highlighting indicates the opposite arm of the stem loop contains a compensating mutation that preserves the predicted hairpin structure. Yellow highlighting indicates that the mutation is not compensated, but still allows pairing with its predicted partner. Red highlighting indicates a mutation to a base unable to pair with the opposite arm. Note that as *S. bayanus* anchors the tree, it is not possible to determine whether differences in its sequence arose along its lineage or along the lineage shared by the other members; those differences are notated as occurring along the *S. bayanus* lineage for simplicity. A similar caveat applies to the mutations at -11 and +11. **(C)** Immunoblot using anti-HA and anti-Pgk1 antibodies. Lanes are in duplicate. Density of anti-HA and anti-Pgk1 bands was quantified, and ratios are shown below the immunoblot in red. **(D)** Cultures in (c) were split, allowing RNA extraction. Expression of *MET30-HA* was measured by rt-qPCR, and normalized to *ACT1* (arbitrary units).

Cho2 function is required for proper regulation of the MET *regulon*

To find novel components of the machinery controlling the expression of the *MET* regulon, a genetic screen was performed to uncover mutants that fail to downregulate *MET3* expression in the presence of methionine. Cells carrying *MET3* tagged in frame with *GFP* in *MET3*'s native genomic context (denoted *MET3-GFP*) were mutagenized with ethyl methanesulfonate (EMS) or ultraviolet (UV) radiation and grown on plates containing 1 mM methionine. These plates were then scanned for mutant colonies fluorescing despite the presence of methionine. To protect against the possibility that overproduction of methionine in such a mutant might cause sickness or lethality, two distinct strains were mutagenized, one of which was a methionine auxotroph due to deletion of the *MET15* gene.

Several strategies were employed to identify causative mutations in the mutants, and mutations were recovered in *MET30* and *CYS3*, as expected from previous studies (Hansen, Johannesen 2000, Menant et al. 2006, Thomas et al. 1995). In addition, a mutation was recovered in *CHO2,* a methyltransferase described as carrying out the first of three methylations involved in phosphatidylcholine synthesis from phosphatidylethanolamine (Summers et al. 1988, Kodaki, Yamashita 1987). The mutation recovered (A493T) caused a nonsense mutation that shortened the 870 amino acid native protein to a 165 amino acid truncated form. Similar shortenings of *CHO2* have previously been demonstrated to completely remove Cho2 function (Summers et al. 1988). This result indicated that the mutation caused the *MET3* overexpression phenotype through loss of Cho2 function rather than gain of novel function, which was confirmed when deletion of *CHO2* caused the same phenotype (figure 3.5a).

Cho2's known activity involves the conversion of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, coupled to the conversion of SAM to SAH. In theory, loss of *CHO2* could induce *MET* regulon transcription by loss of either of those reactions, or through the loss of some unknown function of Cho2. To test the possibility that disruption of the phospholipid synthesis is the causative effect, *cho2Δ* cells were grown with monomethylethanolamine, dimethylethanolamine, or choline, which can be used to produce each of the phospholipids downstream of Cho2 in phosphatidylcholine synthesis (Kennedy, Weiss 1956). Both monomethyl- and dimethylethanolamine were able to repress *MET3-GFP* overexpression, whereas choline was not (figure 3.5b).

The possibility that loss of SAM consumption caused *MET* regulon induction was also tested. The mutants were isolated on plates containing methionine and no cysteine, so cysteine synthesis (in the absence of sulfur assimilation) required the consumption of SAM by cellular methylation reactions, each of which produces SAH. Approximately half the phospholipids in *S. cerevisiae* are phosphatidylcholine (Summers et al. 1988), so its synthesis may involve a significant fraction of the total cellular SAM consumption. So, perhaps without Cho2 activity the methionine provided wasn't being converted to

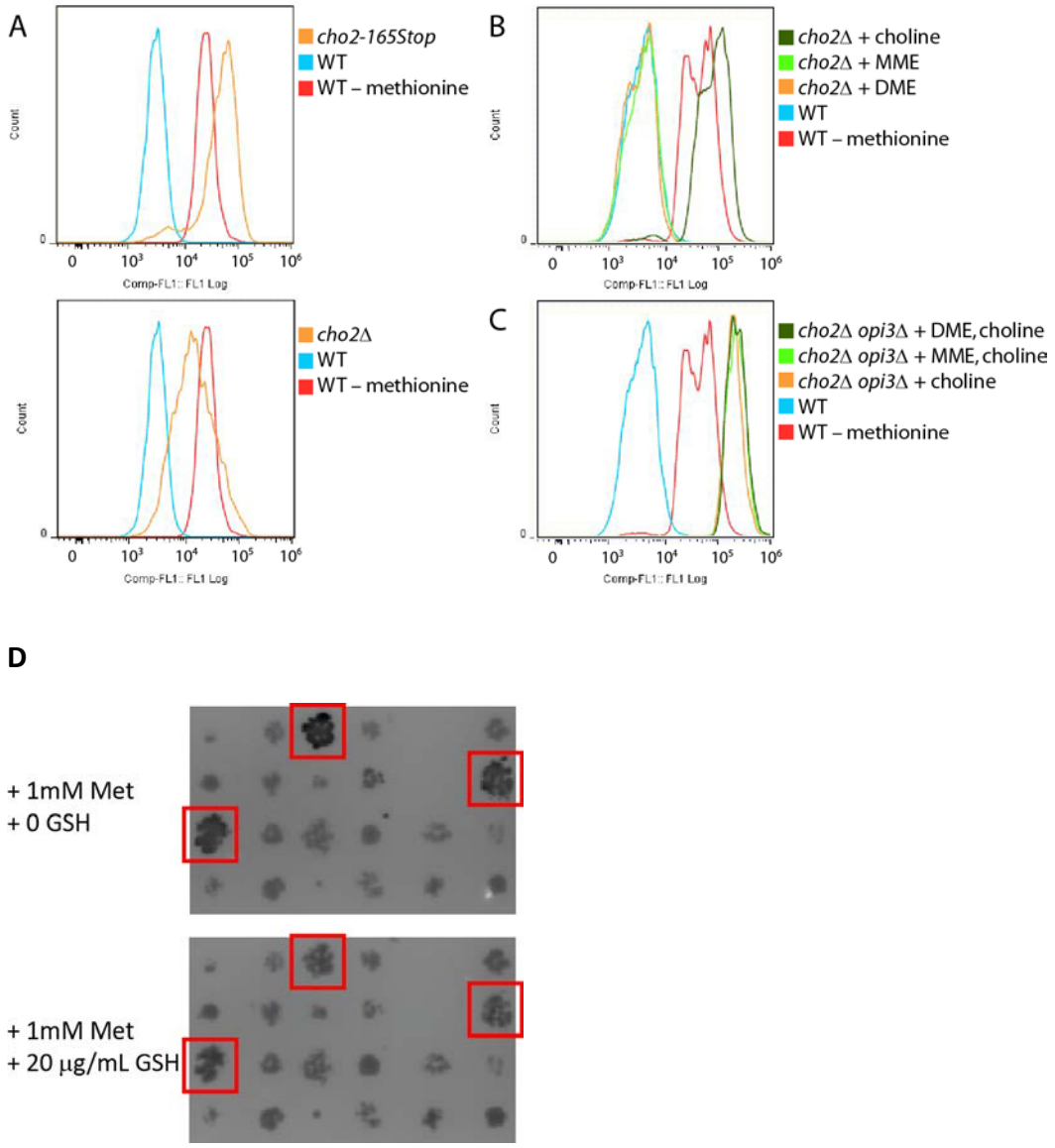


Figure 3.5: *CHO2* mutations induce *MET3-GFP.* All histograms are of GFP-fluorescence from cells carrying *MET3-GFP* under its native promoter, $\log_{10}$ scale. WT is a methionine prototroph, grown overnight with or without methionine. **(A)** Effect on *MET3-GFP* fluorescence by a nonsense mutation in *CHO2* or a complete deletion of *CHO2*. **(B)** Effect on *MET3-GFP* fluorescence in *cho2Δ* of nutritional remediation of compounds that form phospholipids downstream of Cho2's action. **(C)** Effect on *MET3-GFP* fluorescence in *cho2Δ opi3Δ* of nutritional remediation of compounds that form phospholipids downstream of Cho2's action. **(D)** Scan of a tetrad dissection of a *cho2Δ/CHO2 met4Δ/MET4 MET3-GFP/MET3* diploid, replica-plated either to media containing 1 mM methionine and no glutathione (GSH, which is readily taken up by *S. cerevisiae* cells as a cysteine source (Mayfield et al. 2012)), or 1 mM methionine and 20 µg/mL glutathione. Boxed colonies have the genotype *cho2Δ MET4 MET3-GFP.*

cysteine in sufficient quantity. The cysteine deficiency, then, stimulated *MET* regulon transcription. This model predicted that supplementation of monomethyl- and dimethylethanolamine downregulated *MET3-GFP* expression in *cho2Δ* by the capacity of those molecules to be methylated by Opi3, the enzyme that follows Cho2 in phosphatidylcholine synthesis (Kodaki, Yamashita 1987, McGraw, Henry 1989). Indeed, in contrast to the result with the *cho2Δ* single mutant cells, supplementation of *opi3Δ cho2Δ* double mutant cells with monomethyl-or dimethylethanolamine no longer downregulated *MET3-GFP* (figure 3.5c) (The *cho2Δ opi3Δ* cells were fed choline in addition to the pathway intermediates, as they require choline for growth). Furthermore, supplementation of cysteine downregulated *MET3-GFP* in *cho2Δ* (figure 3.5d). Thus, the synthesis of phosphatidylcholine seems to be the main use of cellular SAM pools, so much so that in the absence of phosphatidylcholine synthesis, conversion of methionine to cysteine with a SAH intermediate is impaired.

Discussion

Methionine deficiency can induce members of the MET *regulon*

The *MET* regulon is the set of genes whose transcription is induced by simultaneous starvation for the sulfur-containing amino acids, cysteine and methionine. Previous work indicates that cysteine deficiency alone is necessary and sufficient for transcriptional induction of all tested genes in the *MET* regulon, whereas methionine deficiency alone did not affect their transcription (Hansen, Johannesen 2000, Menant et al. 2006). However, one of the untested members of the *MET* regulon was *STR3,* which codes for a protein involved in the synthesis of methionine from cysteine.

To see if *STR3*'s induction was regulated in the same manner as previously tested members of the *MET* regulon, *STR3* expression was measured in *met13Δ* and *met15Δ* cells grown in media lacking all sulfur-containing amino acids. Although neither strain is capable of synthesizing methionine, *met13Δ* cells are capable of synthesizing cysteine whereas *met15Δ* cells are not. The two strains had an equivalent, large induction of *STR3*. Thus, it appeared that cysteine does not control *STR3* expression and methionine does. This result contrasts with past results for other members of the *MET* regulon that are controlled by cysteine and not methionine. The biological role of Str3 is different from these other members of the *MET* regulon, as Str3 enzyme activity would help remediate methionine deficiency but exacerbate cysteine deficiency. In contrast, all previously tested genes were involved in homocysteine synthesis and thus would help remediate deficiency of both sulfur-containing amino acids.

One possible mechanism for this novel aspect of *STR3*'s expression is that *STR3* was under the control of a different, unknown transcriptional program that responded

specifically to methionine. However, this possibility seems unlikely given that cells deleted for the master transcription factor for the *MET* regulon, *MET4,* do not induce *STR3* when grown without sulfur-containing amino acids (Lee et al. 2010). So, if *STR3*'s induction requires the action of an unknown transcription factor complex, this complex must also require Met4 activity.

