Dissection of Combinatorial Control by the Met4 Transcriptional Complex

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Met4 is the transcriptional activator of the sulfur metabolic network in *Saccharomyces cerevisiae*. Lacking DNA-binding ability, Met4 must interact with proteins called Met4 cofactors to target promoters for transcription. Two types of DNA-binding cofactors (Cbf1 and Met31/Met32) recruit Met4 to promoters and one cofactor (Met28) stabilizes the DNA-bound Met4 complexes. To dissect this combinatorial system, we systematically deleted each category of cofactor(s) and analyzed Met4-activated transcription on a genome-wide scale. We defined a core regulon for Met4, consisting of 45 target genes. Deletion of both Met31 and Met32 eliminated activation of the core regulon, whereas loss of Met28 or Cbf1 interfered with only a subset of targets that map to distinct sectors of the sulfur metabolic network. These transcriptional dependencies roughly correlated with the presence of Cbf1 promoter motifs. Quantitative analysis of in vivo promoter binding properties indicated varying levels of cooperativity and interdependency exists between members of this combinatorial system. Cbf1 was the only cofactor to remain fully bound to target promoters under all conditions, whereas other factors exhibited different degrees of regulated binding in a promoter-specific fashion. Taken together, Met4 cofactors use a variety of mechanisms to allow differential transcription of target genes in response to various cues.

INTRODUCTION

Combinatorial control of transcription enables integration of extracellular and intracellular signals to elicit an appropriate gene expression response. Typical examples of this type of regulation include the binding of different members of a transcription factor family to the same DNA binding site within a promoter, the use of different DNA-binding sites within a promoter to recruit different classes of transcription factors, and protein–protein interactions among transcription factors that allow promoters lacking certain DNA-binding sites to still be bound by those transcription factors. The Met4 transcriptional system, which regulates sulfur metabolism in *Saccharomyces cerevisiae*, is a simple model system to study these forms of combinatorial control. Met4 is the sole activator of the sulfur metabolic network but it is devoid of intrinsic DNA-binding ability. To reach its target promoters, Met4 interacts with DNA-binding cofactor proteins. Met4 can bind either one of two highly similar zinc finger pro-

teins, Met31 and Met32, or a homodimer of the basic-helix-loop-helix protein Cbf1. The Cbf1 homodimer binds the consensus sequence CACGTGA (referred to as a Cbf1 site), whereas Met31 and Met32 individually bind the consensus AAACTGTGGC motif (referred to as a Met31/Met32 site; Thomas *et al.,* 1989; Kuras and Thomas, 1995; Blaiseau *et al.,* 1997). Cbf1 and Met31/Met32 sites are frequently found in promoters of sulfur metabolism genes (Thomas *et al.,* 1989, 1992; Kuras and Thomas, 1995; Kuras *et al.,* 1996; Blaiseau *et al.,* 1997; Blaiseau and Thomas, 1998). Another cofactor, Met28, further stabilizes DNA-bound Met4 complexes (Kuras *et al.,* 1997; Blaiseau and Thomas, 1998). All Met4 cofactor proteins (Met31, Met32, Cbf1, Met28) lack intrinsic transcriptional activation ability and appear to act solely as adaptors for recruiting Met4 to appropriate promoters (Kuras *et al.,* 1996; Blaiseau *et al.,* 1997).

The yeast sulfur metabolic network manages the synthesis of methionine and cysteine, glutathione (an essential antioxidant for cadmium detoxification), and *S*-adenosylmethionine or AdoMet (a main cellular methyl donor that serves a precursor for the biosynthesis of polyamines, vitamins, and modified nucleotides). These compounds are synthesized through distinct branches of the sulfur biosynthetic network (see Figure 5). The sulfate assimilation pathway reduces sulfate into sulfide, the immediate precursor of the organic compound, homocysteine. Homocysteine is then utilized in two diverging biosynthetic pathways: the methyl cycle produces methionine and AdoMet, and the transsulfuration pathway produces cysteine and glutathione. Sulfur metabolism is thus involved in multiple facets of cellular metabolism and accordingly, was found to be regulated at the transcriptional level in response to a variety of environ-

mental and intracellular cues (Kent *et al.,* 1994; Thomas and Surdin-Kerjan, 1997; Fauchon *et al.,* 2002; Aranda and del Olmo, 2004; Barbey *et al.,* 2005; Yen *et al.,* 2005; Chandrasekaran *et al.,* 2006).

An important regulator of Met4 is the SCF^{Met30} ubiquitin ligase, which targets Met4 for ubiquitylation. Unusually, ubiquitylation controls Met4 activity by degradation-dependent and -independent mechanisms. When yeast cells are grown in sulfur-limited minimal medium and subsequently exposed to a high concentration of methionine, Met4 becomes polyubiquitylated and is targeted for degradation by the 26S proteasome (Rouillon *et al.,* 2000). In contrast, when cells are grown in rich medium that contains an excess of sulfur-containing compounds, Met4 is oligo-ubiquitylated such that a chain of one to four ubiquitins is added to Met4. This modification does not result in the destruction of Met4 protein (Kaiser *et al.,* 2000; Kuras *et al.,* 2002; Flick *et al.,* 2004). Instead, oligo-ubiquitylated Met4 is selectively excluded from most but not all target promoters (Kuras *et al.,* 2002). These ubiquitin-modified forms of Met4 control are lost upon exposure to the toxic heavy metal cadmium, which interferes with the ability of Met30 to target Met4 to the core E3 complex and allows activation of Met4 targets (Barbey *et al.,* 2005; Yen *et al.,* 2005). Although SCF^{Met30} may potentially target other substrates for ubiquitylation (Schumacher *et al.,* 2002; Su *et al.,* 2005), restraint of Met4 activity is the only essential function of Met30 (Patton *et al.,* 2000). Indeed, the growth arrest and lethality that results from Met30 loss is bypassed by deletion of the transactivation domain of Met4. Deletion of *MET32*, but not of *CBF1*, *MET28*, or *MET31*, also rescues *met30Δ* lethality. These findings strongly suggest that the Met4 cofactors perform distinct roles, with Met32 playing a prominent role, in Met4-activated transcription (Patton *et al.,* 2000; Su *et al.,* 2005, 2008).

Despite evidence that different Met4-cofactor complexes activate different targets, the functional roles of Met4 cofactors with respect to the entire sulfur metabolic network had remained unexplored. We thus investigated the molecular basis of combinatorial control of Met4 using a genome-wide approach. We first identified Met4-dependent transcripts that were induced under two dramatically different activating conditions for Met4: 1) Met4 hyperactivation and 2) sulfur limitation. We then compared transcriptional profiles of cells that contain all Met4 cofactors with those that lack Cbf1, Met28, or both Met31 and Met32. Met4-activated transcription relied entirely on both Met31 and Met32, whereas only a subset of genes was dependent on Cbf1 and Met28. Dependency on Cbf1 or Met28 mapped to distinct sulfur metabolic processes, separating assimilation of inorganic sulfate from the synthesis of organic compounds.

