

Multiple inputs control sulfur-containing amino acid synthesis in *Saccharomyces cerevisiae*

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ABSTRACT In *Saccharomyces cerevisiae*, transcription of the *MET* regulon, which encodes 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 repression is accomplished by ubiquitination of the transcription factor Met4, which is carried out by the SCF(Met30) E3 ubiquitin ligase. Mutants defective in *MET* regulon repression reveal that loss of Cho2, which is required for the methylation of phosphatidylethanolamine to produce phosphatidylcholine, leads to induction of the *MET* regulon. This induction is due to reduced cysteine synthesis caused by the Cho2 defects, uncovering an important link between phospholipid synthesis and cysteine synthesis. Antimorphic mutants in *S*-adenosyl-methionine (SAM) synthetase genes also induce the *MET* regulon. This effect is due, at least in part, to SAM deficiency controlling the *MET* regulon independently of SAM's contribution to cysteine synthesis. Finally, the Met30 protein is found in two distinct forms whose relative abundance is controlled by the availability of sulfur-containing amino acids. This modification could be involved in the nutritional control of SCF(Met30) activity toward Met4.

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INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is capable of synthesizing the sulfur-containing amino acids methionine and cysteine de novo and can also synthesize each from the other (Figure 1A; Thomas and Surdin-Kerjan, 1997). The transcription of the genes encoding proteins involved in the synthesis of sulfur-containing amino acids, known as the *MET* regulon, rapidly shifts from a low basal level of transcription to a high induced level if methionine and cysteine are limiting for growth. This induction is mediated by the transcription factor Met4. Unlike most transcription factors of yeast, Met4 does not bind DNA directly. Instead, Met4 is recruited to specific sites in the regulatory region of genes by direct interactions with DNA-binding proteins. One of these is Cbf1, which uses a basic

helix-loop-helix binding domain. Other recruiters of Met4 are Met31 and Met32, two highly similar zinc finger-domain proteins with highly similar binding sites.

The sites bound by Cbf1, Met31, and Met32 have been mapped genome wide in *S. cerevisiae* (Lee *et al.*, 2010). Many *MET* regulon genes have binding sites for both Cbf1 and Met31/Met32 in their promoters, although several genes have binding site(s) for only one or the other. Genes encoding proteins involved in the uptake of sulfur and its assimilation into amino acids are more likely to have the Cbf1 binding site(s) in their promoters, whereas genes encoding proteins involved in the uptake of methionine, *S*-adenosyl-methionine (SAM), and synthesis of SAM from methionine are more likely to be regulated by Met31/Met32 independently of Cbf1.

The synthesis of a third important metabolite, SAM, is coregulated with methionine and cysteine. SAM is required for essentially all methylation reactions in the cell, including methylation of proteins, nucleic acids, and lipids (Cantoni, 1977). Every donation of a methyl from SAM produces a molecule of *S*-adenosyl-homocysteine (SAH). Moreover, the methionine-to-cysteine conversion requires this hydrolysis of SAM to SAH (Figure 1A), from which cysteine can be made in three enzymatic steps.

The identity of the metabolites sensed to control the *MET* regulon remains elusive. If the synthesis of SAM from methionine is blocked by deletion of *SAM1* and *SAM2*, methionine supplementation no longer represses the *MET* regulon (Thomas *et al.*, 1988).

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Abbreviations used: SAH, *S*-adenosyl-homocysteine; SAM, *S*-adenosyl-methionine.

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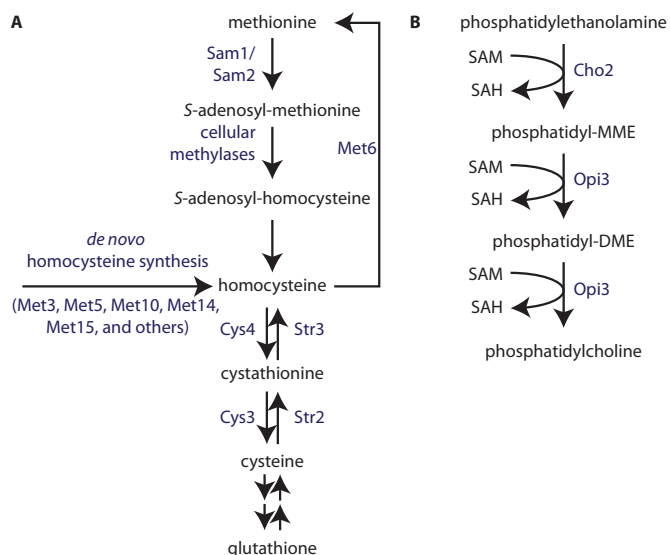


FIGURE 1: Metabolic pathways. (A) Synthesis of sulfur-containing amino acids in *S. cerevisiae*. The conversion of SAM to SAH occurs through myriad cellular methylation reactions, of which only three are shown in B. (B) Synthesis of phosphatidylcholine in *S. cerevisiae*. In addition, ethanolamine, monomethylethanolamine, dimethylethanolamine, and choline can be converted to phosphatidylethanolamine, phosphatidylmonomethylethanolamine (phosphatidyl-MME), phosphatidyl dimethylethanolamine (phosphatidyl-DME), and phosphatidylcholine, respectively, through the action of the Kennedy pathway (not shown).

Thus deficiency of SAM or a metabolite derived from SAM, including possible methylated molecules, induces the *MET* regulon. If the enzymes required to synthesize cysteine from homocysteine, Cys3 or Cys4, are limiting for growth, the *MET* regulon is highly transcribed even when grown in medium with abundant methionine (Hansen and Johannesen, 2000; Menant *et al.*, 2006). This result suggests that cysteine deficiency, or the deficiency of some compound synthesized from cysteine, is sufficient to induce *MET* regulon transcription independently of methionine or SAM levels. Because the synthesis of cysteine from exogenously provided methionine passes through SAM as an intermediate, it was possible that induction of the *MET* regulon in *sam1Δ sam2Δ* cells was also due to cysteine deficiency. Indeed, *MET* regulon expression is reduced in *sam1Δ sam2-12* cells provided with homocysteine, a direct precursor to cysteine (Hansen and Johannesen, 2000). Nonetheless, the repression of the *MET* regulon by homocysteine supplementation in these cells does not achieve the low basal level of wild-type cells. Thus, although cysteine clearly has a role in *MET* regulon repression, the roles of SAM and other metabolites need explanation.

Inactivation of Met4 when sulfur-containing amino acids are plentiful requires its ubiquitination (Rouillon *et al.*, 2000), which is also the case for Met31, Met32, and Cbf1 (Ouni *et al.*, 2010). Ubiquitination can affect Met4 in either of two ways. Depending on the nutritional state, Met4 ubiquitination can lead to its degradation, or ubiquitinated Met4 can remain stable with altered activity (Kuras *et al.*, 2002). Met4 is ubiquitinated by the ubiquitin ligase complex SCF(Met30), in which the F-box subunit Met30 is responsible for targeting SCF(Met30) to Met4. How these ubiquitination events are confined to conditions of sulfur-containing amino acid sufficiency is unknown.

In this study, a genetic screen for mutants affecting transcription of the *MET* regulon uncovered a homeostatic link between phospholipid biosynthesis and the *MET* regulon. The screen also

uncovered several unusual dominant mutants in SAM synthesis and led to an investigation of how the Met30 protein functioned in control of the *MET* regulon.

RESULTS

To understand how the expression of the *MET* regulon is regulated, we performed a genetic screen to uncover mutants that fail to down-regulate *MET* regulon expression in the presence of adequate methionine in the growth medium. Cells with green fluorescent protein (GFP) fusions to the *MET3*, *MET5*, *MET6*, *MET10*, and *MET14* genes were evaluated to find a suitable reporter of *MET* regulon activation. *MET3* was chosen, as it had >80-fold dynamic range of expression as a function of methionine level, and its expression in the repressed state was the lowest tested (Supplemental Figure S1). The *MET3* promoter contains binding sites for both Cbf1 and Met31/Met32 (Lee *et al.*, 2010). Independent cultures of cells carrying *MET3* fused in-frame with *GFP* integrated at the *MET3* locus (denoted *MET3-GFP*) were mutagenized with ethyl methanesulfonate (EMS) or ultraviolet radiation and grown on plates containing 1 mM methionine, a level sufficient to repress the *MET* regulon in wild-type cells. These plates were then scanned for mutant colonies expressing *MET3-GFP* despite the presence of methionine. To protect against the possibility that overproduction of methionine or cysteine in such a derepressed mutant might cause sickness or lethality, we mutagenized two different strains. One strain was a methionine auxotroph due to deletion of the *MET15* gene (JRY9356), which is required for de novo synthesis of homocysteine, the precursor to both methionine and cysteine (Figure 1A). The other had a *lys2Δ* mutation instead of the *met15Δ* mutation and hence was a methionine prototroph (JRY9355).