A different possibility is that *STR3*'s induction reflects the particular transcription factor binding sites in its promoter, specifically the absence of a Cbf1 binding site. The promoters of all four genes previously described as responding only to cysteine deficiency are bound by Cbf1. Two of the four also contain binding sites for Met31/Met32. Thus, perhaps Met31/Met32 mediates a transcriptional response specifically to methionine deficiency. Meanwhile, Cbf1 acts as an activator in cases of cysteine deficiency, but in cases of cysteine availability Cbf1, or some other protein that binds the Cbf1 binding site, acts as a repressor.

The activity of the transcription factor Met4 is controlled by the ubiquitin ligase SCF^{Met30} (Rouillon et al. 2000). Ubiquitination of Met4 does not always lead to its degradation – previous studies report that in cells grown in rich media (YPD) Met4 is oligoubiquitinated and stable, though its activity is different from that seen in cells grown in defined media lacking sulfur-containing amino acids (Kuras et al. 2002). Interestingly, in cells grown in YPD the oligoubiquitinated Met4 is recruited to the *SAM1* promoter and induces *SAM1* transcription. *SAM1*'s promoter has binding sites for Met31/Met32 only and not Cbf1, much like *STR3* (Lee et al. 2010). Several other genes, all containing Cbf1 promoter binding sites, do not show Met4 binding, or transcriptional induction, in cells grown in rich media. Thus, a possible model is that under specific conditions, Met4 is oligoubiquitinated by SCF^{Met30} rather than being polyubiquitinated. This oligoubiquitinated Met4 can interact with Met31 or Met32 to activate transcription of genes with Met31/Met32 promoter binding sites, but cannot overcome transcriptional repression associated with Cbf1 promoter binding sites.

Control of the MET *regulon by pathway intermediates*

Many transcription factors that regulate the abundance of an end-product metabolite are, in fact, controlled by the concentration of a pathway intermediate. The pathway intermediate, in turn, has its abundance controlled by the end-product metabolite, by way of inhibition of the enzyme that produces the pathway intermediate. This method of control has been described for the *LEU, LYS,* and *ADE* regulons in *S. cerevisiae* (Chin et al. 2008, Jones, Fink 1982, Feller et al. 1994). To determine if methionine or cysteine controlled the *MET* regulon in the same manner, *MET* regulon induction was examined in a mutant unable to synthesize intermediates of the homoserine synthesis branch of the methionine and cysteine synthesis pathway. *MET3* induction, but not *STR3* induction, was lower in this mutant than in *met15Δ* cells. This result suggested that cysteine blocked some step of the homoserine synthesis pathway,

and the lifting of this block produced an intermediate that upregulated the *MET* regulon. One candidate for this regulatory intermediate is o-acetyl-homoserine, since *MET2*, which encodes the protein that synthesizes o-acetyl-homoserine, has been described as being under post-transcriptional control by some end product metabolite of the sulfur-containing amino acid synthesis pathway (Baroni et al. 1986).

Differential states of Met30 dependent on nutritional conditions

There are many possibilities for how these metabolites affect Met30's activity towards Met4. Either of these proteins, or an unknown protein that mediates their interaction, may have a conformational change, differential modification, or differential translation between environmental conditions with and without sulfur-containing amino acids. To see if Met30 is differentially modified or translated, epitope-tagged Met30 isolated from cells grown with or without sulfur-containing amino acids was examined by immunoblot. There was a large increase in Met30 abundance in cells grown without sulfur-containing amino acids, concomitant with an increase in *MET30* mRNA. Thus, there was no effect of methionine and cysteine deficiency on the translational efficiency of the *MET30* transcript. This suggests that $tRNA^{Met}$ deficiency likely does not control Met30 activity by impinging upon translation of the Met30 transcript. Consistent with this possibility, growth of cells harboring a temperature sensitive allele of the $tRNA^{Met}$ synthase, Mes1, at high temperature only led to a slight induction of the *MET* regulon (data not shown), which was more consistent with an effect mediated by the general amino acid control pathway involving Gcn5 (Natarajan et al. 2001) than a *MET* regulon-specific effect.

Met30 isolated from cells lacking methionine migrated at a lower position on an immunoblot, indicating it either was a shorter protein, or that it was differentially modified to migrate faster. One possibility is that Met30 was ubiquitinated in media containing methionine. SCF^{Met30} ubiquitinates Met4 specifically in media containing methionine (Rouillon et al. 2000), so perhaps the complex also ubiquitinates Met30 under these conditions. This ubiquitination could serve as a signal to rapidly degrade active Met30. It is still not clear how Met30 becomes activated, but it is possible the activation is not readily reversible, for instance, if the protein is cleaved. If the activation of Met30 is irreversible, the active protein would need to have a short half-life to ensure the cell's ability to quickly induce the *MET* regulon in response to starvation for sulfur-containing amino acids. In support of this hypothesis, Met30 is among the most rapidly degraded proteins in the *S. cerevisiae* proteome (Belle et al. 2006).

The abundance of an N-terminally-tagged Met30 overexpressed from a *GAL1-10* promoter does not change between cells grown with or without methionine (Rouillon et al. 2000). This tagged Met30 migrates on immunoblots at two different positions, but both forms are present in extracts from cells grown with methionine, as well as from

extracts of cells grown without methionine, in contrast to the migration of bands observed in this study. So, it is possible that either the native 5'-UTR, the native transcriptional context, or the native level of expression is required for Met30 protein to be differentially affected between inducing and non-inducing conditions. Interestingly, Met30 did not migrate as a doublet when isolated from cells in which other members of the SCF^{Met30} complex were mutated, supporting the hypothesis that Met30 is ubiquitinated by the SCF complex.

Cho2 activity is required for synthesis of cysteine from methionine

A screen was performed to find more components of the machinery controlling the *MET* regulon. One of the mutants had a compromised *CHO2* allele. *CHO2* encodes the protein responsible for the first step of phosphatidylcholine synthesis from phosphatidylethanolamine, in a reaction consuming SAM (Summers et al. 1988, Kodaki, Yamashita 1987). The induction of the *MET* regulon in cells lacking Cho2 activity was due to an inability to convert the methionine taken from the environment into cysteine. This result indicates that an enormous fraction of cellular SAM consumption happens during phosphatidylcholine synthesis. The expression of *OPI3* is significantly different between cells grown with or without choline, whereas *CHO2* expression is unaffected (Jesch et al. 2005). This is curious, because these proteins act consecutively to carry out phosphatidylcholine synthesis. One possible explanation is that the intermediate produced by Cho2, phosphatidylmonomethylethanolamine, plays an important cellular role, and thus Cho2 activity is continuously required, regardless of the phosphatidylcholine level. This study suggests another possibility – that the consumption of SAM by Cho2 to produce SAH is required even when choline is plentiful, in order to produce homocysteine from methionine.

Materials and Methods

Strains, plasmids and oligonucleotide sequences

Strains used are listed in table 1, and sequences of oligonucleotide used are listed in table 2. Strains JRY9355 and JRY9356 were segregants from a cross of *MET3::GFP::HIS* from the yeast GFP clone collection (Invitrogen 95702) and BY4742. Strains JRY9348-9353 were segregants of a cross between *hom3Δ* from the MATα knockout collection and BY4741. JRY9360 and JRY9361 were segregants resulting from crosses amongst JRY9356, BY4741, and strains of the MATa and MATα knockout collections (Open Biosystems YSC1053 and YSC1054). Diploids were sporulated by growth in liquid YPD, pre-sporulation overnight in YP-acetate, and then incubation for at least 3 days in 2%

potassium acetate supplemented with the nutrients required because of the auxotrophies of the diploid. A modified lithium acetate protocol was used to transform pJR3170 (*MET30-TAP*) into JRY6330 to produce JRY9354.

To create pJR3170, a genomic region containing the *MET30* ORF, 617 bp upstream of the start codon, and a TAP epitope tag was cloned from the *MET30-TAP* strain from the TAP-tagged collection (Open Biosystems YSC1178) onto the pBY011-D123 (URA, cen-ars) plasmid backbone by gap repair. The TAP tag was then replaced with 3xHA epitope tags immediately downstream, in frame, to make pJR3170, also by gap repair.

Media

To starve for sulfur-containing amino acids, cells were first grown overnight to mid-log phase in SD media + CSM (complete supplement mix) (Amberg, Burke & Strathern 2005), which contains methionine. Then, cells were pelleted and residual media was pipetted off, after which cells were resuspended in SD media + CSM – methionine dropout mix, lacking both methionine and cysteine, for 60 minutes.

RNA extraction, cDNA preparation, RT-qPCR

RNA was purified using hot acid phenol and chloroform. 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 were normalized to *ACT1* amplification values.

Whole cell extract preparation, immunoblotting

Protein extracts were precipitated using 20% trichloroacetic acid and solubilized in SDS loading buffer. Immunoblotting was done with standard procedures and blots were imaged using the LiCOR odyssey imager. Antibodies used in the immunoblots were Anti-Pgk1 (Invitrogen) and Anti-HA (Sigma Aldrich H9658).

Screen, mutant identification

Strains JRY9355 and JRY9356 were mutagenized either with ethyl methanesulfonate (EMS) (Amberg, Burke & Strathern 2006), or UV radiation using a Stratalinker apparatus set to 70 microjoules. Cells were plated at a target density of 500 cells per plate on CSM + 1 mM methionine thin agar plates, and grown at room temperature in the dark. After colonies appeared, plates were scanned, face up, with a Typhoon imager, set to scan 3

mM above the platen, scanning with 488 nm laser and 520 nm emission filter, to identify fluorescent colonies.

Mutations in *MET30* were identified by Sanger sequencing of the *MET30* ORF. Causative mutations in *CHO2* and *CYS3* were identified by transformation with a genomic library (Jauert, Jensen & Kirkpatrick 2005) generated on a plasmid with a Kanamycin selection marker, kindly provided by Jeremy Thorner. Mutants were transformed with the library using a modified lithium acetate protocol (Becker, Lundblad 2001), and, after 30 minutes rescue in YPD, plasmids carrying dominant genes suppressing the mutant phenotype were selected for with CSM plates containing 5 mM sodium selenate (Thomas et al. 1995) and kanamycin.

Flow cytometry

Cells were grown to mid-log phase in CSM + 1 mM methionine (except for the "–methionine" control), and then washed once and resuspended in PBS. Fluorescence was determined in approximately 10,000 cells per experiment, using an FC500 flow cytometer.

Chapter 4: Characterization of an ancient duplication in fungal methylenetetrahydrofolate reductase

Introduction

The final step in the biosynthesis of methionine is the methylation of homocysteine, using methyltetrahydrofolate as the methyl donor (Thomas, Surdin-Kerjan 1997). This reaction is conserved in all branches of life. Even organisms that are methionine auxotrophs, such as humans, methylate homocysteine to produce methionine – in these organisms homocysteine is produced by consumption of the universal methyl donor, s-adenosyl-methionine (SAM) to s-adenosyl-homocysteine (SAH), which is then hydrolyzed to homocysteine (Townsend, Tew & Tapiero 2004). SAM itself is synthesized from methionine (Thomas, Surdin-Kerjan 1991), so the production of methionine by re-methylation of homocysteine is required for methionine homeostasis.