We next investigated in vivo recruitment of Met4 and its cofactors to promoters in cells grown in minimal medium under inducing and repressing conditions. Although the binding of Met4, Met31, and Met32 to target promoters is severely decreased under conditions of transcriptional repression, Cbf1 was strongly associated with the core promoter in both inducing and repressing conditions. This constitutive binding of Cbf1 appeared to promote residual binding of Met31 and Met32 at proximal sites. To further examine relationships between cofactors, we analyzed how loss of each cofactor affects remaining components with respect to mRNA/protein levels, promoter binding, and target gene transcription. Under inducing conditions, lack of Met31 and Met32 resulted in the complete loss of Met4 promoter tethering despite strong DNA binding by Cbf1 and the presence of the transcriptionally active form of Met4. In reciprocal

Table 1. Yeast strains

Strain	Relevant genotype	Source
yMT-235	*MATa, ade2-1 can1-100, his3-1,15 leu2-3,112 trp1-1 ura3*	K. Nasmyth
yMT-1465	*MATa, met31:TRP1, met32::HIS3*	Blaiseau *et al.* (1997)
yMT-1693	*MATa, met4::GAL-MET4, met30::LEU2*	Patton *et al.* (2000)
yMT-1782	*MATa, met28::LEU2*	This study
yMT-1813	*MATa, met4::TRP1*	Rouillon *et al.* (2000)
yMT-1885	*MATα, met4::GAL-MET4*	Barbey *et al.* (2005)
yMT-1886	*MATa, met4::GAL-MET4, met30::URA3, met31::LEU2, met32::TRP1*	This study
yMT-1946	*MATa, met4::GAL-MET4, met30::LEU2, cbf1::URA3*	This study
yMT-1947	*MATa, cbf1::TRP1*	Rouillon *et al.* (2000)
yMT-2029	*MATα, met4::GAL-MET4, met30::LEU2, met28::LEU2*	This study
yMT-2450	*MATa, CBF1::3HA-HIS3, sua7::SUA7^{9MYC}-TRP1*	This study
yMT-2453	*MATa, met4:: ^{HA3}MET4, sua7::SUA7^{9MYC}-TRP1*	This study
yMT-2566	*MATa, RPB3::3HA-TRP1, MET28::13MYC-HIS3*	This study
yMT-2567	*MATα RPB3::3HA-TRP1, MET31::11MYC-HIS3*	This study
yMT-2568	*MATa RPB3::3HA-TRP1, MET32::11MYC-HIS3*	This study

manner, Met32 was not bound to target promoters in the absence of Met4. Taken together, these results demonstrate that Met4 cofactors use a variety of mechanisms to allow differential transcription of target genes in response to various environmental and intracellular cues.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

All yeast strains used in this study (Table 1) are in the W303 background (*ade2-1 can1-100, his3-1,15 leu2-3112 trp1-1 ura3*). For Met4 overexpression microarray studies, wild-type, *met4::GAL-MET4,* and *met4::GAL-MET4 met30Δ, met4::GAL-MET4 met30Δ met31Δ met32Δ, met4::GAL-MET4 met30Δ cbf1Δ, met4::GAL-MET4 met30Δ met28Δ* were grown in YEP + 2% raffinose. An aliquot of cells was harvested for a t = 0 time point, and galactose was added to the remaining culture, to a final concentration of 2–3%. Cells were harvested after 15, 30, 60, and 90 min in galactose. For sulfur limitation microarray studies, wild-type, *met4Δ, met31Δ met32Δ, cbf1Δ,* and *met28Δ* strains were grown in minimal B-media (see Cherest and Surdin-Kerjan, 1992 for the composition of B-medium) supplemented with 0.5 mM methionine as the sole sulfur source. An aliquot of cells was harvested for a t = 0 time point, and the remainder were filtered through a 0.22-μm Stericup filter (Millipore, Bedford, MA) and then washed and resuspended in prewarmed (30°C) B-media lacking any source of sulfur. Cells were harvested after 20, 40, and 80 min. Strains used for chromatin immunoprecipitation were grown in 200 ml B-media supplemented with 0.05 mM methionine, filtered through a 0.22-μm Stericup filter (Millipore), and then washed and resuspended in 200 ml prewarmed (30°C) B-media lacking any source of sulfur and allowed to grow at 30°C for 1 h at which time, 100 ml of culture was cross-linked using formaldehyde (see below). Methionine was added to the remaining 100-ml culture to a final concentration of 1 mM, and the culture was cross-linked at 40 min after addition.

Microarray Analysis

Microarray analyses were conducted as previously described (Breitkreutz *et al.,* 2001a,b). The raw and normalized data are available at http://www.ebi.ac.uk/microarray-as/ae/ under accession numbers E-MEXP-2427 and E-MEXP-2429.

Western Analysis

Western analyses were conducted as previously described (Rouillon *et al.,* 2000). Standard procedures were used for SDS-PAGE, semidry transfer to
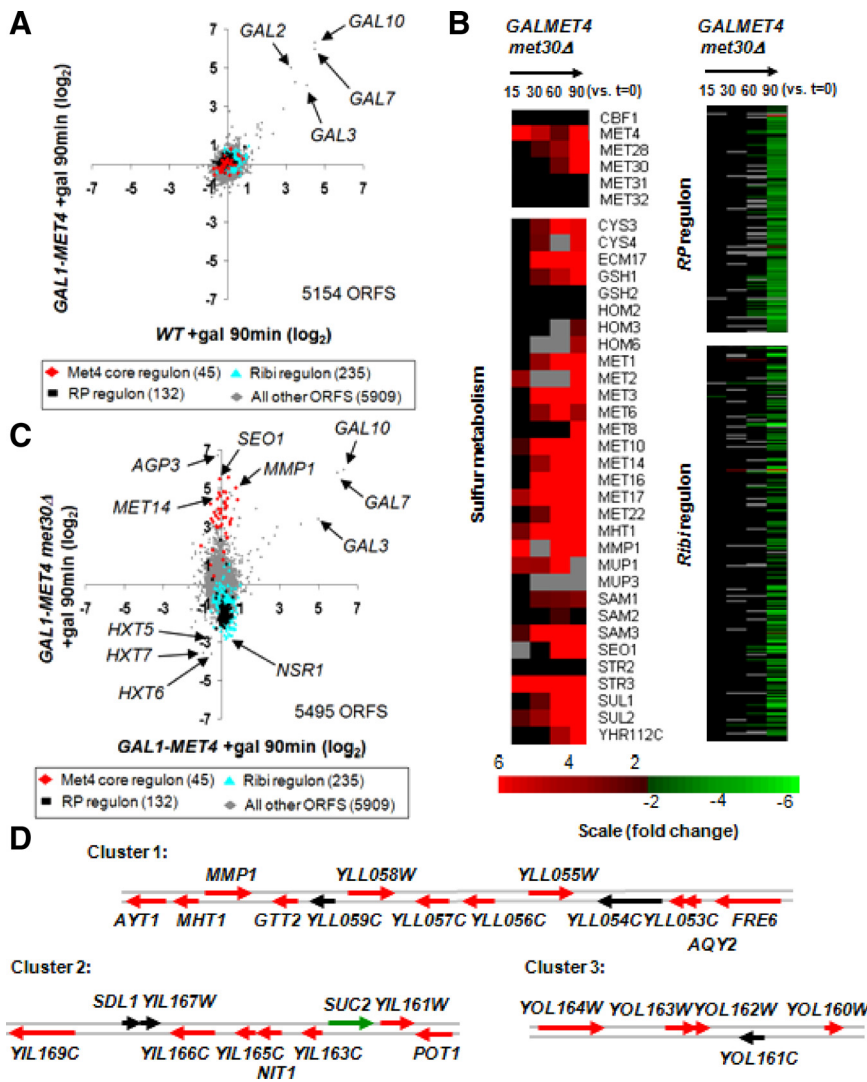
Figure 1. Functional gene clusters affected by Met4 hyperactivation. (A) Scatter plot comparing microarray profiles of wild-type and *met4::GAL1-MET4* cells after 90 min of galactose induction in rich media. Expression data are represented on a log$_2$ scale. The number of ORFs compared is indicated at bottom right. (B) Microarray profiles of *met4::GAL1-MET4 met30Δ* cells harvested at 15, 30, 60, and 90 min after galactose induction compared with cells harvested before galactose induction. Inductions and repressions greater than twofold are marked by red and green boxes, respectively. Less than twofold changes in transcription are represented by black boxes, and unreliable measurements are marked by gray boxes. Note: Partial *MET30* transcripts are detected because the *met30Δ* strain was constructed by an internal disruption of the gene (Thomas *et al.,* 1995). (C) Scatter plot comparing microarray profiles of *met4::GAL1-MET4* and *met4::GAL1-MET4 met30Δ* cells after 90 min of galactose induction in rich media. (D) Schematic of three chromosomal clusters induced in the *met4::GAL1-MET4 met30Δ* strain. Red arrows, at least a twofold induction; green arrows, at least a twofold repression; black arrows, no significant change.