Approximately 10,000 colonies from 12 independent mutageneses were screened for *MET3-GFP* expression, leading to the isolation of 34 mutants. Complementation group assignments were only partially successful, as many of the mutants were partially dominant. Several strategies were used to identify the causative mutations. As a test of the screen, the *MET30* gene was directly sequenced in several mutants, as the original *met30* mutants were found using a similar approach (Thomas *et al.*, 1995). One of the mutants had a mutation in *MET30* that changed cysteine 95 to tyrosine. When recreated in an otherwise unmutagenized strain, this mutation led to greater-than-fivefold increased expression of *MET3* as measured by quantitative real-time PCR (qRT-PCR; see later discussion), giving confidence in the screen.

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

For a subset of mutants, plasmid-library complementation was used to determine causative mutations. This strategy identified a mutation in *CHO2* in a mutant derived from the *met15Δ* parent strain (JRY9356). Cho2 is a methyltransferase that carries out the first of three methylations involved in phosphatidylcholine synthesis from phosphatidylethanolamine (Kodaki and Yamashita, 1987; Summers *et al.*, 1988). The recovered nonsense mutation (*cho2-K165Stop*) shortened the 870-amino acid native protein to a 164-amino acid truncated form. Similar truncation mutations of *CHO2* completely remove Cho2 function (Summers *et al.*, 1988). Thus the *MET3* overexpression phenotype likely resulted from loss of Cho2 function rather than gain of a novel function. Indeed, deletion of *CHO2* in the parental strain caused the same phenotype (Figure 2A), confirming the causality of the *cho2-K165Stop* allele for elevated *MET3* expression. The expression was somewhat higher in the nonsense than the deletion mutant, which could

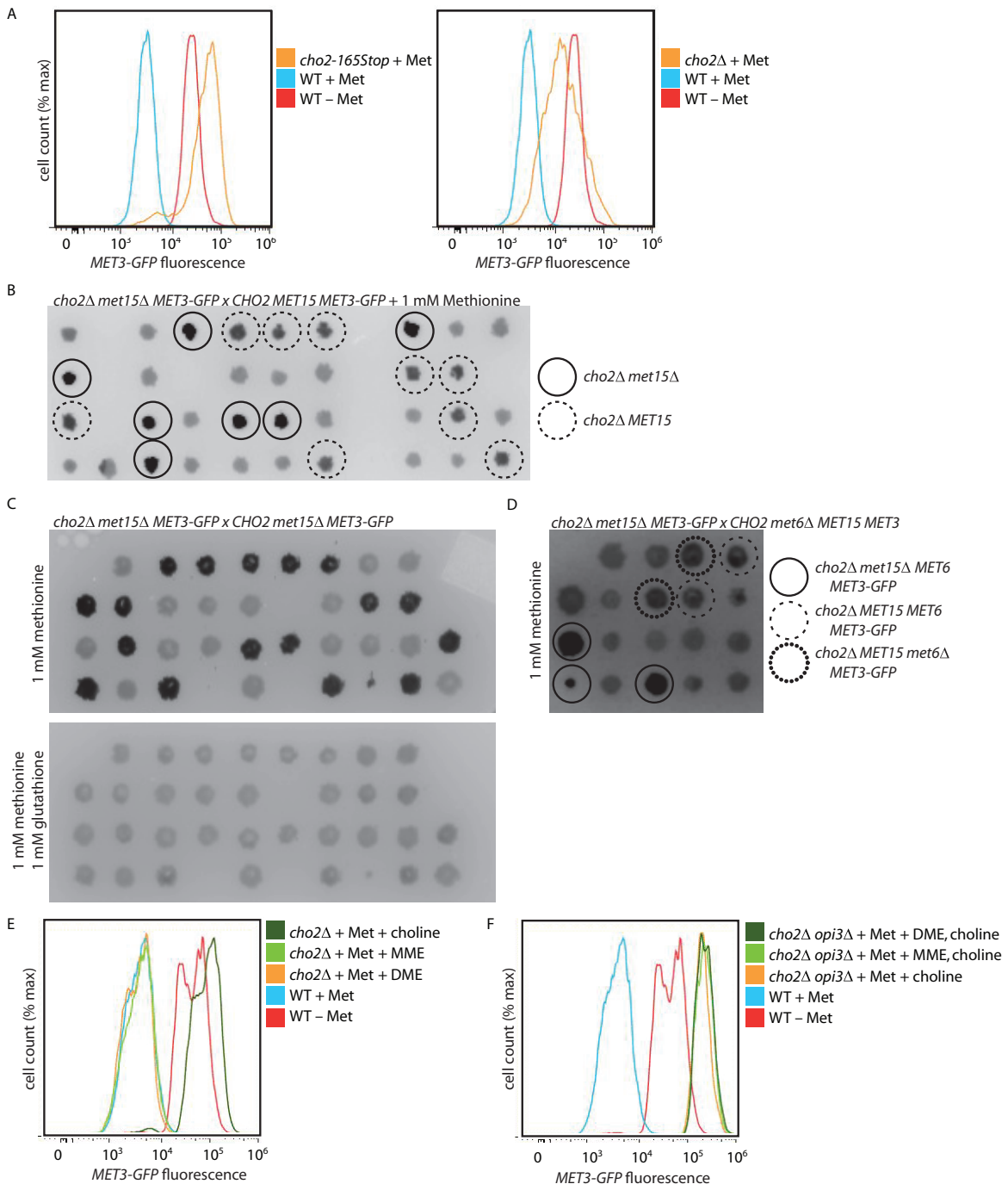


FIGURE 2: CHO2 mutations induce MET3-GFP All histograms are of GFP fluorescence from cells carrying *MET3-GFP* under its native promoter, on a log₁₀ scale, as determined by flow cytometry. For all histograms, WT refers to a methionine prototrophic (*MET15*) strain with *MET3-GFP* (JRY9355), grown overnight with or without methionine as indicated. (A) *MET3-GFP* fluorescence in *met15Δ* cells carrying a nonsense mutation in *CHO2* (JRY9359; left) or a complete deletion of *CHO2* (JRY9360; right), grown in media containing 134 μM (20 μg/ml) methionine. (B) Fluorescence imaging of a tetrad dissection of a *cho2Δ/CHO2 met15Δ/MET15 MET3-GFP/MET3-GFP* diploid grown on plates with 1 mM methionine. The darkness of a colony corresponds to its intensity of GFP fluorescence. Colonies with a heavy circle are *met15Δ*, whereas colonies with a dotted circle are *MET15*. (C) Fluorescence imaging of a tetrad dissection of *cho2Δ met15Δ* and *CHO2 met15Δ* colonies with *MET3-GFP*, replica plated to a plate with 1 mM methionine and no glutathione (top) or a plate with 1 mM methionine + 1 mM glutathione (bottom). (D) Fluorescence imaging of a tetrad dissection of *cho2Δ met15Δ MET3-GFP* (JRY9360) crossed to *met6Δ* from the MAT α Yeast Knockout Collection grown on plates with 1 mM methionine. (E) *MET3-GFP* fluorescence in *cho2Δ* cells with nutritional remediation by compounds that form phospholipids downstream of Cho2's action. MME, monomethylethanolamine; DME, dimethylethanolamine. (F) *MET3-GFP* fluorescence in *cho2Δ opi3Δ* cells (JRY9361) with nutritional remediation by compounds that form phospholipids downstream of Cho2's action. (The *cho2Δ opi3Δ* cells were fed choline in addition to the pathway intermediates, as they required choline for growth.)

suggest that the truncated allele somehow disrupted the activity of Opi3 (see later discussion).

The *cho2-K165Stop met15Δ MET3-GFP* mutant was crossed to the *MET15 MET3-GFP* strain (JRY9355), and the resultant diploid, which had a near-wild-type *MET3-GFP* expression phenotype, was sporulated and dissected. If the mutation in *CHO2* were solely responsible for the *MET3-GFP* induction, then two colonies from each tetrad would have high *MET3-GFP* induction and two colonies would have no *MET3-GFP* induction, corresponding to segregants inheriting *cho2-K165Stop* and *CHO2*, respectively. Qualitatively, *MET3-GFP* induction segregated 2:2 in each of 13 tetrads analyzed. Quantitatively, among the colonies showing *MET3-GFP* induction there were two classes: half of these colonies had high *MET3-GFP* induction and half had lower, but detectable, induction (Figure 2B). These two classes corresponded to colonies inheriting *met15Δ* and *MET15*, respectively, suggesting that full induction of *MET3-GFP* in cells compromised for *CHO2* required a defect in homocysteine or methionine biosynthesis, caused in this cross by *met15Δ*. This explanation was confirmed in a similar cross in which the *met15Δ* mutation was homozygous and high-level *MET3-GFP* induction segregated 2:2 in each of six complete tetrads analyzed (Figure 2C, top).