The enzyme methylenetetrahydrofolate reductase (MTHFR), conserved in all branches of life, produces the methyltetrahydrofolate substrate used in methionine synthesis, using the cofactors FAD and NADH (or NADPH) (Raymond, Kastanos & Appling 1999). Fungal and animal MTHFR proteins can also bind SAM, which allows inhibition of catalytic activity when methionine and SAM are plentiful (Roje et al. 2002). Mutations in MTHFR in humans have been implicated in homocysteinemia, the accumulation of excess homocysteine in the blood, which is a risk factor for cardiovascular disease. One particular mutation, A222V, has an allele frequency of approximately 0.35 (Kim 2005), and reduces enzyme function (Marini et al. 2008).

Genomic sequences of many fungal species reveal two genes predicted to encode MTHFR proteins (Sienko et al. 2007). Sorting of these genes into a phylogenetic tree suggests all the homologs derive from an ancient duplication. Two MTHFR-encoding genes are present in the genome sequences of both *Saccharomyces cerevisiae* (Raymond, Kastanos & Appling 1999) and *Schizosaccharomyces pombe* (Naula et al. 2002), which last shared a common ancestor more than 300 million years ago (Sipiczki 2000), and possibly up to 1 billion years ago (Heckman et al. 2001). Essentially all sequenced genomes of species descended from this ancient yeast still contain two genes predicted to encode MTHFR. The roles of these genes encoding MTHFR have been investigated in several yeast species. In both *S. pombe* and *Aspergillus nidulans* (Sienko et al. 2007) deletion of either gene encoding MTHFR causes methionine auxotrophy. On the other hand, in *S. cerevisiae* deletion of one of the two copies,

MET13, causes methionine auxotrophy, whereas deletion of the other copy, *MET12,* has no overt phenotype (Raymond, Kastanos & Appling 1999). Deletion of *MET13* in *S. cerevisiae* removes all MTHFR activity from cell extracts, suggesting Met12 does not have MTHFR activity under these conditions. Indeed, overexpression of *MET12* does not rescue methionine prototrophy in *met13Δ* cells. However, extracts from *Escherichia coli* cells overexpressing either the *MET12* or *MET13* gene show increased MTHFR activity relative to that seen for wild-type *E. coli*, suggesting Met12 has MTHFR activity under select conditions, though it is also possible Met12 enhances the activity of the native *E. coli* MTHFR.

This study focused on the role of Met12 in *S. cerevisiae*. First, the genesis of the difference between yeast species in terms of their phenotypes after deletion of *MET12* was explored by investigation of the MTHFR homologs in *Saccharomyces bayanus,* a yeast species closely related to *S. cerevisiae* (Scannell et al. 2011). Also, whether Met12 requires Met13 for stability was investigated, as MTHFR proteins are known to form dimers (Brooks et al. 2010). Finally, key regions of the *S. cerevisiae* Met12 and Met13 proteins responsible for the different deletion phenotypes were mapped through the formation of chimeric proteins and assessments of their functions.

Results

Phylogenetic analysis of Met12 function

The role of the duplicated MTHFRs in *S. cerevisiae* is different from those in other yeasts whose MTHFR orthologs have been studied. In *S. pombe* and *A. nidulans* both MTHFR-encoding genes are required for methionine prototrophy, whereas in *S. cerevisiae MET13* is required, and *MET12* is dispensable. Given the phylogenetic relationship of these three species, the ancestral state of MTHFR phenotype is likely to be the same as that of *S. pombe* and *A. nidulans,* and the MTHFR phenotype in *S. cerevisiae* is likely to be derived. To determine where in the lineage leading to *S. cerevisiae* this change occurred, the phenotypes of deletions of the homologs of *MET12* and *MET13* in *S. bayanus* were examined. *S. cerevisiae* is more closely related to *S. bayanus* than to *S. pombe* and *A. nidulans*, with an average of 83% identity between homologous proteins between *S. cerevisiae* and *S. bayanus* (Zill et al. 2010). Homologs of *MET12* and *MET13* in *S. bayanus* were found by sequence homology and conservation of synteny, and are referred to here as *SbMET12* and *SbMET13*, respectively. Deletion of *SbMET12* had no significant effect on growth of *S. bayanus* in medium lacking methionine, as well as in medium containing methionine (figure 4.1a). On the other hand, deletion of *SbMET13* caused an inability to grow without methionine, which could be rescued by the addition of methionine. Thus, the deletion

phenotypes of MTHFR-encoding genes in *S. bayanus* were the same as those in *S. bayanus*. The most parsimonious explanation was that the last common ancestor of *S. cerevisiae* and *S. bayanus* also required function in the ancestor of *MET13* for methionine production, whereas the *MET12* ancestor was dispensable for growth without methionine. At face value, the retention of the MET12 paralog in these species was paradoxical given that they had no overt role in methionine synthesis based upon these assays.

A simple possibility suggested by the lack of phenotype for the deletion of *MET12* in *S. bayanus* and *S. cerevisiae* is that in yeast species descended from the common ancestor of *S. bayanus* and *S. cerevisiae* (known as the *sensu stricto* group) Met12 no longer serves any function whatsoever. A prediction of this possibility is that there

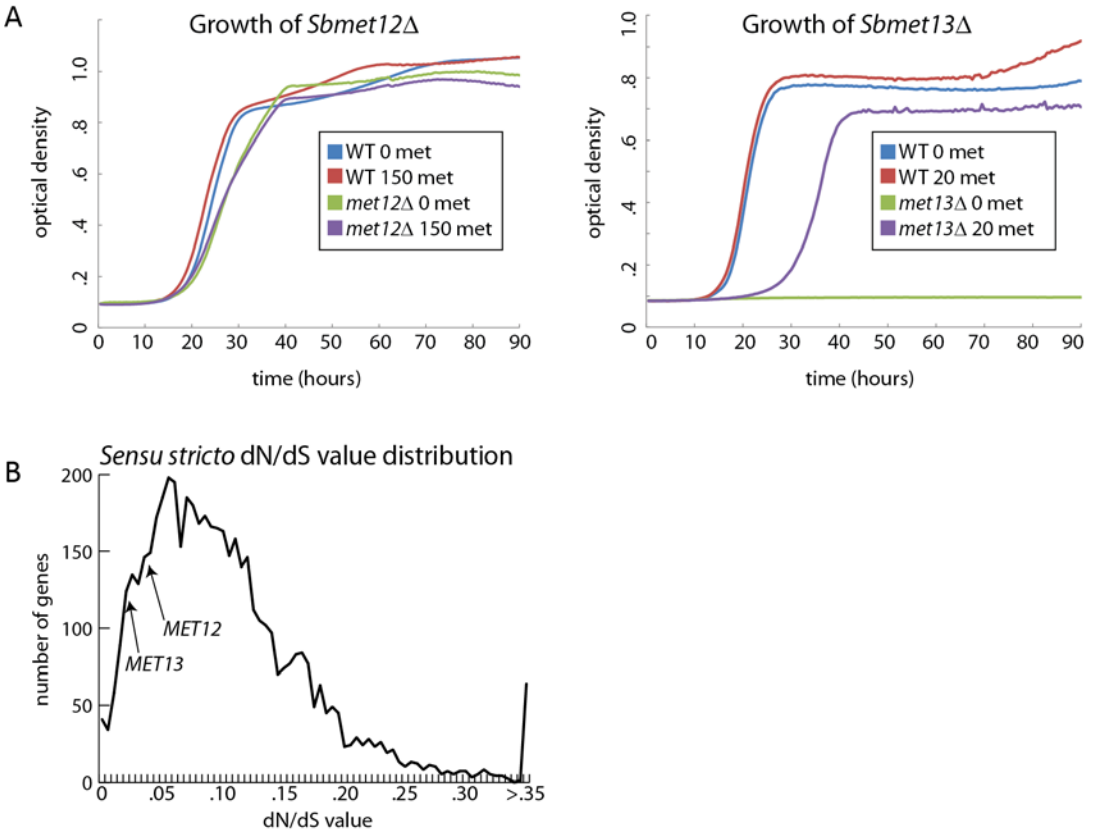


Figure 4.1: Methionine prototrophy of *S. bayanus* deleted for *SbMET12* or *SbMET13.* **(A)** Growth curves of *Sbmet12Δ* and *Sbmet13Δ* in media lacking methionine and media containing methionine (150 µg/mL methionine for *Sbmet12Δ*, and 20 µg/mL for *Sbmet13Δ*). **(B)** Histogram of dN/dS values ("Dataset 1" of (Scannell et al. 2011)) for all genes shared between the five species of the *sensu stricto* group, binned in increments of 0.005. The positions of *MET12* and *MET13* in the histogram are labeled.

would be relaxed conservation of Met12 protein sequence in these species, and the ratio of nonsynonymous to synonymous mutations (dN/dS) in the coding sequence of *MET12* would be higher than the average for other genes in these genomes. However, examination of the dN/dS values within the *sensu stricto* group for *MET12* and *MET13* (Scannell et al. 2011) revealed them to be lower than the dN/dS values for most genes (figure 4.1b). Moreover, the dN/dS values for *MET12* and *MET13* were similar. These observations implied that *MET12* retained some useful function in yeasts of the *sensu stricto* group, despite being dispensable for growth without methionine. Evolutionary pressure to maintain this function presumably restricted *MET12*'s sequence evolution.

Analysis of Met12 protein

The inability of Met12 to compensate for the lack of MTHFR activity in a *met13Δ* strain could result from Met12 requiring Met13's presence to function as an MTHFR. Met12 and Met13 have been shown to heterodimerize (Brooks et al. 2010). Moreover the homomultimerization of MTHFR occurs in animals and prokaryotes: porcine MTHFR is purified as a dimer (Matthews et al. 1984), and bacterial MTHFRs crystallize as either dimers or tetramers. One possibility for the lack of MTHFR enzyme activity in *met13Δ* cells is that Met12 protein requires Met13 binding for stability, and is unstable and degraded in the absence of its binding partner. To test this possibility, Met12 was epitope-tagged and immunoblotted in cell extracts prepared from *MET13* and *met13Δ* strains. Met12 was detected in both cell extracts (figure 4.2). Its abundance may have been lower in extracts made from *met13Δ* cells, but the effect was not statistically significant. Thus, Met12 protein was present in the absence of Met13, yet Met13 may have contributed to the steady state level of Met12.

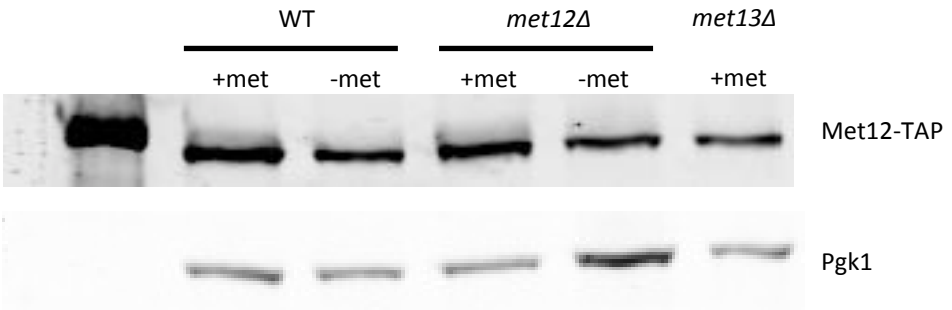


Figure 4.2: Stability of Met12 in the absence of Met13. Met12 epitope-tagged with TAP was expressed under its native promoter on a plasmid. Cell extracts were made from wild type, *met12Δ*, and *met13Δ* cells carrying the plasmid, and immunoblotted with antibodies against TAP and Pgk1. Shown above is a representative replicate, of 3 total experiments.