PVDF membranes, and immunoblotting. 12CA5 and 9E10 monoclonal antibodies were produced as ascites fluid (Kolodziej and Young, 1991) and used at a 1:10,000 dilution. Polyclonal antibodies were raised against Met28, Met32, and Cbf1 (Ausubel *et al.,* 1995).

Chromatin Immunoprecipitation Analysis

Chromatin Immunoprecipitation (ChIP) analyses were conducted as previously described (Kuras *et al.,* 2002). Anti-HA (F-7) and anti-c-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Rpb1 (8WG16) antibodies were purchased from NeoClone Biotechnology (Madison, WI). Multiplex real-time quantitative PCR on ChIP samples was conducted as previously described (Jorgensen *et al.,* 2004). See Supplemental Materials for sequence of real-time primers and probes. Relative promoter binding of each transcription factor was compared as a color scale, with yellow indicating binding and blue, no binding. For each tagged or untagged protein on which ChIP was conducted, ChIP efficiency for all promoters was represented relative to the highest captured promoter, which was assigned a value of 100.

RESULTS

Genome-wide Identification and Characterization of Met4 Transcriptional Targets

To identify a complete set of true Met4 target genes, we generated and analyzed genome-wide expression profiles using two distinct conditions in which Met4 is active: 1) Met4 hyperactivation, in which Met4 is overexpressed in the absence of Met30, and 2) sulfur limitation by growth on defined minimal B-media. These conditions have been established as bona fide activating conditions for Met4 with respect to several target transcripts (Thomas and Surdin-Kerjan, 1997; Patton *et al.,* 2000; Barbey *et al.,* 2005). For Met4 hyperactivation, we induced Met4 expression from the *GAL1* promoter in *met30Δ* cells and profiled samples at 15, 30, 60, and 90 min after induction. In the presence of Met30, overexpression of Met4 in rich media produces a profile that is identical to that of wild-type cells grown under the same conditions (Figure 1A). In rich media, most Met4 target genes are not expressed because of SCF^{Met30}-mediated oligo-ubiquitylation of Met4 (Kuras *et al.,* 2002). In contrast, in the absence of Met30, Met4 expression elicits a robust transcriptional response in rich media, leading to a twofold or greater induction of 400 genes (excluding galactose-responsive genes) and repression of 526 genes, by 90 min of induction (Figure 1, B and C).

As expected, most sulfur metabolism genes are induced upon Met4 hyperactivation with high expression early in the time course (Figure 1B). Genes for ribosomal proteins (RP), ribosome biogenesis (Ribi), and hexose transport (*HXT*) are repressed upon Met4 hyperactivation (Figure 1, B and C). Repression of RP and Ribi genes is characteristic of a stress

response (Gasch *et al.,* 2000; Causton *et al.,* 2001; Jorgensen *et al.,* 2004) and is consistent with Met4 hyperactivation causing a G1 arrest and lethality with defects in translation (Patton *et al.,* 2000). A detailed composite of functional categories for genes in the induced and repressed gene sets from the Met4 hyperactivation profile can be found in Supplemental Materials (Supplemental Figure S1).

The microarray analyses also identified three chromosomal regions that are enriched for Met4-induced genes: 1) *YLL051C- YLL063C* (Ahmed Khan *et al.,* 2000; Zhang *et al.,* 2001), 2) *YIL160C- YIL169C,* and 3) *YOL160W-YOL164W* (Figure 1D). Interestingly, these clusters contain genes involved in processing alternative sources of sulfur such as sulfonates (Supplemental Table S1). The process of sulfonate assimilation is similar in bacteria and yeast (Uria-Nickelsen *et al.,* 1993). Because this peculiar gene organization is reminiscent of a bacterial operon, some of these loci may have bacterial origins. This hypothesis is supported by one gene in the third cluster, *YOL164W* (*BDS1*), which has been shown to be bacterially derived via horizontal transfer (Hall *et al.,* 2005).

We next analyzed the yeast transcriptional response to sulfur limitation. Wild-type cells were grown in minimal B-medium supplemented with 0.5 mM methionine as the sole sulfur source and the cells were profiled at 20, 40, and 80 min after methionine removal. Although the repression of the RP, Ribi, and *HXT* genes found upon Met4 hyperactivation did not occur upon sulfur limitation, both conditions caused a Met4-dependent induction of 45 target genes (Figure 2, A and B), which we define as the Met4 core regulon. As expected, the majority of the core regulon (34 targets) are genes involved in sulfur metabolism, whereas the other targets either relate indirectly to sulfur metabolism (*SER33, BNA3, NIT1, ZWF1*) or are involved in stress response (*RAD59* and *HIT1*). Supplemental Table S2 details the functions of each core regulon gene. Consistent with findings that Met4 is important for sulfur sparing in response to cadmium treatment (Baudouin-Cornu *et al.,* 2001; Fauchon *et al.,* 2002), the core regulon encodes proteins that contain lower sulfur content than the averaged sulfur content of the proteome (Supplemental Figure S2).