Cho2, also known as phosphatidylethanolamine methyltransferase, catalyzes conversion of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, coupled to the consumption of SAM, producing SAH (Figure 1B). This reaction is the first of three methylation reactions required for phosphatidylcholine synthesis from phosphatidylethanolamine, all of which involve consumption of one SAM molecule and production of one SAH molecule. Because Cho2 performs the first step in phosphatidylcholine synthesis, compromising its activity should reduce its consumption of SAM and also compromise the further consumption of SAM by Opi3, which performs the second and third methylations in phosphatidylcholine synthesis (Kodaki and Yamashita, 1987; McGraw and Henry, 1989). Phosphatidylcholine constitutes approximately half of the phospholipids in *S. cerevisiae* (Summers *et al.*, 1988), so its synthesis may consume a significant fraction of the SAM in the cell and hence produce a significant fraction of the SAH in the cell. Therefore cells with compromised Cho2 may have insufficient levels of SAH to support cysteine synthesis from the exogenously available methionine, as each molecule of cysteine produced from methionine requires a conversion of SAM to SAH (Figure 1A). Furthermore, impaired cysteine synthesis induces the *MET* genes (Hansen and Johannesen, 2000; Menant *et al.*, 2006). To test the possibility that compromised Cho2 function induces *MET3-GFP* by causing cysteine deficiency, we tested the effect of exogenous cysteine in the form of glutathione on *MET3-GFP* expression in *cho2Δ* cells (glutathione is readily taken up by *S. cerevisiae* and used as a cysteine source; Mayfield *et al.*, 2012). When added to plates with 1 mM methionine, 1 mM glutathione completely restored *MET3-GFP* expression in *cho2Δ met15Δ* colonies to its low basal level (Figure 2C). Thus the *MET3-GFP* induction more pronounced in *met15Δ* cells than in *MET15* cells because sufficient cysteine was synthesized through de novo homocysteine synthesis in *MET15* cells to repress *MET3-GFP*. Indeed, deletion of *MET6*, which causes a defect in methionine synthesis without compromising de novo homocysteine synthesis (Figure 1A), did not cause greater induction of *MET3-GFP* in a *cho2Δ* background, unlike deletion of *MET15* (Figure 2D).

Monomethylethanolamine can be converted to phosphatidylmonomethylethanolamine by the Kennedy pathway (Kennedy and Weiss, 1956). Phosphatidylmonomethylethanolamine can be methylated by Opi3 to produce first phosphatidylmethylethanolamine

and then, with an additional methylation, phosphatidylcholine, producing two molecules of SAH for every phosphatidylcholine produced. If cells with compromised Cho2 induced *MET3-GFP* due to insufficient SAM consumption and SAH production, then providing these cells with exogenous monomethylethanolamine should reduce the expression of *MET3-GFP*, as the SAH produced by Opi3 could be used for cysteine synthesis. *cho2Δ MET3-GFP* cells were grown with monomethylethanolamine, dimethylethanolamine, or choline. Both monomethylethanolamine and dimethylethanolamine were able to restore low basal expression of *MET3-GFP*, whereas choline was not (Figure 2E). This result was consistent with cysteine levels being sensed in *cho2Δ* cells in controlling the *MET* regulon, as monomethylethanolamine and dimethylethanolamine are both substrates for methylation by Opi3, whereas choline is not. In definitive support of this hypothesis, in contrast to the result with the *cho2Δ MET3-GFP* cells, supplementation of *opi3Δ cho2Δ MET3-GFP* cells with monomethylethanolamine or dimethylethanolamine no longer down-regulated *MET3-GFP* expression (Figure 2F). Thus the capacity of monomethylethanolamine and dimethylethanolamine to reduce *MET3-GFP* expression in *cho2Δ* cells was through their capacity to be methylated by Opi3, producing SAH, and supporting cysteine synthesis in the process. Indeed the induction of *MET3-GFP* expression in the *cho2Δ opi3Δ* double mutants was even greater than in the *cho2Δ* mutant. Therefore the induction of *MET3-GFP* in *cho2Δ* cells was due to cysteine deficiency through insufficient phospholipid methylation.

SAM deficiency also induces MET3-GFP

To identify the causative mutations from additional mutants, we used a strategy of deep sequencing of pooled segregants that circumvented the difficulty of identifying dominant mutations by classic approaches (Birkeland *et al.*, 2010). Briefly, mutants were backcrossed to the wild-type parent and dissected. For four mutants from the screen, each tetrad contained two mutant and two wild-type segregants, and thus the mutant phenotype was caused by a single mutation. For each mutant, the mutation-containing progeny from five to seven tetrads were pooled (Figure 3A), as were the wild-type progeny in a separate pool. The genomes of these pools were sequenced and examined for mutations specific to the mutant pool.

Although many mutations were identified in each pair of mutant and wild-type pools relative to the reference genome, each pair uncovered only one high-confidence mutation that was exclusively in the mutant pool, consistent with the 2:2 segregation observed in the tetrad dissections. Three of the mutants had mutations in *SAM1*: *SAM1-G250S* and *SAM1-G310D*, which was uncovered twice, from two independent mutageneses. One mutant had a mutation in *SAM2*: *SAM2-C93Y*. Because the *SAM1-G310D* allele had the strongest phenotype and was recovered twice, it is the focus of the following analyses. To determine independently whether the *SAM1-G310D* mutation caused the induced expression of *MET3-GFP*, we expressed the mutated and wild-type *SAM1* alleles on plasmids in *sam1Δ* cells carrying *MET3-GFP*. Cells with *SAM1-G310D* induced *MET3-GFP*, whereas those with wild-type *SAM1* did not (Figure 3B).

SAM1 and *SAM2* both encode SAM synthetase genes (Thomas *et al.*, 1988) and are paralogues, produced by the ancient hemiascomycete whole-genome duplication (Wolfe and Shields, 1997). Neither gene is essential, but strains lacking both genes require exogenous SAM to survive; thus both genes individually encode functional SAM synthetase. Thus the mutations recovered here were considered “dominant” mutations, as only one of the two *SAM*