Mapping of critical differences between Met12 and Met13 through chimeric proteins

To determine which domains of Met12 made it incapable of functioning as the lone MTHFR, chimeric genes were constructed between *MET12* and *MET13*. All chimeras were composed of pieces of Met12 (657 total amino acids) and Met13 (600 total amino acids) fused in regions of identical amino acids between the proteins, to ensure structural completeness (figure 4.3a). These chimeras were then overexpressed with a *GAL1-10* promoter in a *met12Δ met13Δ* double mutant, to determine their ability to confer growth to cells grown without methionine. A chimera made of the first 176 amino acids of Met12 and the last 424 amino acids of Met13 (*12/13-176*) conferred growth without methionine (figure 4.3b). This region of Met12 was predicted to contain most of the FAD-binding residues in MTHFR (figure 4.3a), as determined by homology to the crystallized *Escherichia coli* MTHFR, MetF (Guenther et al. 1999). Three residues important for FAD binding fall after the junction point of the *12/13-176* chimera – two were perfectly conserved between Met12 and the *E. coli* MTHFR, whereas one was different in all three of Met12, Met13, and the *E. coli* MTHFR. Thus, Met12 probably retained the ability to bind FAD.

A chimera containing the first 304 amino acids of Met13 and the last 346 amino acids of Met12 (*13/12-304*) also allowed growth of *met12Δ met13Δ* cells without methionine. This region of Met12 is predicted to contain the entire regulatory domain of MTHFR, which downregulates MTHFR activity after binding s-adenosyl-methionine (SAM), which is synthesized from methionine. The domain may contain some activating or stabilizing role as well, as complete deletion of the conserved C-terminal regulatory domain of the human MTHFR compromises its activity (Shan et al. 1999). The ability of the *13/12-304* chimera to confer growth without methionine suggested the activating role of the regulatory domain was conserved in Met12. Additionally, this result implied that the potential repressive activity of the Met12 regulatory domain was not constitutive in these conditions, or that the Met12 regulatory domain was missing repressive activity.

The ability of *S. cerevisiae* carrying these two chimeras to grow without methionine constricted the region that made Met12 unable to function as a lone MTHFR to the 135 amino acids between residues 176 and 311. A chimera containing the first 280 amino acids of Met13 and the last 373 amino acids of Met12 (*13/12-280*) did not restore methionine prototrophy to *met12Δ met13Δ* cells. Combined with the result for the *13/12-304* chimera, this result suggested the presence, between residues 284 and 311 of Met12, of at least one difference between Met12 and Met13 responsible for Met12's inability to function as a lone MTHFR. This region contains two residues predicted to contact methylenetetrahydrofolate. For one of these residues, Met12 is identical to the *E. coli* MTHFR, whereas for the other residue Met12 is identical to Met13.

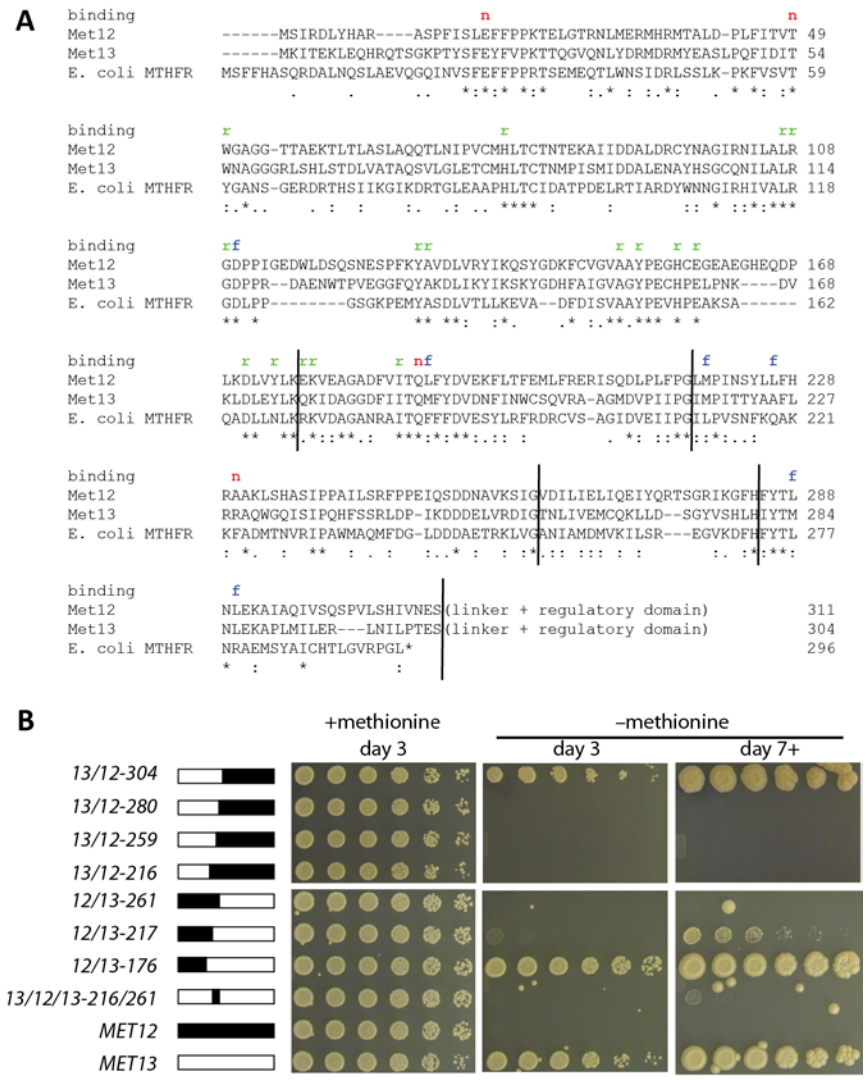


Figure 4.3: Mapping of functional domains of Met12 with chimeric proteins. **(A)** Alignment of Met12 and Met13 catalytic domains to full-length *E. coli* MTHFR, by clustal 2.1. Residues implicated in FAD binding are demarcated with an "r", methylenetetrahydrofolate binding with an "f", and NADH binding with an "n". In addition to the contacts shown, *E. coli* F223 makes contacts with methylenetetrahydrofolate in addition to NADH, and Q183 makes contacts with all three substrates. Junction sites used in chimeric proteins are demarcated with a vertical black line. **(B)** Frogging assay to determine methionine prototrophy of strains carrying chimeric MTHFR proteins. 5-fold serial dilutions were made of *met13Δ met12Δ* strains carrying chimeric Met12-Met13 proteins expressed with a *GAL1-10* promoter. The first column of images shows growth after 3 days on plates containing methionine and containing galactose as the sugar source. The second column shows growth after 3 days on galactose plates lacking methionine, and the third column shows growth on those same plates after 14 days (top panel) or 7 days (bottom panel).

Additionally, a chimera of the first 261 amino acids of Met12 and the last 341 amino acids of Met13 (*12/13-261*) conferred no growth without methionine. Along with the result for the *12/13-176* chimera, this suggests the presence of important differences between Met12 and Met13 between residues 176 and 261 of Met12. To narrow down the region of interest, two chimeras were made that divided this region between them. One was composed of the first 217 residues of Met12 and the last 384 residues of Met13 (*12/13-217*), and the other was composed of the first 216 amino acids of Met13, followed by 45 amino acids of Met12, followed by the last 339 amino acids of Met13 (*13/12/13-216/261*). Both chimeras conferred extremely poor growth without methionine, but neither caused complete methionine auxotrophy. So, the complete auxotrophy seen for the *12/13-261* chimera was presumably due a compound effect or interaction between the regions spanning residues 176 to 216, and 216 to 261.

Discussion

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme required in methionine biosynthesis in all branches of life. Many fungal species carry two genes encoding for MTHFR, all derived from an ancient duplication. The role of the duplicates has been described in a handful of species; in *S. pombe* and *A. nidulans* both genes are required for methionine prototrophy, whereas in *S. cerevisiae* only *MET13* is required for methionine prototrophy, and *MET12* is dispensable. Here, the role of Met12 was investigated.

MET12 *has been subjected to ongoing purifying selection since its loss of phenotype*

Given the phylogeny of *S. pombe*, *A. nidulans*, and *S. cerevisiae*, the phenotypes of the MTHFR-encoding gene deletions in *S. pombe* and *A. nidulans* are likely to be the ancestral state. If the phenotypes of *met12Δ*and *met13Δ* in *S. cerevisiae* represent a derived state, it is important to determine when it arose. In yeast, genes without function are typically deleted very rapidly. Thus, if the current state of MTHFR phenotypes arose very recently, it would be possible that *MET12* had no function and was on its way to being deleted from the genome; if the state arose long ago this possibility would be less likely. To test if the phenotype of *met12Δ* arose recently along the lineage leading to *S. cerevisiae*, a relative of *S. cerevisiae* was investigated. Deletion of the *MET12* homolog in *S. bayanus* did not cause methionine auxotrophy, implying the phenotype of *met12Δ* in *S. cerevisiae* arose before the last common ancestor of *S. bayanus* and *S. cerevisiae*. Moreover, strong purifying selection of *MET12* coding sequence has been maintained since this ancestral yeast, so the protein must play some

role in the cell that was being selected for, keeping the gene from being subjected to genetic drift.

It is possible that Met12 has evolved a completely unrelated cellular function, but several lines of evidence hint that Met12's role is still related to MTHFR activity. The residues important for cofactor and substrate binding for the *E. coli* MTHFR are as conserved in Met12 as in Met13 (figure 4.3b), suggesting Met12 is still involved in the reduction of folate molecules. Furthermore, chemical genomic profiling indicates that heterozygous deletion of *MET12* confers significant sensitivity to the anti-folate drug methotrexate (Hillenmeyer et al. 2008), which is also the case for heterozygous deletion of *MET13* and other genes encoding proteins involved in folate metabolism. Also, a 20-amino acid stretch in the C-terminus of the human protein has been implicated in SAM-binding (Goyette et al. 1994); this region is also highly conserved in both Met12 and Met13 (data not shown). The conservation of a SAM-binding regulatory sequence suggests involvement in methionine synthesis.

Met12 protein is stable in the absence of Met13 protein

This putative cellular role played by Met12 could involve MTHFR activity, but this possibility would need to be reconciled with observations that cell extracts from *met13Δ* cells show no MTHFR activity, and that *met13Δ* cells are methionine auxotrophs, despite having Met12. One possibility is that Met12 is capable of MTHFR activity, but that it requires the presence of Met13 to have this activity, or that it is not expressed in laboratory conditions. Met12 was detectable by immunoblot in wild type and *met13Δ* cell extracts, without a large decrease in abundance between the two, implying Met12 was not unstable in the absence of Met13. MTHFR proteins typically form dimers or tetramers. For the *E. coli* MTHFR oligomerization is necessary for function (Misra, Bhakuni 2003), and the human A222V MTHFR variant has reduced function that is hypothesized to result from an increased propensity to dissociate into monomers (Yamada et al. 2001). Met12 and Met13 form heterodimers *in vivo* (Brooks et al. 2010), although the fraction of either species that forms heterodimers has not been determined. It is possible that Met12 was stable as a monomer, or that it was capable of forming stable homodimers that lack MTHFR activity.