As expected, *MET28* and *MET30,* two known targets of Met4 (Blaiseau *et al.,* 1997; Kuras *et al.,* 1997, 2002; Blaiseau and Thomas, 1998; Rouillon *et al.,* 2000), were induced upon Met4 hyperactivation (a partial *MET30* transcript produced in the *met30Δ* cells allows for measurement of *MET30* mRNA), whereas transcription of the *CBF1, MET31,* and *MET32* genes remained unchanged (Figure 1B). Analysis of the Met4 core regulon promoters (regions −500 to −1 relative to each open reading frame [ORF]) by the MEME motif discovery program (Bailey and Elkan, 1994) revealed the binding motifs for Cbf1 and Met31/Met32 (Figure 3, A and B). The detected Cbf1 motif was an invariant sequence of CACGTGA in 24 of the 45 core regulon promoters that was identical to the previously identified Cbf1 site (Thomas *et al.,* 1989; Kuras and Thomas, 1995). The adenine at the 3′ end the typical E-box CACGTG sequence is consistent with published differences in binding specificities for Cbf1 and Pho4, another bHLH transcription factor (Fisher and Goding, 1992; Shimizu *et al.,* 1997). All 45 core regulon promoters contain a Met31/Met32 binding site that consists of a CTGTGGC motif flanked by highly variable nucleotides. This variability in flanking sequences may reflect either a general promiscuity in DNA binding by this family of transcription factors and/or differences in binding specificities between Met31 and Met32. The core Met31/Met32 binding sequence is an abbreviated version of the previously identified consensus sequence AAACTGTGGC (Thomas *et al.,* 1989; Blaiseau *et al.,*
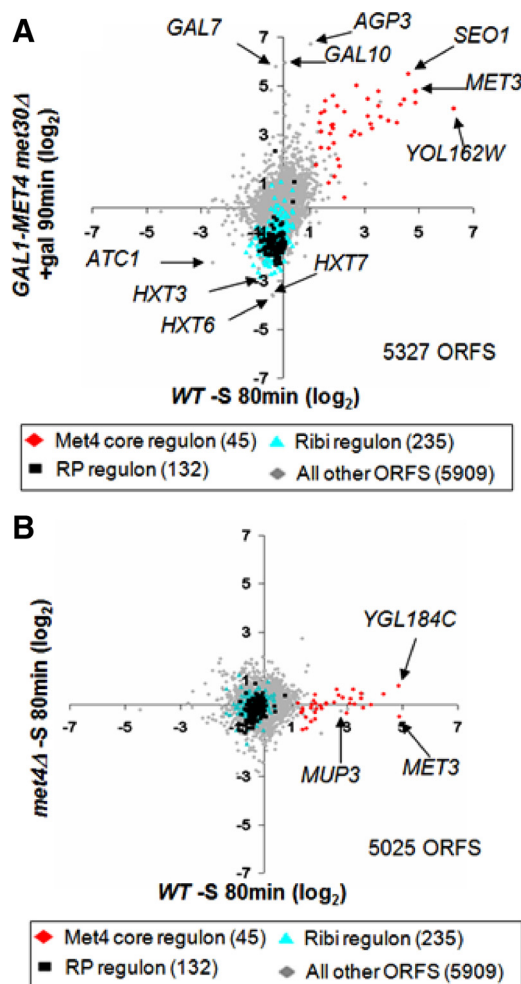

Figure 2. Comparison of microarray profiles. (A) Scatter plot comparing microarray profiles of wild-type cells at 80 min after methionine removal in minimal media and *met4::GAL1-MET4 met30Δ* cells at 90 min after galactose induction in rich media. (B) Scatter plot comparing microarray profiles of wild-type and *met4Δ* cells at 80 min after methionine removal in minimal media.

1997). A comparative genome analysis of related *Saccharomyces* species reported a similar consensus site of SKGTGGSG (where S = C or G; K = G or T; Kellis *et al.,* 2003).

Using the MEME consensus motifs, we next conducted a genome-wide promoter search for Cbf1 and Met31/Met32 sites using the MAST bioinformatics program (Bailey and Gribskov, 1998). With a low stringency E-value cutoff of 500, we identified a maximum of 441 promoters that contain Cbf1 sites and 500 promoters that contain Met31/Met32 sites. Using this MAST criterion, 37 of the 45 core regulon promoters qualified as containing Met31/Met32 motifs (Figure 3C). Because MEME identified Met31/Met32 sites in all 45 core regulon promoters, MAST failed to detect these motifs in eight promoters. Given the high variability of the MEME consensus Met31/Met32 motif (Figure 3B), it is clear that a wide range of sequences allow binding of Met31 and/or Met32. The eight promoters were likely missed by the MAST search because they contain weaker matches to the consensus. The MEME identification of Met31/Met32 motifs in all core regulon promoters is an indicator that true targets of Met4 will contain some version of a Met31/Met32 motif (even if they are not recognized by various search
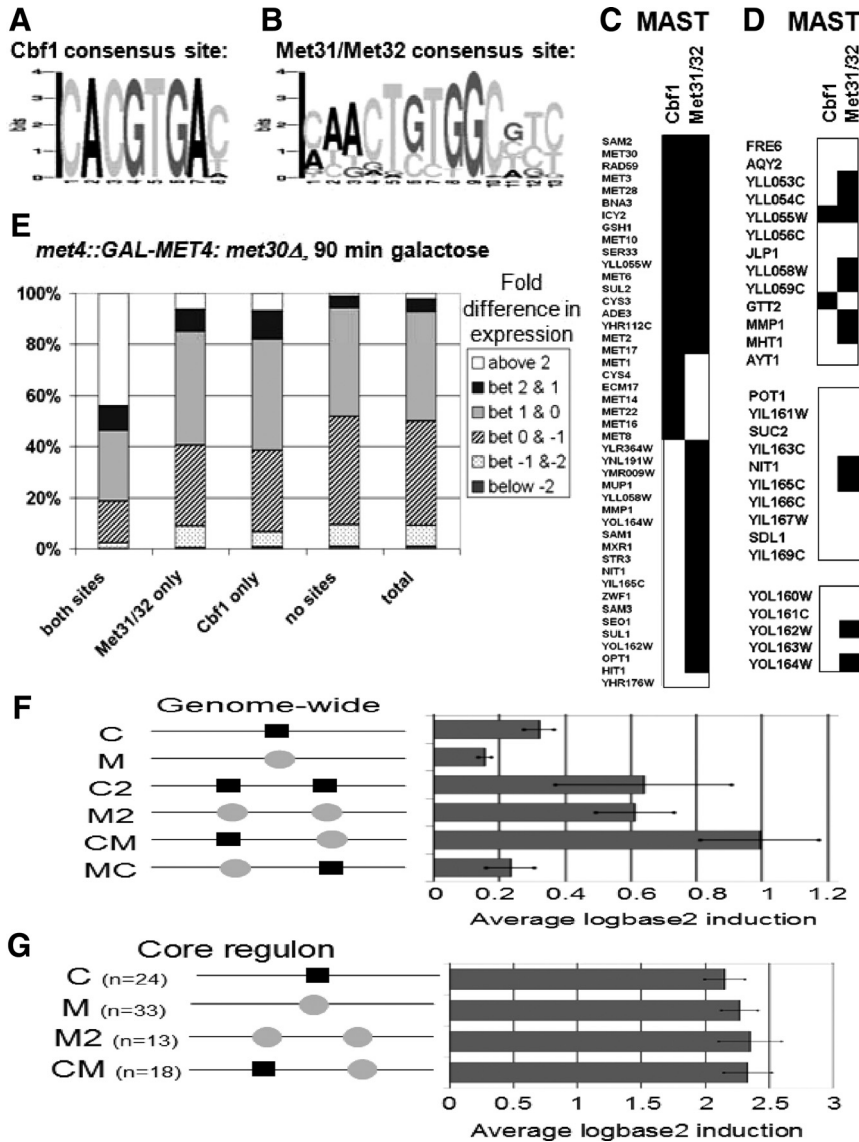
Figure 3. Analysis of Cbf1 and Met31/Met32 binding motifs. MEME PSSMs for (A) Cbf1 and (B) Met31/Met32 binding motifs (determined from regions −500 to −1 relative to each ORF). MAST identification of Cbf1 and Met31/Met32 binding sites (within regions −950 to +50 relative to each ORF) for (C) the Met4 core regulon and (D) the *GAL1-MET4* chromosomal clusters. Genes with MAST matches above an E-value of 500 are marked by black boxes. (E) Correlation between transcriptional induction upon Met4 hyperactivation (as determined by microarray) and promoter presence of Met31/Met32 and Cbf1 sites (as determined by MAST). (F) Averaged $\log_2$ induction levels upon both Met4 hyperactivation and sulfur limitation for six previously identified sets of genes based on promoter composition (Chiang *et al.*, 2006). C and M sites represent exact matches to Cbf1 (TCACGTG) and Met31/32 (TGTGGC) motifs, respectively. (G) Averaged $\log_2$ inductions for core regulon targets categorized in four of six promoter categories upon both Met4 hyperactivation and sulfur limitation.

programs). This false negative problem did not exist for the mostly invariant Cbf1 motif. With respect to the Met4 chromosomal clusters (Figure 1D), MAST detected multiple Met31/Met32 binding sites but only identified two *YLL* promoters with Cbf1 elements (Figure 3D).