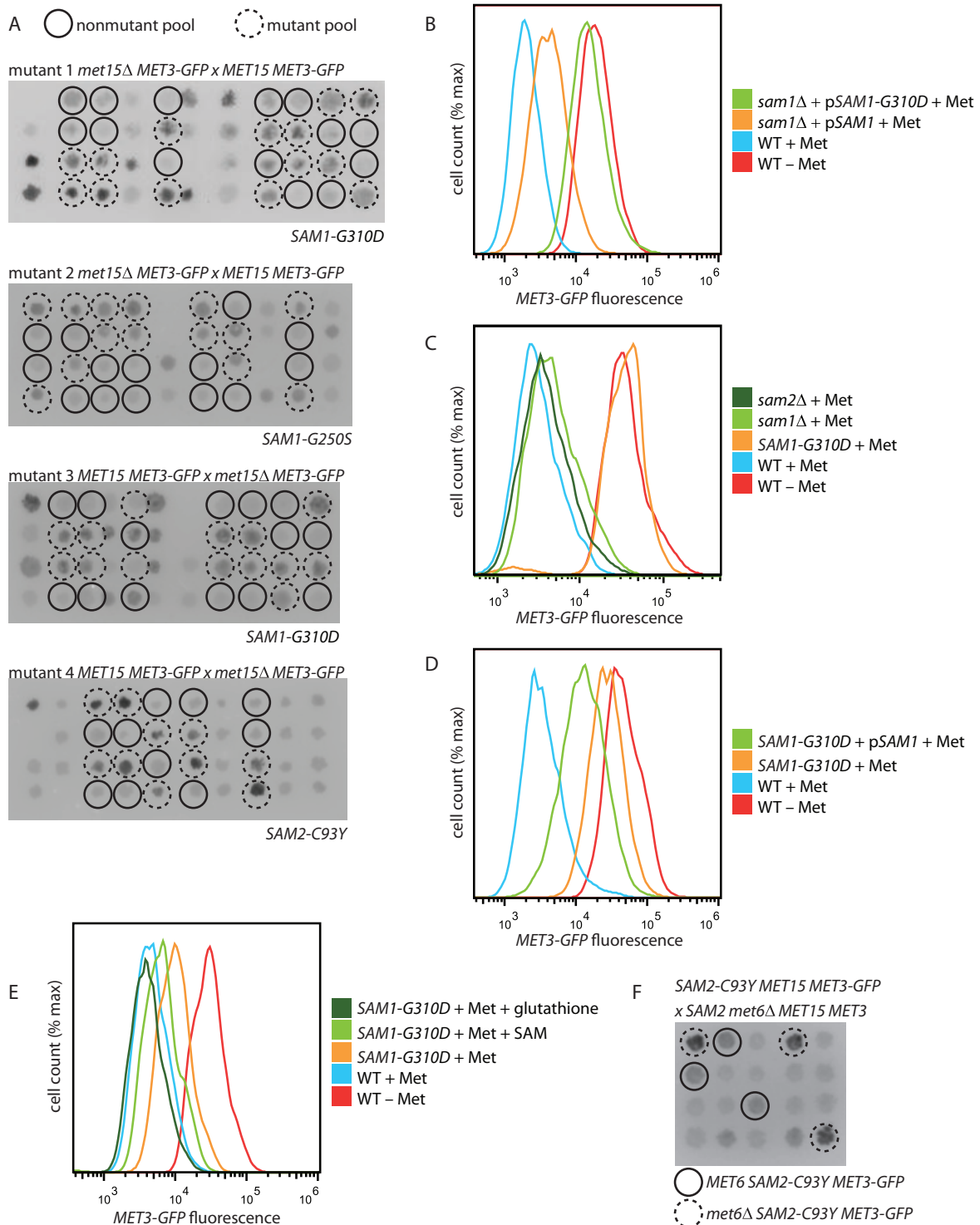


FIGURE 3: SAM synthetase mutations induced *MET3-GFP*. (A) Fluorescence imaging of tetrad dissections of four mutants (JRY9391, JRY9394, JRY9395, and JRY9396) backcrossed to an unmutagenized strain (JRY9355 or JRY9356) and grown on a plate with 1 mM methionine. A total of 10–15 tetrads were analyzed, and the colonies selected for the pooling are marked. (B) Flow cytometry of *sam1Δ MET3-GFP* cells carrying either the wild-type *SAM1* allele (JRY9469) or the *SAM1-G310D* allele (JRY9470) on a single-copy plasmid expressed from the native *SAM1* promoter and grown with 134 μ M methionine. (C) Flow cytometry of *sam1Δ* or *sam2Δ* cells with *MET3-GFP* (JRY9508 and JRY9509, respectively) grown in 134 μ M methionine as compared with *SAM1-G310D* cells. (D) Flow cytometry of *SAM1-G310D* cells with *MET3-GFP* grown with 134 μ M methionine and carrying either the wild-type *SAM1* allele on a single-copy plasmid expressed from the native *SAM1* promoter (JRY9454) or a blank single-copy plasmid with no insert (JRY9472). (E) Flow cytometry of *SAM1-G310D* cells with *MET3-GFP* grown in 134 μ M methionine, as well as with additional 0.2 mM SAM or 10 mM glutathione. (F) Fluorescence imaging of a tetrad dissection of *SAM2-C93Y/SAM2 met6Δ/MET6 MET3/MET3-GFP* diploids grown on a plate with 1 mM methionine. Ten tetrads were analyzed.

genes was mutated in each mutant. The dominant mode of action was not through a mechanism analogous to haploinsufficiency, in which the 50% reduction in gene product in a heterozygote is sufficient to cause a phenotype, as neither *sam1Δ* nor *sam2Δ* induced *MET3-GFP* in the presence of methionine to the levels seen in the mutants (Figure 3C). It was also unlikely that the mutants were neomorphs or hypermorphs, as the mutants had somewhat less *MET3-GFP* expression in the presence of methionine when wild-type SAM synthetase was expressed off a plasmid (Figure 3D). The remaining possibility was that the mutations were antimorphs, in which mutant enzymes disrupt the wild-type enzyme's function in addition to their own (colloquially, although ambiguously, referred to as a "dominant-negative" mode of action). In support of this model, addition of SAM substantially repressed *MET3-GFP* induction in the *SAM1-G310D* mutant (Figure 3E). Furthermore, this result indicated that the antimorphic effect was mediated through loss of SAM synthetase activity rather than of some undiscovered function of SAM synthetase.

Antimorphic SAM synthetase alleles would reduce synthesis of SAM from exogenously provided methionine and reduce the synthesis of all other downstream metabolites, including cysteine (Figure 1A). *sam1Δ sam2Δ* cells induce *MET* genes (Thomas *et al.*, 1988), which has been attributed, at least in part, to the reduction in cysteine produced from methionine (Hansen and Johannesen, 2000). As with the *cho2* mutant described earlier, the expression of *MET3-GFP* was tested in the *SAM1-G310D* mutant in medium supplemented with cysteine, in the form of glutathione. *MET3-GFP* remained induced in medium with 1 mM glutathione and 1 mM methionine (our unpublished observations), conditions sufficient to restore basal expression of *MET3-GFP* in a *cho2Δ* mutant. However, growth with 10 mM glutathione and 1 mM methionine repressed *MET3-GFP* (Figure 3E).

This result suggested that the SAM synthetase mutants induced *MET3-GFP* because they experienced cysteine deficiency, as was the case with the *cho2* mutant. However, in contrast to the *cho2* mutant, SAM synthetase mutants that had functional de novo homocysteine synthesis still showed substantial *MET3-GFP* induction (Figure 3, A and B, and our unpublished observations). Thus, in the SAM synthetase mutants, both the synthesis of cysteine from methionine was impaired, as was the case with the *CHO2* mutant, and de novo homocysteine synthesis was not capable of providing sufficient cysteine to reduce *MET3-GFP* expression. One possible explanation for the difference in behavior of SAM synthetase and *cho2* mutants was that SAM deficiency caused a deficiency in the synthesis of cysteine from homocysteine separate from its effect on the synthesis of cysteine from methionine, thus compromising both sources of cysteine. This possibility resonated with the known regulatory role of SAM in cysteine synthesis, via allosteric control of cystathionine β-synthase activity (Taoka *et al.*, 1999). Alternatively, SAM deficiency could have been directly sensed to induce *MET3-GFP*, independently of cysteine sensing. In both of these hypotheses, exacerbating the SAM deficiency in the SAM synthetase mutants without directly affecting cysteine synthesis would be predicted to enhance induction of *MET3-GFP*. This prediction was tested through deletion of *MET6*, which produces methionine from homocysteine, in the SAM synthetase mutants. Importantly, although *MET6* deletion compromises SAM synthesis, it should not reduce cysteine synthesis, as the reduction of SAM levels is paired with a precise stoichiometric increase in homocysteine (Figure 1A). Indeed, *Met6* diverts homocysteine that could be used for cysteine synthesis toward methionine synthesis. Surprisingly, for the *SAM1-G310D* mutant, deletion of *MET6* caused a strong synthetic sickness that could

not be rescued with methionine and glutathione, thus precluding proper analysis. Because *met15Δ* did not cause synthetic sickness in combination with SAM synthetase mutants, the synergism with *met6Δ* suggested that proper cycling of homocysteine to methionine was required for sufficient SAM synthesis in this mutant, even when 1 mM methionine was provided exogenously. Strains with *met6Δ* in combination with one of the weaker SAM synthetase mutants, *SAM2-C93Y*, were successfully obtained. *MET3-GFP* expression was strongly enhanced in these strains relative to strains with *SAM2-C93Y* alone (Figure 3F), consistent with SAM deficiency inducing *MET3-GFP* independently of reducing flux from methionine to homocysteine. Thus, either through compromising both de novo cysteine synthesis and cysteine synthesis from methionine, or by being independently sensed, specific deficiencies in SAM led to increased *MET3* gene expression.

Effects of sulfur-containing amino acid availability on Met30 protein

Ultimately, the metabolites controlling the *MET* regulon exert their effect by changing the activity of Met30 toward the *Met4* transcription factor. An epitope-tagged Met30 that provided full Met30 function was evaluated by immunoblotting to ask whether there were detectable changes in the Met30 protein itself in response to starvation for sulfur-containing amino acids. *met15Δ* cells carrying epitope-tagged *MET30* on a plasmid expressed from its own promoter were grown in medium with methionine and then shifted to medium without methionine or cysteine for 1 h (Figure 4A). Met30 abundance increased in the sulfur amino acid-starved culture, as was expected given that *MET30*'s transcription is induced by *Met4* during growth without methionine and cysteine (Rouillon *et al.*, 2000). In addition to the induction of Met30 protein abundance, there was a shift in the mobility of Met30 under inducing conditions—in addition to a species of Met30 present in both cultures, there was a faster-migrating, more-abundant species specifically in the culture grown without methionine and cysteine. The protein extracts were prepared under reducing conditions, and thus the mobility shift was not due to differential formation of a disulfide bond.