Met12 contains several independent differences from Met13 that interfere with MTHFR function

Ultimately, the difference between *MET12* and *MET13* that allows only Met13 to function as a lone MTHFR must be due to a difference in the coding regions of these genes, since expression of *MET12* from a *GAL1-10* overexpression promoter, with non-native 5'- and 3'-UTRs, also does not compensate for the absence of *MET13,* whereas

overexpression of *MET13* with the same construct does. Chimeric proteins between Met12 and Met13 were created to map critical differences. Eukaryotic MTHFRs are composed of an N-terminal catalytic domain and a C-terminal regulatory domain, with a linker of variable length in between. Much of Met12 was successfully substituted into Met13 to produce chimeras capable of supporting methionine prototrophy – the regulatory region and linker could be incorporated into a functional MTHFR, as well as over half of the catalytic domain. The functional half of the catalytic region contains most of the FAD cofactor-binding residues. Thus, Met12's inability to function as a lone MTHFR likely is not caused by an inability to bind the FAD cofactor.

The region of Met12 that could not be substituted into Met13 to give a functional MTHFR was further subdivided, which implicated a 20-amino acid region at the very end of the catalytic domain of Met12 as completely abolishing methionine prototrophy. While this region does contain two residues predicted to interact with the folate substrate, Met12 has identical residues at these positions to either the *E. coli* MTHFR or Met13. This region does contain many residues that are diverged between all three of Met12, Met13, and the *E. coli* MTHFR. Perhaps this region is important in relaying the regulation conferred by the C-terminal domain, which the *E. coli* MTHFR lacks.

A separate region of Met12, 85 amino acids in length, was also implicated in Met12's inability to support growth without methionine, suggesting there were multiple reasons Met12 failed to function as a lone MTHFR. This second region contained half the residues predicted to contact methylenetetrahydrofolate based on the crystal structure of *E. coli* MTHFR. However, there is poor conservation of these residues for both Met12 and Met13 as compared to the crystalized MTHFR. So, it was possible that Met12 was unable to bind methylenetetrahydrofolate, but other differences in the same region that do not affect methylenetetrahydrofolate binding could instead be responsible.

Model for Met12's role

The MTHFR of the bacteria *Thermus thermophilus* has been characterized as a homodimer with one active subunit bound to FAD, and one inactive subunit that is not FAD-bound (Igari et al. 2011). FAD can be added to purified *T. thermophilus* MTHFR dimers such that both subunits become FAD-bound, but this does not increase the enzyme activity. If half-of-the-site reactivity also applies to *S. cerevisiae* MTHFR, one possibility for Met12's role is that it forms heterodimers with Met13 in which Met13 is the active subunit. Several enzymes have been shown to bind to catalytically inactive paralogous proteins that act as regulatory subunits, such as the *de novo* DNA methyltransferases in plants and mammals (Henderson et al. 2010). So, Met12's evolutionarily conserved role could involve regulation of the Met13 subunit's activity. By analogy, if the common human MTHFR minor allele, A222V, confers some beneficial function when dimerized to the wild type MTHFR, it may be maintained in the population by balancing selection, rather than genetic drift.

Materials and Methods

Strains, plasmids, and oligonucleotide sequences

Strains used are listed in table 1, and sequences of oligonucleotide used are listed in table 2. Deletion of *SbMET12* (Sbay_16.295, as annotated in (Scannell et al. 2011)) and *SbMET13* (Sbay_7.143) was done with a lithium acetate protocol, modified for *S. bayanus* as described previously (Scannell et al. 2011).

JRY9369 was created by crossing JRY9368, created by a modified lithium acetate protocol (Becker, Lundblad 2001), to *met13Δ*, taken from the MATa knockout collection (YSC1054), followed by sporulation on 1% potassium acetate plates supplemented with 200 µg/mL CSM mix. The modified lithium acetate protocol was also used for cloning of genes by gap repair of cut plasmids, as well as for transformation of plasmids into yeast strains.

Chimeric MTHFR genes were made by modification of the *MET12* or *MET13* clone from the HIP FLEXGene collection, using gap repair. Gap repair was also used to create *MET12* epitope-tagged with TAP from the plasmid carrying TAP-tagged *MET30* described in chapter 3.

Media, Growth curves, and serial dilution spotting

S. bayanus strains were grown on standard *S. cerevisiae* media. To generate growth curves, cells were first grown overnight in SD + CSM (complete supplement mix) (Amberg, Burke & Strathern 2005), then were diluted to an optical density (OD) at 600 nm wavelength of 0.01 in 200 uL of media, in triplicate, in a 96-well format. A SpectraMax Plus384 96-well plate reader, kept at 24^{o}C, was used to measure the OD at 595 nm wavelength every 30 minutes. For the growth curves, the media used was SD with CSM – methionine dropout mix, with methionine added back for the cultures grown with methionine.

For the serial dilution plating assay, 5-fold dilutions were made from cells starting at 10^8 cells/mL after growth overnight in SD + CSM. Serial dilution spotting was done with a 48-pin replicator (Dan-Kar corporation). Plates used were modified SD + CSM (with or without methionine, as appropriate) plates, with 2% galactose + 0.1% glucose as the sugar source.

Whole cell extract preparation, immunoblotting

Protein extracts were precipitated using 20% trichloroacetic acid and solubilized in SDS loading buffer. Immunoblotting was done with standard procedures and blots were imaged using the LiCOR odyssey imager. Antibodies used in the immunoblots were Anti-TAP (Thermo Scientific CAB1001) and Anti-Pgk1 (Invitrogen A6457).

Table 1: Yeast strains used

Strains used in chapter 2

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

Strains used in chapter 3

Name	***Genotype***	***Source***
JRY6330	MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1* (BY4741)	Jef Boeke
JRY6331	MATα *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1* (BY4742)	Jef Boeke
JRY9347	*met13Δ::KanMX* MATα *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	Open Biosystems
JRY9348	*hom3Δ::KanMX* MATα *leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9349	*hom3Δ::KanMX* MATa *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9350	*hom3Δ::KanMX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9351	*hom3Δ::KanMX* MATa *met15Δ0 lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study

JRY9352	MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9353	MATa *met15Δ0 lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9354	JRY6330 [pJR3170]	this study
JRY9355	*MET3::GFP::HIS3MX* MATa *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9356	*MET3::GFP::HIS3MX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9357	*met30(C95Y) MET3::GFP::HIS3MX* MATa *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9358	*cys3(S25P) MET3::GFP::HIS3MX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9359	*cho2(165Stop) MET3::GFP::HIS3MX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9360	*cho2Δ::KanMX MET3::GFP::HIS3MX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study
JRY9361	*cho2Δ::KanMX opi3Δ::KanMX MET3::GFP::HIS3MX* MATα *met15Δ0 leu2Δ0 ura3Δ0 his3Δ1*	this study

Strains used in chapter 4

Name	***Species***	***Genotype***	***Source***
JRY9362	*S. bayanus*	MATa *hoΔ::loxP his3 ura3 lys2 Cyh2-12*	this study
JRY9363	*S. bayanus*	MATα *hoΔ::loxP his3 ura3 lys2 Cyh2-12*	this study
JRY9364	*S. bayanus*	MATa *hoΔ::loxP his3 ura3 lys2 Cyh2-12 met12Δ*	this study
JRY9365	*S. bayanus*	MATα *hoΔ::loxP his3 ura3 lys2 Cyh2-12 met13Δ*	this study
JRY6331	*S. cerevisiae*	MATα *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1* (BY4742)	Jef Boeke
JRY9366	*S. cerevisiae*	MATα *lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1* [pJR3181]	this study
JRY9367	*S. cerevisiae*	met13Δ::KanMX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 [pJR3181]	this study
JRY9368	*S. cerevisiae*	met12Δ::His3MX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 [pJR3181]	this study
JRY9369	*S. cerevisiae*	met12Δ::HIS3MX met13Δ::KanMX MATα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1	this study
JRY9370	*S. cerevisiae*	JRY9369 [pJR3171]	this study
JRY9371	*S. cerevisiae*	JRY9369 [pJR3172]	this study
JRY9372	*S. cerevisiae*	JRY9369 [pJR3173]	this study
JRY9373	*S. cerevisiae*	JRY9369 [pJR3174]	this study
JRY9374	*S. cerevisiae*	JRY9369 [pJR3175]	this study
JRY9375	*S. cerevisiae*	JRY9369 [pJR3176]	this study
JRY9376	*S. cerevisiae*	JRY9369 [pJR3177]	this study
JRY9377	*S. cerevisiae*	JRY9369 [pJR3178]	this study
JRY9378	*S. cerevisiae*	JRY9369 [pJR3179]	this study
JRY9379	*S. cerevisiae*	JRY9369 [pJR3180]	this study

Table 2: Oligonucleotides used

Oligonucleotides used in chapter 2

strain construction

set1KO	TATTTGTTGAATCTTTATAAGAGGTCTCTGCGTTTAGAGACGGATCCCCGGGTTAATTAA TGTTAAATCAGGAAGCTCCAAACAAATCAATGTATCATCGCGATGAATTCGAGCTCGTTT
fol3KO	GACCGATAAGGTCACCTGTTAGTAAGTTGATCTAGTGTATCGGATCCCCGGGTTAATTAA AGTTTCTCAAGTGACTATTGTGAATCCGTAATAATTCACCCGATGAATTCGAGCTCGTTT
S. pombe fol1KO	GGGAAGTTACTTCATCAACGCTGAC tgacctacaggaaagagttactATGTCGTAGAGATGAGAAGCG atcaggggataacgcaggaaaATCTTTCGTCGTTGTAGACCC GGCTACTTTACCAAAGGTACGGG

qPCR

ACT1	GGCATCATACCTTCTACAACGAATTG GTTTTGTCCTTGTACTCTTCCGGTAG
BNA2	GACGTTGCATTTCCATGCTG CCCTGCACTGCTTTAAGAAG
IRC18	TGATCTGGTTGCCAAGAAGG GCCGAGACTAAGCATAGACT
PER33	TTCCCAGGAATCGTCCCATG CGATGGCATATGTAACGGAT
SFT2	GTTCTAGCCGCTAAACCAAG TCTTGCCGAATTGACACCAG
THI12	TCAAGAAGGCAACCGACTAC GAAAGTACCTTCCTGTCTGC
TIP1	AGGCTGCATCTTCTTCCAAG CAACAACAGCACCGAAAGAG

Oligonucleotides used in chapter 3

pJR3170 construction

MET30 cloning	AGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGACAAACGGCACCCAAAATC CGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTCACTGATGATTCGCGTCTA
HA tagging	ATTTGGGTGCGTAAAAATGTACAAATTCGATCTCAATGATAACATCTTTTACCCATACGA CAGATTACGCTGCTCAGTGCTGAATCATGGTCA TAG CTGTTTCCTGTGTGAAATTGTTAT

qPCR

ACT1	GGCATCATACCTTCTACAACGAATTG GTTTTGTCCTTGTACTCTTCCGGTAG
MET3	TTCTTGCAATTCGGTGGTGG GACTCTAGCTGAATATCGGC
MET30-3xHA	GGAAGTGTATGCACACGTTC CATAGGGATAGCCCGCATAG
STR3	GTCCCATGCTTCCATTGATC CTTATGCAGGTCATCGATGG

Oligonucleotides used in chapter 4

Strain construction

Sbay MET12KO	GGGATATTTAGACGATACGCCTTGATAGAATTCCTTAAACCGGATCCCCGGGTTAATTAA TCGCATTTTTTTTTGTATTTATTTTTAGTAGGAGAGGTTACGATGAATTCGAGCTCGTTT
Sbay MET13KO	CGGAAATTCTGTTAAAGAGTTGTTGTTCCGGGCGCTATTACGATGAATTCGAGCTCGTTT CCACTACCAACCAGAACTCTCCTCACCACCACCACTCGACCGGATCCCCGGGTTAATTAA
Scer Met12KO	AAGCGTGTTGGACGGGACAGGTTGATTACATTTTTTAAACCGGATCCCCGGGTTAATTAA ACATTATTTTCGCATTTTTGTATTTATATTCAGTGAAATACGATGAATTCGAGCTCTTT