Of the ∼400 transcripts induced by Met4 hyperactivation, only 84 genes contained promoters with Cbf1 sites, and only 90 genes had promoters with Met31/Met32 sites (Supplemental Figure S4, A and B). Moreover, only 49% of promoters containing Cbf1 and Met31/Met32 sites as determined by the MAST algorithm were induced upon Met4 hyperactivation (Supplemental Figure S4C). Yeast transcription factor binding occurs within the first 600 bp upstream of the start of the ORF (Lee *et al.*, 2002). Other studies indicate that Met4 binding occurs between −100 and −450 with respect to the gene start (Chiang *et al.*, 2006; Shultzaberger *et al.*, 2007). Because MAST automatically searches −950 to +50, some promoters identified by MAST as hits will be false positives because their motifs are located in physiologically irrelevant positions. In support of this reasoning, MAST hits that contain the Met31/Met32 motif between −100 and −450 were significantly (p = 4.89×10^{-7}) more induced

upon Met4 hyperactivation than MAST hits that do not contain a Met31/Met32 motif within this region (Supplemental Figure S5). Despite these false positives and false negatives, we analyzed our transcriptional profiles with respect to different promoter compositions based on MAST. Eighteen of the 45 core regulon promoters contained both Cbf1 and Met31/Met32 sites (Figure 3C). Consistently, a comparative genome study indicated 46% of regions containing Met31/Met32 motifs also contain Cbf1 motifs (Kellis *et al.*, 2003). As expected, promoters identified by MAST to contain both Met31/Met32 and Cbf1 motifs have the strongest correlation with Met4-dependent transcription compared with promoters that contain only Met31/Met32 or only Cbf1 sites (Figure 3E). Previous studies of endogenous and synthetic promoters have determined that transcription is highest when the Cbf1 site is upstream of Met31/Met32 site (Chiang *et al.*, 2006; Shultzaberger *et al.*, 2007). To determine if we observed the same phenomena with our microarrays, we used the same promoter groupings from Chiang *et al.* (Figure 3F). These promoter categories are based on genome-wide searches within a 500-base pair region upstream of the gene start and are overlapping with the broadest
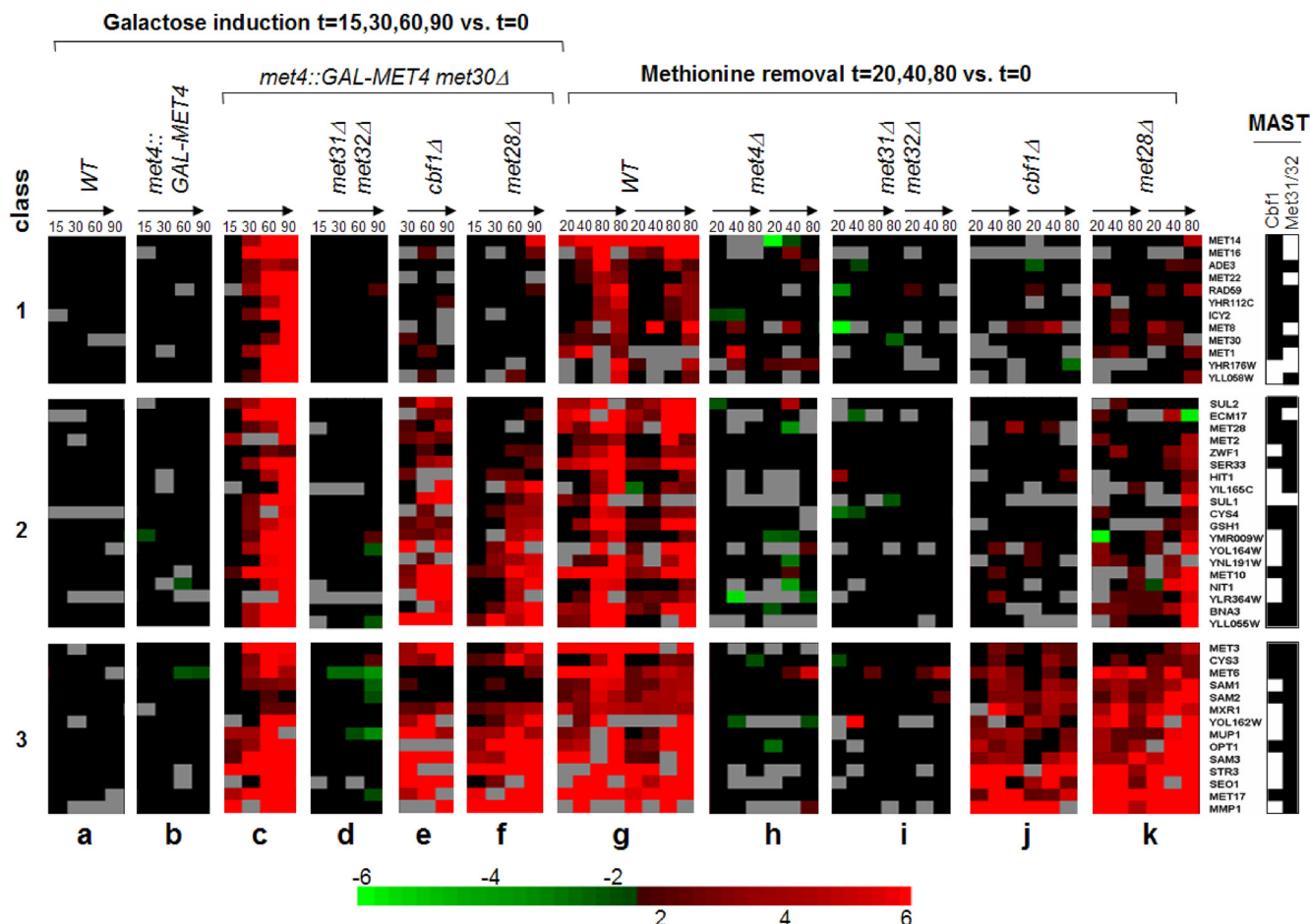
Figure 4. Microarray profiles of Met4 core regulon transcripts in wild-type and cofactor deletion strains upon Met4 hyperactivation and sulfur starvation. Inductions and repressions greater than twofold are marked by red and green boxes, respectively. Black boxes, less than twofold changes in transcription; gray boxes, unreliable measurements. Core regulon genes are clustered into three classes (far left) based on Cbf1/Met28-dependency. Columns a–f were harvested at 15, 30, 60, and 90 min after a shift from raffinose to galactose in rich media. Columns g–k were harvested at 20, 40, and 80 min after removal of methionine from minimal media. Microarray profiles represent fold-change over transcript levels found before galactose shift or methionine removal. Microarray profiles for two independent time courses are shown for the methionine-removal studies. Rightmost column uses black boxes to represent promoters with matches to Met31/Met32 and Cbf1 motifs as determined by MAST.

categories, termed C and M, containing at least one exact match to a TCACGTG Cbf1 (C) site or a TGTGGC Met31/Met32 (M) site, respectively. The C2 and M2 categories contain at least two exact matches to TCACGTG or TGT-GGC, respectively. The CM and MC categories contain one exact match for one sequence upstream of another. Like the previous studies, the CM category exhibited the highest induction upon Met4 hyperactivation and sulfur limitation (Figure 3F). However, when we limited the analysis to the core regulon, the CM promoters did not differ from other promoter categories that are well represented in the regulon (Figure 3G). An explanation for this discrepancy may be that the genome-wide set of CM promoters contains the highest fraction of true targets, causing the highest averaged induction under Met4 inducing conditions. The CM configuration is found in many regulon promoters (n = 18), whereas the MC configuration is found in only one regulon promoter.