This differential mobility could reflect a posttranslational modification or a change in the composition of the protein itself. We noticed that the *MET30* mRNA had the capacity to form a large hairpin toward the 5'-end whose stability was estimated using mfold (Zuker, 2003) at -26.2 kcal/mol (Figure 4B); a larger, imperfect hairpin can be drawn with extra 5' nucleotides with a predicted stability of -35.2 kcal/mol, well within the range of stabilities that mediate attenuation in bacterial biosynthetic operons (Lee and Yanofsky, 1977; Zurawski *et al.*, 1978; Lynn *et al.*, 1985). Moreover, this hairpin was conserved in all the *sensu stricto* yeast species through a pattern of compensatory mutations (Figure 4C). mRNA secondary structure can affect eukaryotic translation in many ways, including by causing alternative splicing (Meyer *et al.*, 2011), alternative frames of translation (Ivanov *et al.*, 2000), and alternative translation start-site selection (Hinnebusch, 2005; Reineke *et al.*, 2008), any of which could potentially explain the changed mobility of Met30 in response to methionine limitation. To test the possible significance of the potential RNA hairpin, we created a mutant *MET30* allele with seven nucleotides in the hairpin mutated, such that the protein sequence was maintained, whereas the stability of the potential hairpin formation was disrupted (Figure 4D). However, this mutant allele produced the same two species of Met30 protein with the same differential mobilities, relative abundances, response to growth conditions, and effect on *MET3* expression as wild-type *MET30* (Figure 4, E and F).

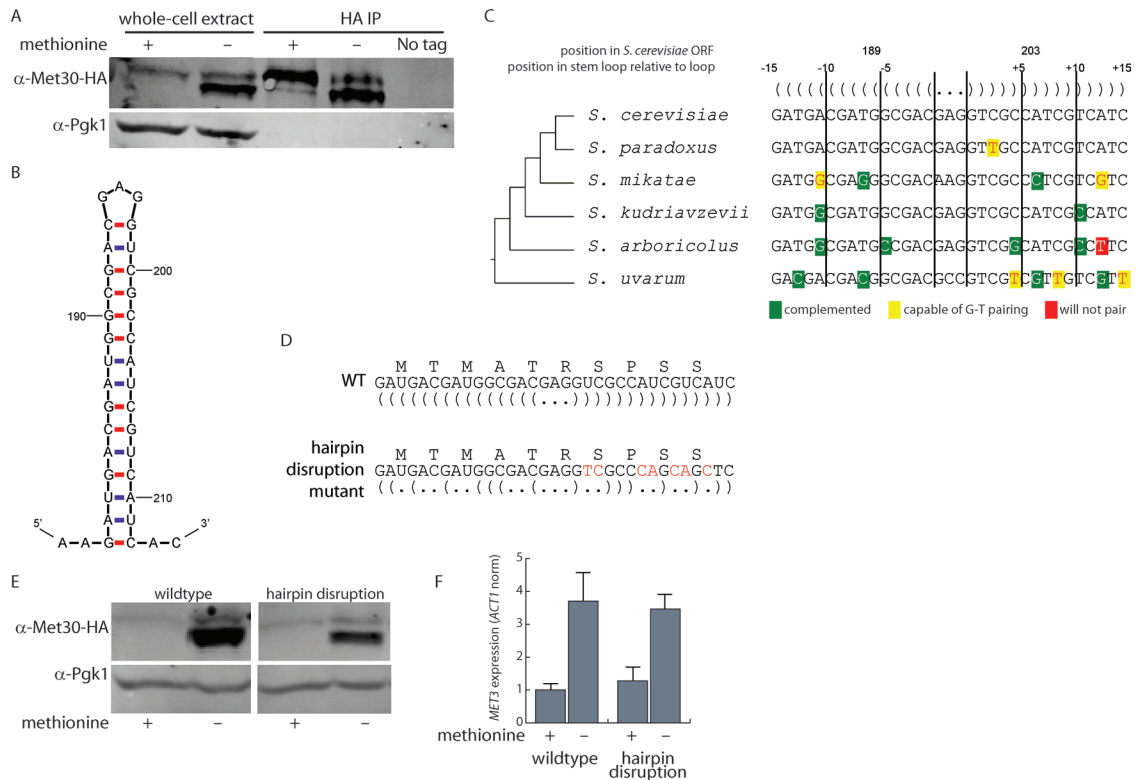


FIGURE 4: The Met30 protein had two isoforms. (A) Immunoblot of Met30-3xHA expressed from a single-copy plasmid in *met15Δ* cells (JRY9354) grown with 134 μM methionine or after growth for 1 h in medium containing neither methionine nor cysteine, prepared either as a whole-cell extract or by direct immunoprecipitation of HA. Detection of Pgk1 was used as a loading control, and an isogenic strain lacking HA-tagged Met30 was used as a negative control. (B) RNA structure, as predicted using mfold (Zuker, 2003), in a section of the *MET30* mRNA (bases 178–214). (C) Conservation of a predicted RNA structure in *MET30* of other species in the sensu stricto clade. 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 (Scannell et al., 2011; Liti et al., 2013), with mutations highlighted. (*Saccharomyces uvarum* is also referred to as *S. bayanus* var. *uvarum* or *S. bayanus*; Libkind et al., 2011.) Green highlighting indicates that the opposite arm of the stem loop contains a compensating mutation that preserves the predicted hairpin structure. Yellow highlighting indicates that the mutation was 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 because *S. uvarum* 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. uvarum* lineage for simplicity. A similar caveat applies to the mutations at –11 and +11. (D) Mutational scheme to disrupt potential hairpin formation. The translation of the targeted segment is shown above the sequence. (E) Immunoblot of *met15Δ* cells carrying either wild-type Met30-3xHA (JRY9354) or Met30-3xHA with hairpin-disrupting mutations (JRY9510) expressed from single-copy plasmids and grown either in medium with 134 μM methionine or after growth for 1 h in medium lacking both methionine and cysteine. (F) *MET3* expression determined by RT-PCR in *MET15* cells with wild-type or hairpin-disrupted *MET30* alleles (JRY9511 and JRY9512, respectively) expressed off single-copy plasmids and grown overnight with or without 134 μM methionine. All cDNA values were internally normalized to *ACT1* cDNA values from the same cDNA preparation and normalized to wild type grown with methionine afterward. *n* = 2; error bars show SE.

To determine whether posttranslational modifications of Met30 could explain the mobility difference of Met30 in repressing versus inducing conditions, we applied multidimensional protein identification technology (MudPIT) to Met30 immunoprecipitated from a culture starved for sulfur-containing amino acids. Overall, peptides were detected that covered 81% of Met30. Multiple peptides were also identified from coimmunoprecipitated Skp1, Hrt1, and Cdc53, the members of the SCF(Met30) E3 ubiquitin ligase complex that, along with Met30, ubiquitinates Met4 (Supplemental Table S1). None of these proteins was detected in a mock immunoprecipitation using untagged Met30. Cdc34, the E2 associated with the SCF complexes, was not detected in the Met30 immunoprecipitate, nor was Met4.

Multiple modified residues of Met30 were detected. Phosphorylations were detected on serines 47, 70, and 105 and trioxidations on cysteines 95 and 455 (Supplemental Figures S2–S6). Peptides containing the unphosphorylated forms of serines 47, 70, and 105 were also detected. In contrast, cysteines 95 and 455 were found only in the trioxidized form. Twelve other cysteines in Met30, and all other cysteines detected on other peptides identified in the immunoprecipitation, were detected solely in the unoxidized form. It is possible that the trioxidations occurred during the protein purification, but this possibility would require that these particular cysteines were particularly prone to oxidation compared with the other cysteines in the sample. Conversely, trioxidation of cysteine is