Plasmid construction

pJR3181	ACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCAAAATAACTGAGACATCTGG TTCTCTTCCATGGATTAATTAACCCGGGGATCCGTCGACCGCTTGGTCGACCGGCTTGGTCGAGTAACATTT
pJR3171	CATGATTCTGGAAAGATTGAACATTCTACCTACGGAATCAAGCGAAGAAGAGGGAGAAGA ACGACAGGTTTCCCGACTGG
pJR3172	CAATTTGAGCAATAGCCTTTTCCAAATTTAATGTATAGAAGTGCAAGTGAGAAACGTAAC GCCTGGTATCTTTATAGTCCT
pJR3173	TTTGATATATTTCCTGAATCAATTCGATAAGAATGTCCACTCCGATATCACGGACCAGCT GCCTGGTATCTTTATAGTCCT
pJR3174	TGTGGAAAAGCAGATAGGAGTTAATAGGCATCAACCCAGGAATAATGGGCACGTCCATGC GCCTGGTATCTTTATAGTCCT
pJR3175	TGTCGAGCAATTTTTGACACATTTCCACGATCAAGTTAGTACCAATGGACTTCACGGCAT GCCTGGTATCTTTATAGTCCT
pJR3176	CCCTTCTCAAGAAGGCCGCGTAGGTAGTGATCGGCATGATCCCAGGGAAAAGGGGCAAAT ACGACAGGTTTCCCGACTGG
pJR3177	GATGAAGTCGCCGCCGGCGTCGATCTTCTGCTTCAAATACTCGAGATCCTTCAATGGGTCTTGCT GCCTGGTATCTTTATAGTCCT
pJR3178	CCCCCCAGAAATCCAATCGGATGATAATGCCGTGAAGTCCATCGGAACTAACTTGATCGT ACGACAGGTTTCCCGACTGG

Table 3: Plasmids used

Plasmids used in chapter 2

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

Plasmids used in chapter 3

plasmid	***backbone***	***yeast selection***	***insert***	***source***
pJR3170	pBY011-D123	*URA3*	*MET30*(-670-1923)3xHA	this study

Plasmids used in chapter 4

plasmid	***name***	***backbone***	***yeast selection***	***insert***	***source***
pJR3171	13/12-304	pBY011-D123	*URA3*	*MET13*(1-912) *MET12*(934-1971)	this study
pJR3172	13/12-280	pBY011-D123	*URA3*	*MET13*(1-840) *MET12*(853-1971)	this study
pJR3173	13/12-259	pBY011-D123	*URA3*	*MET13*(1-777) *MET12*(784-1971)	this study
pJR3174	13/12-216	pBY011-D123	*URA3*	*MET13*(1-648) *MET12*(652-1971)	this study
pJR3175	12/13-261	pBY011-D123	*URA3*	*MET12*(1-783) *MET13*(778-1800)	this study
pJR3176	12/13-217	pBY011-D123	*URA3*	*MET12*(1-651) *MET13*(649-1800)	this study
pJR3177	12/13-176	pBY011-D123	*URA3*	*MET12*(1-528) *MET13*(529-1800)	this study
pJR3178	13/12/13-216/261	pBY011-D123	*URA3*	*MET13*(1-648) *MET12*(652-783) MET13(778-1800)	this study
pJR3179		pBY011-D123	*URA3*	*MET12*(1-1971)	HIP flexgene
pJR3180		pBY011-D123	*URA3*	*MET13*(1-1800)	HIP flexgene
pJR3181		pBY011-D123	*URA3*	*MET12*(-400-1971)TAP	this study

All plasmids contain an AMP bacterial selection marker. Numbers in parentheses correspond to genomic coordinates relative to the ORF start codon; negative numbers indicate the inclusion of the native ORF promoter.

References

Alkins, S.A., Byrd, J.C., Morgan, S.K., Ward, F.T. & Weiss, R.B. 1996, "Anaphylactoid reactions to methotrexate", *Cancer,* vol. 77, no. 10, pp. 2123-2126.

Amberg, D.C., Burke, D. & Strathern, J.N. 2005, *Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual,* Cold Spring Harbor Laboratory Pr.

Amberg, D.C., Burke, D.J. & Strathern, J.N. 2006, "Ethyl Methane Sulfonate (EMS) Mutagenesis", *Cold Spring Harbor Protocols,* vol. 2006, no. 1, pp. pdb.prot4180.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. & Kouzarides, T. 2001, "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain", *Nature,* vol. 410, no. 6824, pp. 120-124.

Baroni, M., Livian, S., Martegani, E. & Alberghina, L. 1986, "Molecular cloning and regulation of the expression of the MET2 gene of Saccharomyces cerevisiae", *Gene,* vol. 46, no. 1, pp. 71-78.

Bayly, A.M., Berglez, J.M., Patel, O., Castelli, L.A., Hankins, E.G., Coloe, P., Hopkins Sibley, C. & Macreadie, I.G. 2001, "Folic acid utilisation related to sulfa drug resistance in Saccharomyces cerevisiae", *FEMS microbiology letters,* vol. 204, no. 2, pp. 387-390.

Becker, D.M. & Lundblad, V. 2001, "Introduction of DNA into yeast cells", *Current protocols in molecular biology,* Chapter 13, Unit13.7.

Belle, A., Tanay, A., Bitincka, L., Shamir, R. & O'Shea, E.K. 2006, "Quantification of protein half-lives in the budding yeast proteome", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 35, pp. 13004-13009.

Bernstein, B.E., Humphrey, E.L., Erlich, R.L., Schneider, R., Bouman, P., Liu, J.S., Kouzarides, T. & Schreiber, S.L. 2002, "Methylation of histone H3 Lys 4 in coding regions of active genes", *Proceedings of the National Academy of Sciences,* vol. 99, no. 13, pp. 8695-8700.

Boa, S., Coert, C. & Patterton, H.G. 2003, "Saccharomyces cerevisiae Set1p is a methyltransferase specific for lysine 4 of histone H3 and is required for efficient gene expression", *Yeast,* vol. 20, no. 9, pp. 827-835.

Borde, V., Robine, N., Lin, W., Bonfils, S., Geli, V. & Nicolas, A. 2009, "Histone H3 lysine 4 trimethylation marks meiotic recombination initiation sites", *EMBO J,* vol. 28, no. 2, pp. 99-111.

Botto, L.D. & Yang, Q. 2000, "5, 10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review", *American Journal of Epidemiology,* vol. 151, no. 9, pp. 862-877.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F. & Allis, C.D. 2001, "Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae.", *Genes & development,* vol. 15, no. 24, pp. 3286-3295.

Brooks, M.A., Gewartowski, K., Mitsiki, E., Létoquart, J., Pache, R.A., Billier, Y., Bertero, M., Corréa, M., Czarnocki-Cieciura, M. & Dadlez, M. 2010, "Systematic bioinformatics and experimental validation of yeast complexes reduces the rate of attrition during structural investigations", *Structure,* vol. 18, no. 9, pp. 1075-1082.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S. & Zhang, Y. 2002, "Role of histone H3 lysine 27 methylation in Polycomb-group silencing", *Science,* vol. 298, no. 5595, pp. 1039-1043.

Chang, L.F., Cunningham, T.S., Gatzek, P.R., Chen, W.J. & Kohlhaw, G.B. 1984, "Cloning and characterization of yeast Leu4, one of two genes responsible for alpha-isopropylmalate synthesis", *Genetics,* vol. 108, no. 1, pp. 91-106.

Chartrand, P., Meng, X.H., Huttelmaier, S., Donato, D. & Singer, R.H. 2002, "Asymmetric sorting of ash1p in yeast results from inhibition of translation by localization elements in the mRNA.", *Molecular cell,* vol. 10, no. 6, pp. 1319-1330.

Cheng, X., Kumar, S., Klimasauskas, S. & Roberts, R. 1993, "Crystal structure of the HhaI DNA methyltransferase", *Cold Spring Harbor Symposia on Quantitative Biology*, pp. 331.

Cherest, H. & Surdin-Kerjan, Y. 1992, "Genetic analysis of a new mutation conferring cysteine auxotrophy in Saccharomyces cerevisiae: updating of the sulfur metabolism pathway", *Genetics,* vol. 130, no. 1, pp. 51-58.

Chin, C., Chubukov, V., Jolly, E.R., DeRisi, J. & Li, H. 2008, "Dynamics and design principles of a basic regulatory architecture controlling metabolic pathways.", *PLoS Biology,* vol. 6, no. 6, pp. e146.

Cooper, A.J.L. 1983, "Biochemistry of sulfur-containing amino acids", *Annual Review of Biochemistry,* vol. 52, no. 1, pp. 187-222.

Cordero, A., Mulinare, J., Berry, R., Boyle, C., Dietz, W. & Johnston, R. 2010, "CDC Grand Rounds: additional opportunities to prevent neural tube defects with folic acid fortification", *MMWR Morb Mortal Wkly Rep,* vol. 59, no. 31, pp. 980-984.

Crider, K.S., Quinlivan, E.P., Berry, R.J., Hao, L., Li, Z., Maneval, D., Yang, T.P., Rasmussen, S.A., Yang, Q. & Zhu, J.H. 2011, "Genomic DNA Methylation Changes in Response to Folic Acid Supplementation in a Population-Based Intervention Study among Women of Reproductive Age", *PLoS ONE,* vol. 6, no. 12, pp. e28144.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. & Pirrotta, V. 2002, "*Drosophila* Enhancer of Zeste/ESC Complexes Have a Histone H3 Methyltransferase Activity that Marks Chromosomal Polycomb Sites", *Cell,* vol. 111, no. 2, pp. 185-196.

Drinnenberg, I.A., Fink, G.R. & Bartel, D.P. 2011, "Compatibility with killer explains the rise of RNAi-deficient fungi", *Science,* vol. 333, no. 6049, pp. 1592-1592.

Edwards, C.R., Dang, W. & Berger, S.L. 2011, "Histone H4 lysine 20 of Saccharomyces cerevisiae is monomethylated and functions in subtelomeric silencing", *Biochemistry,* vol. 50, no. 48, pp. 10473-10483.

Esteller, M. 2007, "Cancer epigenomics: DNA methylomes and histone-modification maps", *Nature Reviews Genetics,* vol. 8, no. 4, pp. 286-298.

Feller, A., Dubois, E., Ramos, F. & Piérard, A. 1994, "Repression of the genes for lysine biosynthesis in Saccharomyces cerevisiae is caused by limitation of Lys14-dependent transcriptional activation", *Molecular and cellular biology,* vol. 14, no. 10, pp. 6411-6418.

Fingerman, I.M., Wu, C., Wilson, B.D. & Briggs, S.D. 2005, "Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in Saccharomyces cerevisiae", *The Journal of biological chemistry,* vol. 280, no. 31, pp. 28761-28765.

Friso, S., Choi, S.W., Girelli, D., Mason, J.B., Dolnikowski, G.G., Bagley, P.J., Olivieri, O., Jacques, P.F., Rosenberg, I.H. & Corrocher, R. 2002, "A common mutation in the 5, 10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status", *Proceedings of the National Academy of Sciences,* vol. 99, no. 8, pp. 5606.