To further evaluate the relative importance of each cofactor in Met4-activated transcription, we profiled cells that lacked Cbf1, Met28, or both Met31 and Met32 under the same growth conditions. In the absence of both Met31 and Met32, the induction of Met4 target genes was completely

lost under both activating conditions (Figure 4, columns d and i). In contrast, the core Met4 regulon exhibited varying levels of dependency on Cbf1 and Met28 (Figure 4, columns e, f, j, and k). We therefore sorted the Met4 core regulon into three classes based on the level of dependency for Met28 and Cbf1 (Figure 4, far left). Class 1 comprised genes whose transcription was strictly dependent on Cbf1 and Met28 in both growth conditions. Class 2 genes displayed intermediate dependency upon Met28 and Cbf1. In general, activation of class 2 transcripts required functional Cbf1 and Met28 cofactors under the conditions of sulfur limitation but did not require Met28 and Cbf1 under the stronger activating conditions of Met4 hyperactivation. Class 3 target genes were induced independently of either Cbf1 or Met28, regardless of the inducing conditions.

Mapping these three classes of Cbf1 dependency with respect to their biochemical pathways yielded the following distinctions between sulfur metabolic processes: i) genes required for the uptake and reduction of inorganic sulfate were Cbf1/Met28-dependent, ii) genes required for the biosynthesis of homocysteine, methionine, and AdoMet (the methyl cycle) were Cbf1/Met28-independent, and iii) genes
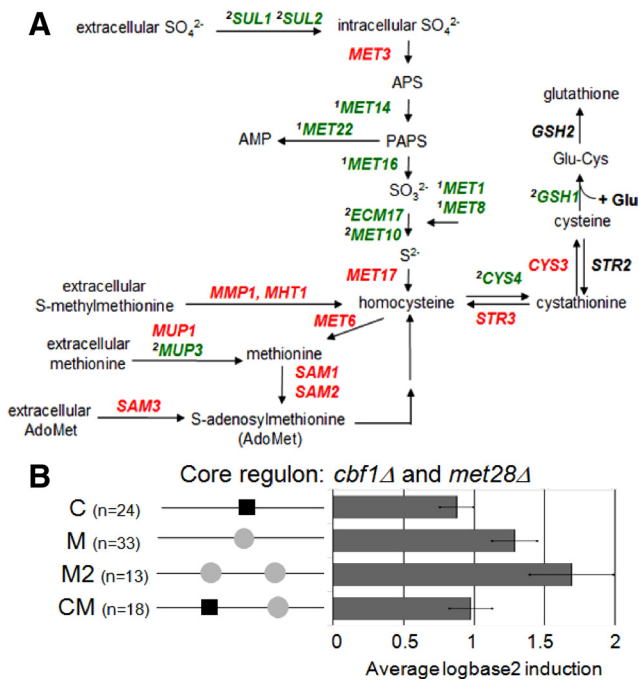
Figure 5. (A) Schematic of the sulfur assimilation pathway (adapted from Thomas and Surdin-Kerjan, 1997). Cbf1-dependent class 1 and class 2 genes are green, with the class number listed as a superscript, and Cbf1-independent class 3 genes are red. Nontarget genes are black. (B) Averaged $\log_2$ inductions for core regulon targets categorized in four of six promoter categories upon both Met4 hyperactivation and sulfur limitation in cells with a *cbf1Δ* or *met28Δ* background.

required for the biosynthesis of cysteine and glutathione (the transsulfuration pathway) comprised of both Cbf1/Met28-dependent and -independent genes (Figure 5A). These distinctions indicate that Cbf1 and Met28 are required for the

sulfate assimilation portion of yeast sulfur metabolism. This hypothesis is further supported by studies that show that *cbf1Δ* and *met28Δ* cells are unable to grow in the presence of inorganic sulfur as the sole sulfur source (Thomas *et al.*, 1992; Thomas and Surdin-Kerjan, 1997).

Dependence on Cbf1 correlated with, but did not strictly correspond to, the presence of Cbf1-binding motifs (Figure 4, far right). Class 1 promoters comprised the highest percentage of promoters with Cbf1 motifs and class 3 promoters contained the lowest. These data suggest that, although direct binding of Cbf1 to its motif is important for Cbf1-dependent promoters, other factors affect whether a promoter is Cbf1-dependent. One factor appears to be the presence of a low stringency Met31/Met32 motif. Of the eight promoters in which MAST did not recognize Met31/Met32 motifs, six are Cbf1-dependent class 1 targets (Figure 4, far right). The two other false negatives are in class 2. As expected, the M and M2 categories of regulon targets exhibited higher averaged inductions in *cbf1Δ* and *met28Δ* cells than the C and CM categories (Figure 5B). All four analyzed categories of regulon promoters contain relatively equal distributions of the three Cbf1-dependency classes (data not shown).

Of all Met4 cofactors, only deletion of *MET32* bypasses *met30Δ* lethality (Patton *et al.*, 2000; Su *et al.*, 2008). We found that deletion of *MET32* alone, or both *MET31* and *MET32*, partially rescued the growth inhibition from combined Met30 loss and nonphysiologically high levels of active Met4 (Figure 6A and data not shown). Loss of both Met31 and Met32 abolished the signature of the Met4 hyperactivation profile with respect to the core Met4 regulon, the RP regulon, and the Ribi regulon (Figure 6B). The only transcriptional feature retained in the absence of Met31/Met32 was the repression of the hexose transport (*HXT*) genes. Interestingly, when the *met4::GAL1-MET4, met30Δmet31Δmet32Δ* profile was compared with a *met4::GAL1-MET4* profile, glycolysis genes were selectively repressed when Met30, Met31, and Met32 were absent (Figure 6C). Repression of glycolysis may be a response to stress (Gasch, 2002). It is possible that
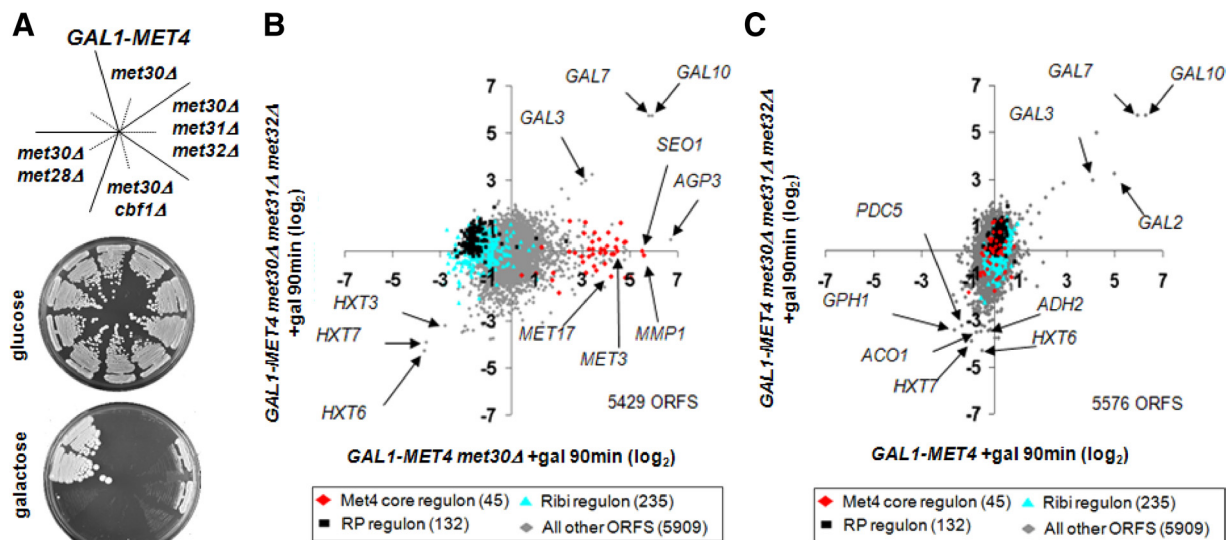