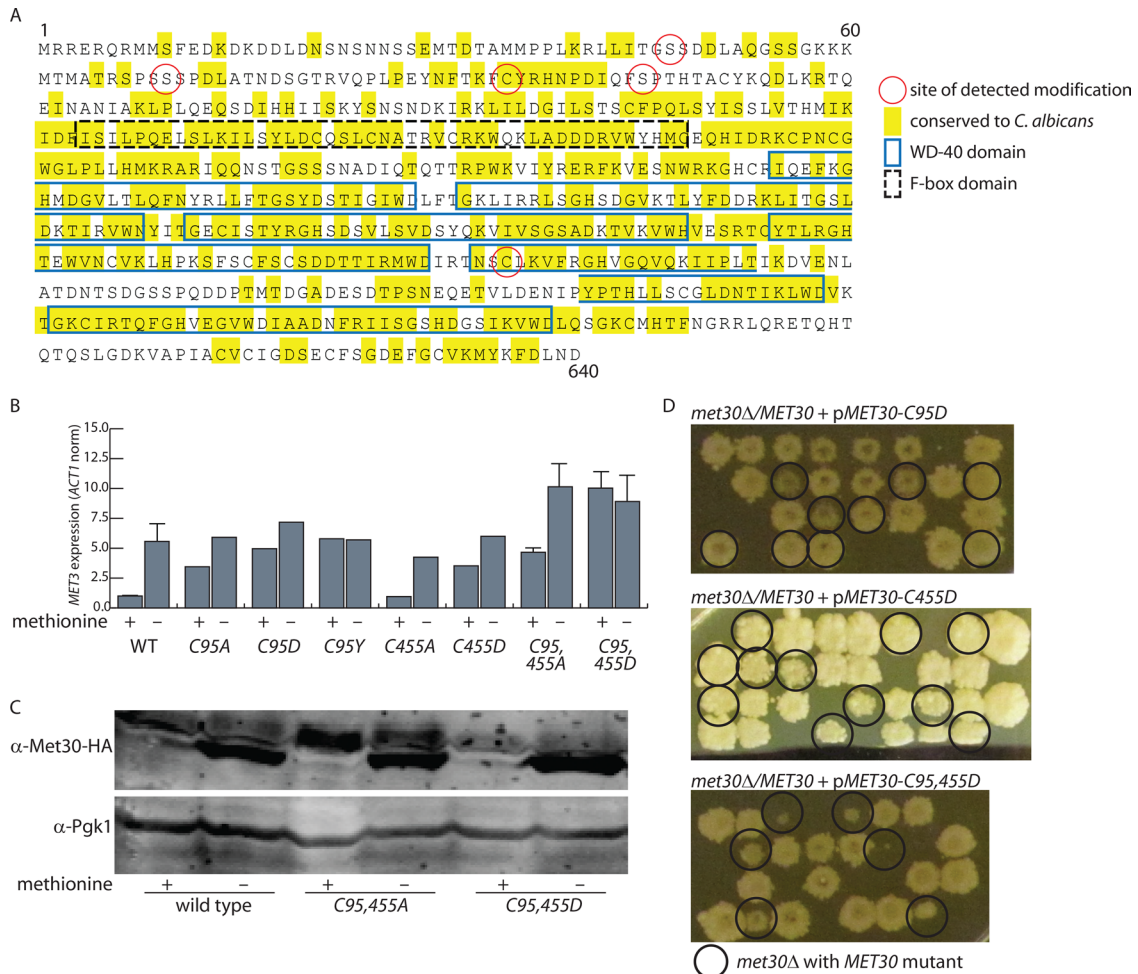


FIGURE 5: Posttranslational modification profiling of Met30. (A) Diagram of the Met30 amino acid sequence with known domains and residues conserved to *C. albicans* highlighted. *C. albicans* sequence was obtained from the Saccharomyces Genome Database (<http://yeastgenome.org>), and alignment was done with ClustalW2 (Goujon *et al.*, 2007). Residues detected as modified are circled. (B) *MET3* expression determined by RT-PCR in *met30Δ MET15* cells carrying single-copy plasmids with wild-type or mutant *MET30* alleles grown overnight with 134 μ M methionine or in medium lacking both methionine and cysteine. WT refers to JRY9490, C95A to JRY9491, C95D to JRY9492, C95Y to JRY9505, C455A to JRY9494, C455D to JRY9495, C95,455A to JRY9497, and C95,455D to JRY9498. *MET3* cDNA values were internally normalized to *ACT1* cDNA values from the same cDNA preparation and then normalized to wild type grown with methionine. $n = 2$ for wild type, C95,455A, and C95,455D; $n = 1$ for the others. Error bars show SE. (C) Immunoblot of wild-type or mutant Met30-3xHA proteins expressed from single-copy plasmids in *met15Δ* cells either grown with 134 μ M methionine or after growth for 1 h in medium without methionine or cysteine. Wild type refers to JRY9477, C95,455A to JRY9484, and C95,455D to JRY9485. $n = 2$; the increased intensity of Met30-3xHA in C94,455A did not replicate. (D) Dissections of *met30Δ/MET30 met15Δ/MET15 ura3Δ/ura3Δ* diploids (JRY9451) carrying single-copy *URA3* plasmids carrying mutant alleles of *MET30*.

irreversible (Hamann *et al.*, 2002) and thus not susceptible to reducing agents used during sample preparation.

Of the modified residues, only cysteines 95 and 455 were conserved as far as *Candida albicans* (Figure 5A). Cysteine 95 lies in a conserved segment (residues 90–112) that does not match any annotated domains, whereas cysteine 455 lies in one of Met30's WD-40 repeats. The phosphorylations all fell in Met30's poorly conserved N-terminus. Because cellular oxidation is connected to the *MET* regulon, as the primary cellular antioxidant, glutathione, is synthesized from cysteine, we explored whether the specific oxidized species of Met30 had *in vivo* significance. Moreover, a mutation affecting the position of one of the oxidized cysteines, *met30-C95Y*, was recovered in the screen for mutants that induced *MET3-GFP*,

hinting at the potential significance of this modification. In contrast, there is no direct connection between sulfur-containing amino acids and phosphorylation.

Mutant alleles were created with either cysteine 95 or 455 mutated to aspartic acid (*met30-C95D* and *met30-C455D*), as aspartic acid is more similar in volume and polarity to trioxidized cysteine than unoxidized cysteine is, and with cysteines 95 and 455 mutated to alanine in an effort to mimic permanently unoxidized cysteines (*met30-C95A* and *met30-C455A*), with the obvious and notable exception that alanine is unable to form disulfide bonds. In addition, alleles were made with both cysteine 95 and 455 mutated to alanine or aspartic acid (*met30-C95,455A* and *met30-C95,455D*). As *MET30* is an essential gene, plasmids carrying the mutant alleles were

transformed into *met30Δ/MET30* diploids, which were then sporulated. Mutation of cysteine 95 to either alanine or aspartate increased *MET3* expression in cells grown with methionine-supplemented medium (Figure 5B). These amino acids imperfectly mimic opposite oxidation states and yet had the same effect on Met30 function. Indeed, mutation of cysteine 95 to tyrosine, a mutation recovered from the screen, had the same effect on *MET3* expression, indicating that cysteine 95 plays an important role in Met30's repressive activity. If the regulatory role of Met30 involved disulfide bond formation involving cysteine 95, then, like the alanine, aspartic acid, and tyrosine substitutions, trioxidized cysteine would be unable to perform this function, and thus targeted oxidation of cysteine 95 in conditions lacking sulfur-containing amino acids would lead to increased *MET* regulon expression.

Mutation of cysteine 455 to alanine had little effect on *MET3* expression, and mutation to aspartic acid increased *MET3* expression. Both mutations seemed to enhance the effect of the cysteine 95 mutations, as *met30-C95,455A* had a stronger *MET3* expression phenotype than *met30-C95A*, and likewise for *met30-C95,455D* compared with *met30-C95D*.

Given that the C95 mutants induced *MET3-GFP*, an effect enhanced in C95, 455 double mutants, we determined the effect of the double mutations on Met30's apparent mobility. Met30 is itself up-regulated by Met4, and hence defects in Met30 would be expected to result in overproduction of mutant Met30 protein. To avoid the potential complication of mutant *met30* alleles affecting the protein modification indirectly through this feedback loop, we evaluated the epitope-tagged mutant Met30 proteins in extracts from cells carrying an additional wild-type untagged *MET30*. The mutant Met30 proteins had the same mobility pattern on acrylamide gels as the wild-type Met30, implying that the two sites of oxidized cysteine on Met30 did not play a significant role in the regulated mobility shift in Met30 in response to methionine availability (Figure 5C), although it remained possible that the mobility shift played a role in the oxidation. Of interest, *met30Δ* colonies carrying the *met30-C95,455D* allele grew very poorly, even though *met30Δ* colonies carrying *met30-C95D* or *met30-C455D* were not particularly sick (Figure 5D). Deletion of *MET30* is lethal but can be rescued through deletion of *MET4* (Patton *et al.*, 2000), suggesting that Met30's essential function is down-regulation of the *MET* regulon. Consistent with that hypothesis, the slowly growing double mutant showed the highest induction of *MET3* and presumably genes of this regulon.