Fujita, Y., Ukena, E., Iefuji, H., Giga-Hama, Y. & Takegawa, K. 2006, "Homocysteine accumulation causes a defect in purine biosynthesis: further characterization of

Schizosaccharomyces pombe methionine auxotrophs", *Microbiology,* vol. 152, no. 2, pp. 397-404.

Goll, M.G. & Bestor, T.H. 2005, "Eukaryotic cytosine methyltransferases", *Annu.Rev.Biochem.,* vol. 74, pp. 481-514.

Goyette, P., Sumner, J.S., Milos, R., Duncan, A.M.V., Rosenblatt, D.S., Matthews, R.G. & Rozen, R. 1994, "Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification", *Nature genetics,* vol. 7, no. 2, pp. 195-200.

Green, E., Mas, G., Young, N., Garcia, B. & Gozani, O. 2012, "Methylation of H4 lysines 5, 8 and 12 by yeast Set5 calibrates chromatin stress responses", *Nat Struct Mol Biol,* vol. advance online publication.

Gu, S.G. & Fire, A. 2010, "Partitioning the C. elegans genome by nucleosome modification, occupancy, and positioning", *Chromosoma,* vol. 119, no. 1, pp. 73-87.

Guenther, B.D., Sheppard, C.A., Tran, P., Rozen, R., Matthews, R.G. & Ludwig, M.L. 1999, "The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia", *Nature Structural & Molecular Biology,* vol. 6, no. 4, pp. 359-365.

Hansen, K.H., Bracken, A.P., Pasini, D., Dietrich, N., Gehani, S.S., Monrad, A., Rappsilber, J., Lerdrup, M. & Helin, K. 2008, "A model for transmission of the H3K27me3 epigenetic mark", *Nature cell biology,* vol. 10, no. 11, pp. 1291-1300.

Hansen, J. & Johannesen, P.F. 2000, "Cysteine is essential for transcriptional regulation of the sulfur assimilation genes in Saccharomyces cerevisiae.", *Molecular & general genetics : MGG,* vol. 263, no. 3, pp. 535-542.

Heckman, D.S., Geiser, D.M., Eidell, B.R., Stauffer, R.L., Kardos, N.L. & Hedges, S.B. 2001, "Molecular Evidence for the Early Colonization of Land by Fungi and Plants", *Science,* vol. 293, no. 5532, pp. 1129-1133.

Henderson, I.R., Deleris, A., Wong, W., Zhong, X., Chin, H.G., Horwitz, G.A., Kelly, K.A., Pradhan, S. & Jacobsen, S.E. 2010, "The De Novo Cytosine Methyltransferase DRM2 Requires Intact UBA Domains and a Catalytically Mutated Paralog DRM3 during RNA–Directed DNA Methylation in Arabidopsis thaliana", *PLoS Genetics,* vol. 6, no. 10, pp. e1001182.

Hillenmeyer, M.E., Fung, E., Wildenhain, J., Pierce, S.E., Hoon, S., Lee, W., Proctor, M., Onge, R.P.S., Tyers, M. & Koller, D. 2008, "The chemical genomic portrait of yeast: uncovering a phenotype for all genes", *Science,* vol. 320, no. 5874, pp. 362-365.

Honein, M., Paulozzi, L., Mathews, T., Erickson, J. & Wong, L. 2001, "Impact of Folic Acid Fortification of the US Food Supply on the Occurrence of Neural Tube Defects", *JAMA: The Journal of the American Medical Association,* vol. 285, no. 23.

Igari, S., Ohtaki, A., Yamanaka, Y., Sato, Y., Yohda, M., Odaka, M., Noguchi, K. & Yamada, K. 2011, "Properties and Crystal Structure of Methylenetetrahydrofolate Reductase from Thermus thermophilus HB8", *PloS one,* vol. 6, no. 8, pp. e23716.

Jackson, J.P., Lindroth, A.M., Cao, X. & Jacobsen, S.E. 2002, "Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase", *Nature,* vol. 416, no. 6880, pp. 556-560.

Janošík, M., Kery, V., Gaustadnes, M., Maclean, K.N. & Kraus, J.P. 2001, "Regulation of human cystathionine β-synthase by S-adenosyl-L-methionine: evidence for two catalytically active conformations involving an autoinhibitory domain in the C-terminal region", *Biochemistry,* vol. 40, no. 35, pp. 10625-10633.

Jauert, P.A., Jensen, L.E. & Kirkpatrick, D.T. 2005, "A novel yeast genomic DNA library on a geneticin-resistance vector", *Yeast,* vol. 22, no. 8, pp. 653-657.

Jeltsch, A. 2010, "Phylogeny of methylomes", *Science,* vol. 328, no. 5980, pp. 837-838.

Jesch, S., Zhao, X., Wells, M. & Henry, S. 2005, "Genome-wide Analysis Reveals Inositol, Not Choline, as the Major Effector of Ino2p-Ino4p and Unfolded Protein Response Target Gene Expression in Yeast", *Journal of Biological Chemistry,* vol. 280, no. 10.

Jia, D., Jurkowska, R.Z., Zhang, X., Jeltsch, A. & Cheng, X. 2007, "Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation", *Nature,* vol. 449, no. 7159, pp. 248-251.

Jones, E.W. & Fink, G.R. 1982, "Regulation of amino acid and nucleotide biosynthesis in yeast", *Cold Spring Harbor Monograph Archive,* vol. 11, no. 0, pp. 181-299.

Kagan, R.M. & Clarke, S. 1994, "Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes", *Archives of Biochemistry and Biophysics,* vol. 310, no. 2, pp. 417-427.

Kalisch, W.E. & Rasmuson, B. 1974, "Changes of zeste phenotype induced by autosomal mutations in Drosophila melanogaster", *Hereditas,* vol. 78, no. 1, pp. 97-103.

Kennedy, E.P. & Weiss, S.B. 1956, "The function of cytidine coenzymes in the biosynthesis of phospholipides", *Journal of Biological Chemistry,* vol. 222, no. 1, pp. 193.

Kim, Y.I. 2005, "5, 10-Methylenetetrahydrofolate Reductase Polymorphisms and Pharmacogenetics: A New Role of Single Nucleotide Polymorphisms in the Folate Metabolic Pathway in Human Health and Disease", *Nutrition reviews,* vol. 63, no. 11, pp. 398-407.

Kirmizis, A., Santos-Rosa, H., Penkett, C., Singer, M., Vermeulen, M., Mann, M., Bahler, J., Green, R. & Kouzarides, T. 2007, "Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation", *Nature,* vol. 449, no. 7164, pp. 928-932.

Klose, R.J., Gardner, K.E., Liang, G., Erdjument-Bromage, H., Tempst, P. & Zhang, Y. 2007, "Demethylation of Histone H3K36 and H3K9 by Rph1: a Vestige of an H3K9 Methylation System in Saccharomyces cerevisiae?", *Molecular and cellular biology,* vol. 27, no. 11, pp. 3951-3961.

Kodaki, T. & Yamashita, S. 1987, "Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes.", *The Journal of biological chemistry,* vol. 262, no. 32, pp. 15428-15435.

Kouzarides, T. 2007, "Chromatin modifications and their function", *Cell,* vol. 128, no. 4, pp. 693-705.

Kuras, L., Rouillon, A., Lee, T., Barbey, R., Tyers, M. & Thomas, D. 2002, "Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment", *Molecular cell,* vol. 10, no. 1, pp. 69-80.

Larsson, J. & Rasmuson-Lestander, A. 1994, "Molecular cloning of the S-adenosylmethionine synthetase gene in Drosophila melanogaster", *FEBS letters,* vol. 342, no. 3, pp. 329-333.

Lee, T.A., Jorgensen, P., Bognar, A.L., Peyraud, C., Thomas, D. & Tyers, M. 2010, "Dissection of combinatorial control by the Met4 transcriptional complex", *Molecular biology of the cell,* vol. 21, no. 3, pp. 456-469.

Liu, C.L., Kaplan, T., Kim, M., Buratowski, S., Schreiber, S.L., Friedman, N. & Rando, O.J. 2005, "Single-nucleosome mapping of histone modifications in S. cerevisiae", *PLoS biology,* vol. 3, no. 10, pp. e328.

Marini, N.J., Gin, J., Ziegle, J., Keho, K.H., Ginzinger, D., Gilbert, D.A. & Rine, J. 2008, "The prevalence of folate-remedial MTHFR enzyme variants in humans", *Proceedings of the National Academy of Sciences,* vol. 105, no. 23, pp. 8055.

Matthews, R.G., Vanoni, M.A., Hainfeld, J.F. & Wall, J. 1984, "Methylenetetrahydrofolate reductase. Evidence for spatially distinct subunit domains obtained by scanning

transmission electron microscopy and limited proteolysis", *Journal of Biological Chemistry,* vol. 259, no. 19, pp. 11647-11650.

Mayfield, J.A., Davies, M.W., Dimster-Denk, D., Pleskac, N., McCarthy, S., Boydston, E.A., Fink, L., Lin, X.X., Narain, A.S. & Meighan, M. 2012, "Surrogate Genetics and Metabolic Profiling for Characterization of Human Disease Alleles", *Genetics,* .

McGraw, P. & Henry, S.A. 1989, "Mutations in the Saccharomyces cerevisiae opi3 gene: effects on phospholipid methylation, growth and cross-pathway regulation of inositol synthesis", *Genetics,* vol. 122, no. 2, pp. 317-330.

Menant, A., Barbey, R. & Thomas, D. 2006, "Substrate-mediated remodeling of methionine transport by multiple ubiquitin-dependent mechanisms in yeast cells", *EMBO J,* vol. 25, no. 19, pp. 4436-4447.

Menant, A., Baudouin-Cornu, P., Peyraud, C., Tyers, M. & Thomas, D. 2006, "Determinants of the ubiquitin-mediated degradation of the Met4 transcription factor", *The Journal of biological chemistry,* vol. 281, no. 17, pp. 11744-11754.

Misra, S.K. & Bhakuni, V. 2003, "Unique holoenzyme dimers of the tetrameric enzyme Escherichia coli methylenetetrahydrofolate reductase: characterization of structural features associated with modulation of the enzyme's function", *Biochemistry,* vol. 42, no. 13, pp. 3921-3928.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E. & Simon, J.A. 2002, "Histone Methyltransferase Activity of a *Drosophila* Polycomb Group Repressor Complex", *Cell,* vol. 111, no. 2, pp. 197-208.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. & Grewal, S.I.S. 2001, "Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly", *Science,* vol. 292, no. 5514, pp. 110-113.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G. & Marton, M.J. 2001, "Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast", *Molecular and cellular biology,* vol. 21, no. 13, pp. 4347-4368.

Naula, N., Walther, C., Baumann, D. & Ernst Schweingruber, M. 2002, "Two non-complementing genes encoding enzymatically active methylenetetrahydrofolate reductases control methionine requirement in fission yeast Schizosaccharomyces pombe", *Yeast,* vol. 19, no. 10, pp. 841-848.

Ng, H.H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y. & Struhl, K. 2002, "Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association", *Genes & development,* vol. 16, no. 12, pp. 1518-1527.

Ng, H.H., Robert, F., Young, R.A. & Struhl, K. 2003, "Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity", *Molecular cell,* vol. 11, no. 3, pp. 709-719.

Noma, K. & Grewal, S.I.S. 2002, "Histone H3 lysine 4 methylation is mediated by Set1 and promotes maintenance of active chromatin states in fission yeast", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. Suppl 4, pp. 16438.