Figure 6. Loss of both Met31 and Met32 blocks the Met4 transcriptional response and partially rescues *met4::GAL1-MET4 met30Δ* lethality. (A) Different strains with *met4::GAL1-MET4* background were streaked onto rich-media plates containing glucose or galactose. (B) Scatter plot comparing microarray profiles of *met4::GAL1-MET4 met30Δ* cells with *met4::GAL1-MET4 met30Δmet31Δmet32Δ* cells after 90 min of galactose treatment in rich media. (C) Scatter plot comparing microarray profiles of *met4::GAL1-MET4* cells with *met4::GAL1-MET4 met30Δmet31Δmet32Δ* cells after 90 min of galactose treatment in rich media.

gross overexpression of active Met4 in the absence of Met31/Met32 promoter platforms causes Met4 to squelch the general transcriptional machinery to cause defects independent of Met4 target gene expression.

Met28, Met31, and Met32 Exhibit Decreased Promoter Occupancy upon Repression, whereas Cbf1 Remains Constitutively Bound

To further characterize the function of each Met4 cofactor, we examined in vivo binding of Cbf1, Met28, Met31, and Met32 to target promoters and how it relates to recruitment of Met4 and the general transcriptional machinery. ChIP experiments were performed under conditions of Met4 activation and repression. Cells were first starved of methionine for 1 h (Met4 activation) and subsequently were exposed to 1 mM methionine for 40 min (Met4 repression). To be certain of promoter occupancy by Met4 and its cofactors, ChIP experiments were performed using two approaches. The first approach used monoclonal anti-hemagglutinin (HA) and anti-Myc antibodies to detect chromosomally-tagged [HA]Met4, Cbf1[HA], Met28[Myc], Met31[Myc], and Met32[Myc], whereas the second approach used polyclonal antibodies raised against Met4, Cbf1, Met28, and Met32 to detect the unmodified transcription factors. Recruitment of the general transcription machinery was assessed by the detection of RNA polymerase II (using an antibody that detects unphosphorylated Ser2 residues within the CTD of Rpb1 that is characteristic of the nonelongating form or Rpb3[HA]) or general transcription factors (TFIIB[Myc]).

Immunoblots showed that Rpb3, TFIIB, Cbf1, Met31, and Met32 protein levels remain unchanged upon activation and repression in minimal medium, whereas both Met4 and Met28 protein levels decrease upon methionine exposure (Figure 7, A and B). The decrease in Met4 and Met28 levels is consistent with previous findings that Met4 is ubiquitylated and rapidly degraded by the proteasome upon methionine exposure after sulfur limitation (Rouillon *et al.*, 2000; Kuras *et al.*, 2002) and that *MET28* is regulated at the level of transcription by Met4 (Blaiseau and Thomas, 1998). We observed that Met4 is phosphorylated in the absence of methionine, (Figure 7A, 7B, data not shown), consistent with this being the transcriptionally active form of Met4 (Kaiser *et al.*, 2000; Flick *et al.*, 2004; Barbey *et al.*, 2005). Met4, Cbf1, Met28, Met31, and Met32 occupancy was measured at 16 different target promoters under both induction and repression conditions. In addition to being functionally diverse, these targets represent all three classes of Cbf1/Met28 dependency and different promoter compositions, induction strengths, and induction kinetics (Figure 4; Supplemental Table S2 and Figures S6 and S7).

We first confirmed the fidelity of our quantitative ChIP assay by measuring promoter recruitment of RNA polymerase II in wild-type, met4Δ, met31Δmet32Δ, cbf1Δ, and met28Δ cells. Under inducing conditions, patterns of Rpb1 recruitment mirrored microarray transcription profiles (Figure 7C; Supplemental Figure S6). As expected, class 3 genes showed the strongest recruitment of Rpb1 in cbf1Δ and met28Δ cells (Figure 7C, class panel). Also, Rpb1 occupancy at the 16 Met4 target promoters was severely decreased upon repression with methionine, whereas Rpb1 occupancy remained unaffected at the constitutively expressed nontarget control promoters *ACT1* and *PGK1* (Figure 8A, second panel; Supplemental Figure S11). Similarly, promoter occupancy for tagged TFIIB[Myc] and Rpb3[HA] correlated strongly with transcriptional induction (Figure 8A, second panel; Supplemental Figure S12). Consistent with our previous ChIP analyses (Kuras *et al.*, 2002; Barbey *et al.*, 2005), high levels of Met4
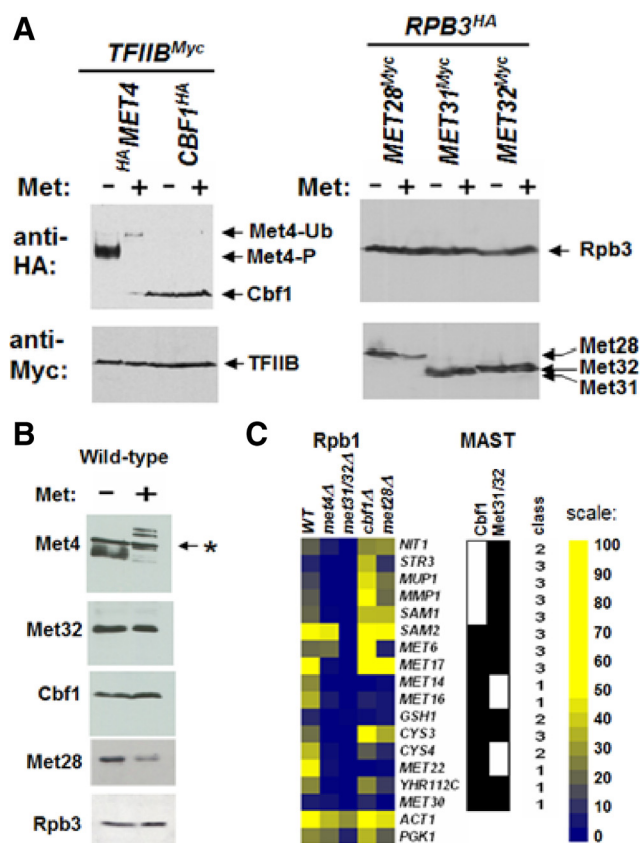