DISCUSSION

A genetic screen for components of the *MET* regulon uncovered deep connections to phospholipid biosynthesis mediated through SAM levels, extended previous ideas about which molecules were sensed by the *MET* regulon, identified a class of antimorphic mutations whose effect extended to effects on paralogous proteins, and eliminated at least some candidate mechanisms for how Met30 activity is regulated.

cho2 mutants resulted in activation of the *MET* regulon. *CHO2* encodes the enzyme responsible for the first step of phosphatidylcholine synthesis from phosphatidylethanolamine, in a reaction consuming SAM and producing SAH (Kodaki and Yamashita, 1987; Summers *et al.*, 1988). 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 due to insufficient cellular SAM consumption. This result strongly implied that phosphatidylcholine synthesis accounted for an enormous fraction of SAM consumption and that cells lacking Cho2 function were unable to

synthesize sufficient cysteine from methionine due to insufficient SAM consumption. When the de novo cysteine synthesis pathway was intact, enough cysteine was synthesized to prevent the full induction of *MET3-GFP* observed in *cho2Δ met15Δ* double mutants.

Previous work established that Opi3 has phosphatidylethanolamine methyltransferase activity (Kodaki and Yamashita, 1987) and that overexpression of *OPI3* can at least partially suppress the phenotype of *cho2* mutants (Preitschopf *et al.*, 1993). The discovery that *opi3* mutations exacerbated the induction of *MET3-GFP* in cells with *cho2Δ* implied that even under physiological levels of Opi3 enzyme, Opi3 consumed sufficient SAM in the methylation of phosphatidylethanolamine, producing enough SAH in the process, to contribute to cysteine synthesis. A significant regulatory overlap between SAM synthesis and phosphatidylcholine synthesis was noted before (Hickman *et al.*, 2011), which established that significant SAM pools are required for methylation reactions in phospholipid synthesis. Our work extends this conclusion by showing that those methylation reactions are also required to produce enough SAH to fully power cysteine synthesis.

Of interest, the expression of *OPI3* is significantly repressed in cells grown with exogenously provided choline, whereas *CHO2* expression is unaffected (Jesch *et al.*, 2005), even though these proteins act consecutively to carry out phosphatidylcholine synthesis. This study suggests that differential regulation allows consumption of SAM by Cho2 to produce SAH required for proper sulfur-containing amino acid homeostasis even when choline is plentiful. Because significant phosphatidylmonomethylethanolamine would be produced under these conditions but not converted into phosphatidylcholine, perhaps there is an undiscovered but important role for phosphatidylmonomethylethanolamine.

Mutations creating antimorphic SAM synthetase alleles also induce *MET* gene expression. This effect is mediated through loss of SAM synthesis in these mutants, as addition of SAM restores repression. Antimorphic enzyme variants can act by disrupting the formation of homo-oligomeric structures, but these alleles of *SAM1* and *SAM2* are of special interest because their phenotype indicates that the individual mutations disrupt the function of both isozymes, since cells with only one of either isozyme make adequate SAM to maintain repression of *MET3*. Studies of *Escherichia coli* SAM synthetase, which is 55–60% identical to the yeast enzymes, offer some insight. *E. coli* SAM synthetase is a homotetramer, which, when inactivated by *N*-ethylmaleimide, dissociates from tetramers to dimers, resulting from modification of the cysteine residues at positions 90 and 240 (Markham and Satishchandran, 1988). Cysteine 90 maps to the interfaces between subunits in the crystal structure (Komoto *et al.*, 2004), and mutation of the *E. coli* SAM synthetase cysteine 90 residue biases the protein toward dimers, which have far less enzymatic activity than the tetramers (Reczkowski and Markham, 1995). Cysteine 90 of *E. coli* SAM synthetase corresponds to cysteine 93 in *S. cerevisiae* Sam2, which was one of the mutated residues recovered here. Our data imply that although homotetramers of Sam1 or of Sam2 must be functional, heterotetramers may account for most of the SAM synthesized in cells with both isozymes in order for the dominant alleles of *SAM1* or *SAM2* to exert a regulatory phenotype.

In *cho2* mutants, full induction of the *MET* regulon requires that cells also carry, for example, a *met15Δ* mutation to disrupt de novo homocysteine synthesis. In contrast, in mutants with compromised SAM synthetases, full *MET* gene induction occurs without disruption of de novo homocysteine synthesis. Because cysteine deficiency is clearly a cause of *MET* regulon induction, as proposed earlier (Hansen and Johannesen, 2000; Menant *et al.*, 2006), the

phenotype of *SAM1* and *SAM2* mutants implies that SAM deficiency compromised the synthesis of cysteine from homocysteine, SAM deficiency itself was sensed to turn on the *MET* genes, or both. The first possibility is most parsimonious because the cystathionine β -synthase (CBS) protein in *S. cerevisiae*, which catalyzes the first step in cysteine synthesis from homocysteine, is predicted to contain a domain that stimulates enzyme activity in response to SAM availability (Taoka *et al.*, 1999). It would appear that the regulatory machinery controlling *MET* gene transcription senses cysteine levels in some way and that SAM deficiency indirectly induces the *MET* regulon by causing cysteine deficiency through inadequate CBS activity, as well as through inadequate flux of methionine to homocysteine.

Although less parsimonious, it remains possible that SAM deficiency is independently sensed, in addition to cysteine deficiency. Although many *MET* regulon members have both Cbf1- and Met31/Met32-binding sites in their promoters, those with only Met31/Met32 promoter binding sites are likely to be involved specifically in methionine and SAM synthesis rather than cysteine synthesis (Lee *et al.*, 2010). Perhaps Cbf1 specifically responds to cysteine deficiency, and Met31/Met32 specifically responds to SAM deficiency. The reporter gene used here, *MET3*, has both binding sites (Lee *et al.*, 2010) and did respond to both SAM deficiency and cysteine deficiency, as would be predicted by this model.

It seems highly likely that the altered mobility of Met30 protein in cells with inadequate sulfur-containing amino acids, along with its increased abundance, is a key factor that remains to be understood. The appearance of two forms of Met30 was previously noted (Rouillon *et al.*, 2000), although the previous study did not reveal the connection between the two species and methionine availability. The difference is presumably due to a difference in the construct used. The previous study used a *GAL1* promoter-driven Met30 with an N-terminal tag replacing the first seven amino acids, whereas in this study, full-length *MET30* was expressed off its native promoter and Met30 was C-terminally tagged. We have not detected any posttranslational modification sufficient to explain the mobility shift. A tantalizing secondary structure in the *MET30* mRNA could, in principle, cause translation to initiate at an internal methionine under conditions in which Met30 activity is not needed. Indeed, the striking density of the codons for cysteine and methionine in this region of the *MET30* open reading frame (ORF) was reminiscent of attenuation mechanisms governing amino acid biosynthesis in bacteria in response to the levels of tRNAs changed with their cognate amino acids (our unpublished observations). Unfortunately, although we detected peptides covering 80% of the Met30 protein, we were unable to detect the most-N-terminal peptides from Met30 mass spectra, precluding a definitive conclusion on this possibility. However, to the extent that the septuple mutation of the putative RNA hairpin had no discernible effect on induction of *MET3-GFP*, RNA secondary structure may not have contributed to the altered mobility of Met30 under inducing conditions. Perhaps the RNA hairpin has been conserved for some other function.

Although we were unable to detect Met30 and its interacting partners by mass spectrometry from cells grown in media containing methionine, which is when Met30 is most active yet least abundant, our observations suggest interesting possibilities. First, the substantial quantitative increase in the faster-running form of Met30 when the SCF(Met30) complex is least active invites speculation that the abundance of the faster-running form enables it to displace the slower-running and presumably active form of Met30 in the SCF complex. Second, if association with either Met4 or the E2 protein,

Cdc34, is the regulated step controlling SCF-Met30 function, then the trioxidized C95 of Met30 might play a pivotal role in controlling this association if cysteine levels, or perhaps glutathione levels, are physiological regulators of that oxidation. Mutations of C95 of Met30 to alanine, aspartic acid, or tyrosine all disrupted Met30 function. If the regulatory role of Met30 involved disulfide bond formation involving cysteine 95, then, like the alanine, aspartic acid, and tyrosine substitutions, trioxidized cysteine would be unable to perform this function, and thus targeted oxidation of cysteine 95 in conditions lacking sulfur-containing amino acids would lead to increased *MET* regulon expression.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotide sequences