Ouni, I., Flick, K. & Kaiser, P. 2010, "A transcriptional activator is part of an SCF ubiquitin ligase to control degradation of its cofactors", *Molecular cell,* vol. 40, no. 6, pp. 954-964.

Papp, B. & Müller, J. 2006, "Histone trimethylation and the maintenance of transcriptional ONand OFF states by trxG and PcG proteins", *Genes & development,* vol. 20, no. 15, pp. 2041.

Pejchal, R. & Ludwig, M.L. 2004, "Cobalamin-independent methionine synthase (MetE): a face-to-face double barrel that evolved by gene duplication", *PLoS biology,* vol. 3, no. 2, pp. e31.

Peters, A.H.F.M., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A.H.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H.A. & Jenuwein, T. 2003, "Partitioning and Plasticity of Repressive Histone Methylation States in Mammalian Chromatin", *Molecular cell,* vol. 12, no. 6, pp. 1577-1589.

Prentice, H.L. 1992, "High efficiency transformation of Schizosaccharomyces pombe by electroporation", *Nucleic acids research,* vol. 20, no. 3, pp. 621-621.

Rafalski, J.A. & Falco, S.C. 1988, "Structure of the yeast HOM3 gene which encodes aspartokinase", *The Journal of biological chemistry,* vol. 263, no. 5, pp. 2146-2151.

Raymond, R.K., Kastanos, E.K. & Appling, D.R. 1999, "Saccharomyces cerevisiae expresses two genes encoding isozymes of methylenetetrahydrofolate reductase", *Archives of Biochemistry and Biophysics,* vol. 372, no. 2, pp. 300-308.

Rébora, K., Laloo, B. & Daignan-Fornier, B. 2005, "Revisiting Purine-Histidine Cross-Pathway Regulation in Saccharomyces cerevisiae", *Genetics,* vol. 170, no. 1, pp. 61-70.

Reineke, L.C., Komar, A.A., Caprara, M.G. & Merrick, W.C. 2008, "A small stem loop element directs internal initiation of the URE2 internal ribosome entry site in Saccharomyces cerevisiae", *The Journal of biological chemistry,* vol. 283, no. 27, pp. 19011-19025.

Ringrose, L. & Paro, R. 2004, "Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins", *Annu.Rev.Genet.,* vol. 38, pp. 413-443.

Roberts, C. & Selker, E. 1995, "Mutations affecting the biosynthesis of S-adenosylmethionine cause reduction of DNA methylation in Neurospora crassa", *Nucleic acids research,* vol. 23, no. 23.

Roguev, A., Schaft, D., Shevchenko, A., Pijnappel, W.W.M.P., Wilm, M., Aasland, R. & Stewart, A.F. 2001, "The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4", *The EMBO journal,* vol. 20, no. 24, pp. 7137-7148.

Roje, S., Chan, S.Y., Kaplan, F., Raymond, R.K., Horne, D.W., Appling, D.R. & Hanson, A.D. 2002, "Metabolic Engineering in Yeast Demonstrates ThatS-Adenosylmethionine Controls Flux through the Methylenetetrahydrofolate Reductase Reaction in Vivo", *Journal of Biological Chemistry,* vol. 277, no. 6, pp. 4056.

Rouillon, A., Barbey, R., Patton, E.E., Tyers, M. & Thomas, D. 2000, "Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCFMet30 complex", *EMBO J,* vol. 19, no. 2, pp. 282-294.

Rusche, L.N., Kirchmaier, A.L. & Rine, J. 2003, "The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae", *Annual Review of Biochemistry,* vol. 72, no. 1, pp. 481-516.

Sawada, K., Yang, Z., Horton, J.R., Collins, R.E., Zhang, X. & Cheng, X. 2004, "Structure of the conserved core of the yeast Dot1p, a nucleosomal histone H3 lysine 79 methyltransferase", *Journal of Biological Chemistry,* vol. 279, no. 41, pp. 43296-43306.

Scannell, D.R., Butler, G. & Wolfe, K.H. 2007, "Yeast genome evolution—the origin of the species", *Yeast,* vol. 24, no. 11, pp. 929-942.

Scannell, D., Zill, O., Rokas, A., Payen, C., Dunham, M., Eisen, M., Rine, J., Johnston, M. & Hittinger, C. 2011, "The Awesome Power of Yeast Evolutionary Genetics: New Genome Sequences and Strain Resources for the Saccharomyces sensu stricto Genus", *G3: Genes, Genomes, Genetics,* vol. 1, no. 1.

Shan, X., Wang, L., Hoffmaster, R. & Kruger, W.D. 1999, "Functional characterization of human methylenetetrahydrofolate reductase in Saccharomyces cerevisiae", *Journal of Biological Chemistry,* vol. 274, no. 46, pp. 32613-32618.

Sienko, M., Natorff, R., Zielinski, Z., Hejduk, A. & Paszewski, A. 2007, "Two Aspergillus nidulans genes encoding methylenetetrahydrofolate reductases are up-regulated by homocysteine", *Fungal Genetics and Biology,* vol. 44, no. 7, pp. 691-700.

Sipiczki, M. 2000, "Where does fission yeast sit on the tree of life", *Genome Biol,* vol. 1, no. 2, pp. 1011.1-1011.4.

Smith, K., Kothe, G., Matsen, C., Khlafallah, T., Adhvaryu, K., Hemphill, M., Freitag, M., Motamedi, M. & Selker, E. 2008, "The fungus Neurospora crassa displays telomeric silencing mediated by multiple sirtuins and by methylation of histone H3 lysine 9", *Epigenetics and Chromatin,* vol. 1, pp. 5.

Strahl, B.D., Grant, P.A., Briggs, S.D., Sun, Z.W., Bone, J.R., Caldwell, J.A., Mollah, S., Cook, R.G., Shabanowitz, J., Hunt, D.F. & Allis, C.D. 2002, "Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression", *Molecular and cellular biology,* vol. 22, no. 5, pp. 1298-1306.

Suh, J.R., Herbig, A.K. & Stover, P.J. 2001, "New perspectives on folate catabolism", *Annual Review of Nutrition,* vol. 21, no. 1, pp. 255-282.

Suliman, H.S., Sawyer, G.M., Appling, D.R. & Robertus, J.D. 2005, "Purification and properties of cobalamin-independent methionine synthase from Candida albicans and Saccharomyces cerevisiae", *Archives of Biochemistry and Biophysics,* vol. 441, no. 1, pp. 56-63.

Summers, E.F., Letts, V.A., McGraw, P. & Henry, S.A. 1988, "Saccharomyces cerevisiae cho2 mutants are deficient in phospholipid methylation and cross-pathway regulation of inositol synthesis", *Genetics,* vol. 120, no. 4, pp. 909-922.

Sze, J.Y., Woontner, M., Jaehning, J.A. & Kohlhaw, G.B. 1992, "In vitro transcriptional activation by a metabolic intermediate: activation by Leu3 depends on alpha-isopropylmalate", *Science,* vol. 258, no. 5085, pp. 1143-1145.

Takezawa, K., Okamoto, I., Okamoto, W., Takeda, M., Sakai, K., Tsukioka, S., Kuwata, K., Yamaguchi, H., Nishio, K. & Nakagawa, K. 2011, "Thymidylate synthase as a determinant of pemetrexed sensitivity in non-small cell lung cancer", *British journal of cancer,* vol. 104, no. 10, pp. 1594-1601.

Tamaru, H. & Selker, E.U. 2001, "A histone H3 methyltransferase controls DNA methylation in Neurospora crassa", *Nature,* vol. 414, no. 6861, pp. 277-283.

Thomas, D. & Surdin-Kerjan, Y. 1997, "Metabolism of sulfur amino acids in Saccharomyces cerevisiae.", *Microbiology and Molecular Biology Reviews,* vol. 61, no. 4, pp. 503-532.

Thomas, D., Kuras, L., Barbey, R., Cherest, H., Blaiseau, P.L. & Surdin-Kerjan, Y. 1995, "Met30p, a yeast transcriptional inhibitor that responds to S- adenosylmethionine, is an essential protein with WD40 repeats", *Molecular and cellular biology,* vol. 15, no. 12, pp. 6526-6534.

Thomas, D. & Surdin-Kerjan, Y. 1991, "The synthesis of the two S-adenosyl-methionine synthetases is differently regulated in *Saccharomyces cerevisiae*", *Molecular and General Genetics MGG,* vol. 226, no. 1, pp. 224-232.

Tolarova, M. & Harris, J. 1995, "Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins", *Teratology,* vol. 51, no. 2, pp. 71-78.

Tollervey, D., Lehtonen, H., Carmo-Fonseca, M. & Hurt, E.C. 1991, "The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast", *The EMBO journal,* vol. 10, no. 3, pp. 573.

Townsend, D.M., Tew, K.D. & Tapiero, H. 2004, "Sulfur containing amino acids and human disease", *Biomedicine & Pharmacotherapy,* vol. 58, no. 1, pp. 47-55.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G. & Reuter, G. 1994, "The protein encoded by the Drosophila position-effect variegation suppressor gene Su (var) 3-9 combines domains of antagonistic regulators of homeotic gene complexes", *The EMBO journal,* vol. 13, no. 16, pp. 3822.

van Leeuwen, F., Gafken, P.R. & Gottschling, D.E. 2002, "Dot1p modulates silencing in yeast by methylation of the nucleosome core", *Cell,* vol. 109, no. 6, pp. 745-756.

Veerappan, C.S., Avramova, Z. & Moriyama, E.N. 2008, "Evolution of SET-domain protein families in the unicellular and multicellular Ascomycota fungi", *BMC evolutionary biology,* vol. 8, no. 1, pp. 190.

Venkatasubrahmanyam, S., Hwang, W.W., Meneghini, M.D., Tong, A.H.Y. & Madhani, H.D. 2007, "Genome-wide, as opposed to local, antisilencing is mediated redundantly by the euchromatic factors Set1 and H2A.Z", *Proceedings of the National Academy of Sciences,* vol. 104, no. 42, pp. 16609-16614.

Wald, N., Sneddon, J., Densem, J., Frost, C. & Stone, R. 1991, "Prevention of neural tube defects: results of the Medical Research Council Vitamin Study", *Lancet,* vol. 338, no. 8760, pp. 131-137.

Xiao, B., Jing, C., Wilson, J.R., Walker, P.A., Vasisht, N., Kelly, G., Howell, S., Taylor, I.A., Blackburn, G.M. & Gamblin, S.J. 2003, "Structure and catalytic mechanism of the human histone methyltransferase SET7/9", *Nature,* vol. 421, no. 6923, pp. 652-656.

Yamada, K., Chen, Z., Rozen, R. & Matthews, R.G. 2001, "Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase", *Proceedings of the National Academy of Sciences,* vol. 98, no. 26, pp. 14853.

Yokoyama, A., Wang, Z., Wysocka, J., Sanyal, M., Aufiero, D.J., Kitabayashi, I., Herr, W. & Cleary, M.L. 2004, "Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate Hox gene expression", *Molecular and cellular biology,* vol. 24, no. 13, pp. 5639-5649.

Zhang, X., Bernatavichute, Y.V., Cokus, S., Pellegrini, M. & Jacobsen, S.E. 2009, "Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in Arabidopsis thaliana", *Genome biology,* vol. 10, no. 6, pp. R62.

Zill, O.A., Scannell, D., Teytelman, L. & Rine, J. 2010, "Co-evolution of transcriptional silencing proteins and the DNA elements specifying their assembly", *PLoS biology,* vol. 8, no. 11, pp. e1000550.