Strains used are listed in Supplemental Table S2, plasmids used are listed in Supplemental Table S3, and sequences of oligonucleotides used are listed in Supplemental Table S4. Strains JRY9384-9 were produced by mating members of the yeast GFP clone collection (95702; Invitrogen, Carlsbad, CA) to BY4742 (JRY6331; Brachmann *et al.*, 1998). Strains JRY9355 and JRY9356 were haploid progeny of JRY9384. JRY9360, JRY9361, JRY9508, and JRY9509 were segregants resulting from crosses among JRY9356, BY4741, and strains of the *MATa* and *MAT α* Yeast Knockout Collections (YSC1053 and YSC1054 from Thermo, Waltham, MA). Diploids were sporulated by growth in liquid yeast extract/peptone/dextrose (YPD), presporulation overnight in YP acetate, and then incubation for at least 3 d in 2% potassium acetate supplemented with the nutrients required by the diploid. A modified lithium acetate protocol (Becker and Lundblad, 2001) was used for all plasmid transformations. Because *MET30* is an essential gene, JRY9490-8 were created by transformation of JRY9451 (derived from the yeast essential heterozygous diploid collection, Thermo YSC1057) with plasmids carrying various *MET30* alleles, followed by sporulation and dissection to isolate appropriate haploids.

pJR3170 consists of a genomic region containing the *MET30* ORF and 617 base pairs upstream of the start codon in the pBY011-D123 (URA, Cen-Ars) plasmid, with triple-hemagglutinin (3xHA) epitope tags fused in-frame to the 3' end of the ORF. pJR3170 was later found to have a substitution, S382L, that had no discernible effect on *MET30* function. pJR3182 is identical to pJR3170 but lacks this substitution; the plasmid carried by JRY9511 is identical to pJR3182 but lacks the HA epitope tag. Site-directed mutagenesis was performed as previously described (Makarova *et al.*, 2000) to create pJR3183-3202 from pJR3182 and to create the *MET30* alleles carried by JRY9510 and JRY9512. The plasmids carried by JRY9469 and JRY9470 were created by ligating a *KpnI-SphI* fragment carrying the *SAM1* alleles of BY4741 and JRY9391, respectively, into pBY011-D123.

Media

Synthetic defined media (SD) plus complete supplement mix (CSM, which lacks cysteine), with supplements dropped from the CSM or added back as appropriate to the experiment, were used for all experiments.

To starve for sulfur-containing amino acids, cells were first grown overnight to mid log phase in SD + CSM – methionine (Amberg *et al.*, 2005) with 134 μ M (20 μ g/ml) methionine added back. Then cells were pelleted and residual media was pipetted off, after which cells were resuspended in SD + CSM – methionine, which lacks all sulfur-containing amino acids, for 60 min. For starvation experiments involving cells carrying plasmids, the medium used lacked uracil but was otherwise identical.

Screen and mutant identification

Strains JRY9355 and JRY9356 were mutagenized with either EMS (Amberg *et al.*, 2006) or ultraviolet radiation using a Stratilinker apparatus (Stratagene, La Jolla, CA) set to 70 μ J to cause 50% killing. Cells were plated at a target density of 500 cells/plate on SD + 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 (GE Healthcare, Little Chalfont, UK) set to scan 3 mM above the platen, scanning with a 488-nm laser and 520-nm emission filter to identify fluorescent colonies. The same settings were used to determine fluorescence in tetrad dissections.

The mutation in *MET30* in JRY9357 was identified by Sanger sequencing of the *MET30* ORF. Select mutants with recessive mutant phenotypes were queried by transformation with a genomic library (Jauert *et al.*, 2005) generated on a plasmid with a kanamycin selection marker. Transformed cells were allowed to recover for 30 min in liquid YPD, after which cells with plasmids carrying dominant genes suppressing the mutant phenotype were selected by plating the transformed mutants on CSM plates containing 1 mM methionine, 5 mM sodium selenate (Thomas *et al.*, 1995), and kanamycin. The uptake of sodium selenate, a toxin, is mediated through sulfate transporters, whose expression is controlled as part of the *MET* regulon. This approach identified a causative mutation in *CHO2* (in JRY9359).

Additional mutants, selected on the basis of clear Mendelian segregation in tetrad dissections, were queried by sequencing of pooled segregants (Birkeland *et al.*, 2010). From a given tetrad dissection of a mutant crossed to the wild-type parent, colonies showing mutant or wild-type *MET3-GFP* expression were separately pooled in 1 ml of YPD on ice. A 0.5-ml amount of each pool was saved, and DNA was extracted from the remaining 0.5 ml by phenol:chloroform:isoamyl alcohol treatment, then sheared with a Covaris AFA (Covaris, Woodburn, MA). Genomic libraries were prepared with a TruSeq DNA sample preparation kit (Illumina, San Diego, CA). The pooled genomes were sequenced with 50-base pair single reads, on an Illumina HiSeq 2000 sequencing system. Sequence data were analyzed using the BWA, SAMtools, and BCFtools software packages (Li, 2011). This approach identified several mutations in the SAM synthetase genes *SAM1* and *SAM2* (in JRY9391, JRY9394, JRY9395, and JRY9396).

Flow cytometry and fluorescence microscopy

Cells were grown overnight to mid log phase in SD + CSM + 1 mM methionine (except for the “–methionine” controls, which were grown in SD + CSM – methionine), with additional supplements as required, and then washed once and resuspended in phosphate-buffered saline, pH 7.4, on ice. Fluorescence was determined in 10,000 cells/culture using an FC500 flow cytometer. Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR). Fluorescence imaging in Supplemental Figure S1 was done as in Denby *et al.* (2012).

Whole-cell extract preparation and 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 (Li-Cor Biosciences, Lincoln, NE). Antibodies used in the immunoblots were anti-Pgk1 (Invitrogen) and anti-HA (H9658; Sigma-Aldrich, St. Louis, MO).

RNA extraction, cDNA preparation, and qRT-PCR

RNA was purified using hot acid phenol and chloroform. Residual DNA was removed by DNase treatment (04716728001; Roche,

Basel, Switzerland), after which the RNA was purified again by use of a Qiagen RNeasy kit (Qiagen, Venlo, Netherlands). cDNAs were prepared with an Invitrogen Superscript III kit and quantified with a Stratagene MX3000 quantitative PCR system. All primer-set amplification values were normalized to *ACT1* amplification values.

Met30-3xHA immunoprecipitation

Cells were grown to a density of 2×10^7 cells/ml in 2 l of SD + CSM – URA + 134 μ M methionine and then pelleted, washed with water, and resuspended for 1 h in 2 l of SD + CSM – URA – methionine. Cells were washed with IP buffer (10% glycerol, 0.75% NP-40, 150 mM NaCl, 1.5 mM magnesium acetate, 50 mM Tris, pH 7.8) plus protease inhibitors (04693116001; Roche) and then pelleted and frozen in liquid nitrogen. Cells were lysed with a Spex SamplePrep Freezer/Mill 6870 (Spex, Metuchen, NJ), after which the ground cell material was resuspended in IP buffer plus protease inhibitors + phosphatase inhibitors (04906837001; Roche). Nonsoluble material was removed by centrifugation, and Met30-3xHA was precipitated from the soluble material by incubation for 75 min at 4°C with 120 μ l of anti-HA conjugated beads (A2095; Sigma-Aldrich) that had been prewashed with IP buffer plus protease and phosphatase inhibitors. Unbound proteins were removed by repeated washing with IP buffer plus protease and phosphatase inhibitors, after which bound protein was eluted by treating beads twice with 0.5 ml 500 mM ammonium hydroxide and 0.5 mM EDTA for 20 min at room temperature.

MudPIT

Immunoprecipitated proteins were reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (C4706; Sigma-Aldrich) and alkylated with 55 mM 2-chloroacetamide (22790; Sigma-Aldrich). Proteins were digested for 18 h at 37°C in 2 M urea, 100 mM Tris, pH 8.5, and 1 mM CaCl₂ with 2 μ g of trypsin (V5111; Promega, Madison, WI).

MudPIT analysis was performed using an Agilent 1200 G1311 quaternary pump (Agilent, Santa Clara, CA) and a Thermo LTQ-Orbitrap XL using an in-house-built electrospray stage (Wolters *et al.*, 2001). Protein and peptide identification and protein quantification were done with Integrated Proteomics Pipeline-P2 (Integrated Proteomics Applications, San Diego, CA.). Tandem mass spectra were extracted from raw files using RawExtract 1.9.9 (McDonald *et al.*, 2004) and were searched against a Uniprot *S. cerevisiae* database with reversed sequences using ProLuCID (Peng *et al.*, 2003; Xu *et al.*, 2006). The search space included all fully tryptic and half-tryptic peptide candidates. Differential modifications considered were 15.9949 MC, 31.989828 C, 47.984744 C, 57.02146 C, and 79.9663 STY. Peptide candidates were filtered using DTASelect, with these parameters: -p 2 -y 1 -trypstat -fp 0.05 -modstat -DM 10 -dm -in -m 1 (Tabb *et al.*, 2002; McDonald *et al.*, 2004). Ascore was determined as in Beausoleil *et al.* (2006).

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