Figure 7. (A) Western analysis of epitope-tagged Met4, Cbf1, Met28, Met31, Met32, TFIIB, and Rpb3 in minimal media at 1 h after methionine removal (−) and at 40 min after subsequent addition of methionine (+). (B) Western analysis of untagged Met4, Cbf1, Met28, Met32, and Rpb3 using same growth conditions. Asterisk (*) indicates a nonspecific cross-reactive protein. (C) ChIP promoter binding properties for Rpb1 at 1 h after methionine removal in wild-type and various deletion strains. Promoter binding is represented on a yellow-blue color scale relative to the highest captured promoter detected for Rpb1, which is set at 100. The MAST panel uses black boxes to identify promoters that were detected by MAST to contain Cbf1 and/or Met31/Met32 motifs. Column labeled class indicates category of Cbf1/Met28 dependency (see text).

occupancy were measured at the 16 target promoters after methionine removal, whereas a dramatic decrease in Met4 occupancy was measured at the same promoters upon methionine exposure (Figure 8A, third and fourth panels; Supplemental Figure S9).

Like Met4, the promoter occupancy for Met28, Met31, and Met32 dramatically decreased upon methionine exposure (Figure 8A, third and fourth panels, and B; Supplemental Figure S10). The decrease in Met28 occupancy levels is expected as a consequence of decreased Met28 protein because *MET28* is a Met4 target gene (Figure 7, A and B). Met31 and Met32 exhibit similar promoter binding profiles in wild-type cells and bind target promoters in a regulated manner (Figure 8A, third and fourth panels). Because Met31 and Met32 protein levels do not appear to be significantly decreased upon repression compared with their levels detected upon induction (Figure 7, A and B), we speculate that posttranslational modification may prevent these factors from binding DNA.

For the 16 target promoters investigated, Cbf1 only bound promoters that contained Cbf1 sites. This finding strongly
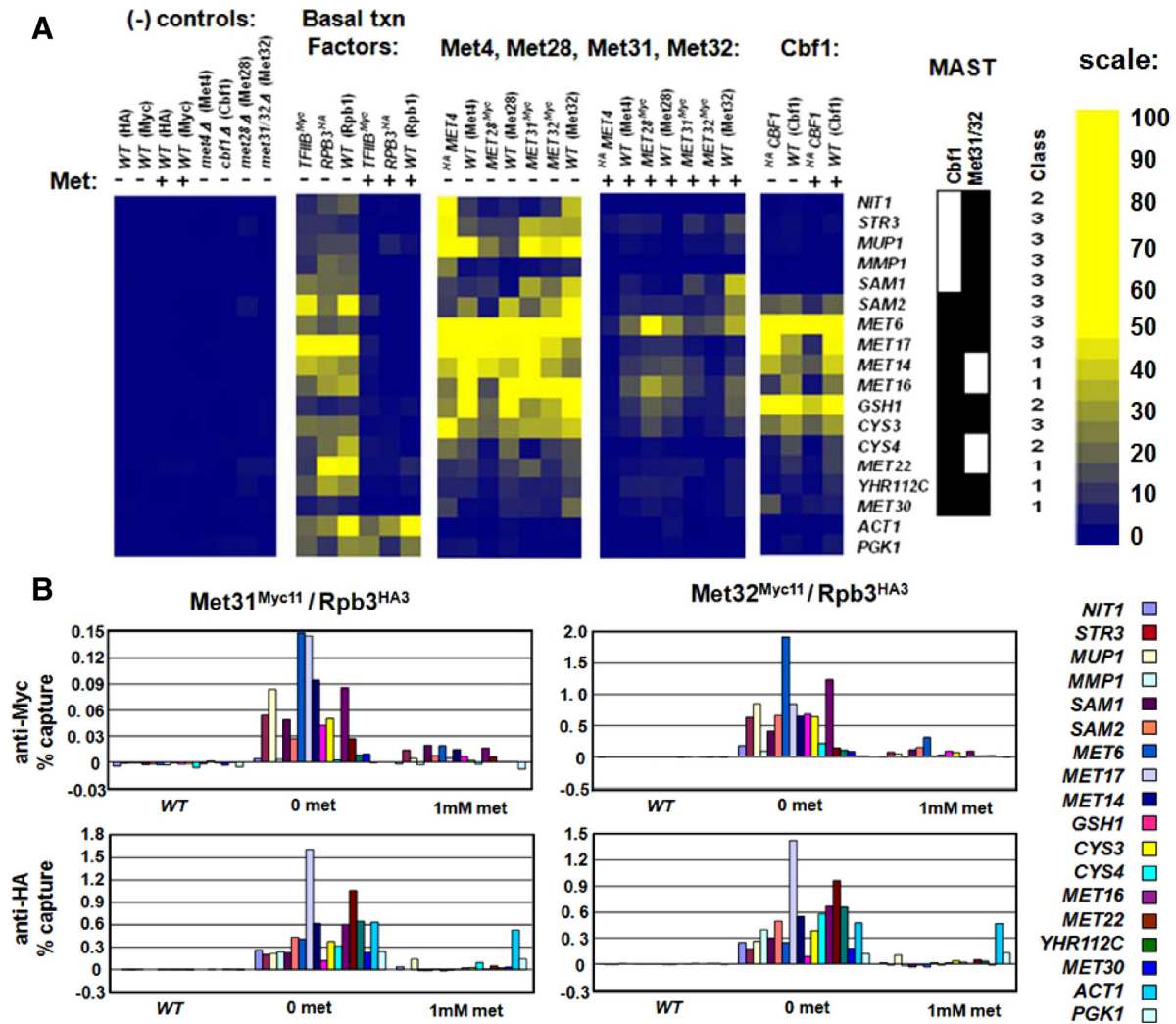
Figure 8. (A) ChIP promoter binding properties for Met4, Met28, Met31, Met32, Cbf1, TFIIB, Rpb3, and Rpb1. A color scale (right column) shows promoter binding relative to the highest percent capture detected for each immunoprecipitated factor (Met4[HA], Met4, Met28, Met28[Myc], Met31[Myc], Met32, Met32[Myc], Cbf1, Cbf1[HA], TFIIB[Myc], Rpb3[HA], and Rpb1), which is arbitrarily set at 100. TFIIB[Myc] binding is the average of TFIIB[Myc] binding values from [HA]Met4/TFIIB[Myc] and Cbf1[HA]/TFIIB[Myc] ChIP, and Rpb3[HA] binding is the average of Rpb3[HA] binding values from Met28[Myc]/Rpb3[HA], Met31[Myc]/Rpb3[HA], and Met32[Myc]/Rpb3[HA] ChIP. The MAST panel uses black boxes to identify promoters that were detected by MAST to contain Cbf1 and/or Met31/Met32 motifs. Column labeled class indicates category of Cbf1/Met28 dependency. (B) ChIP of Met31[Myc]/Rpb3[HA] and Met32[Myc]/Rpb3[HA] cells. Percent capture is represented as the percentage of the total promoter available. Samples were harvested at 1 h after methionine removal from B-media (0 met, second bar grouping) and at 40 min after subsequent treatment with 1 mM methionine (1 mM met, third bar grouping). Background percent capture levels were determined in untagged wild-type cells upon methionine starvation (first bar grouping).

indicates that Cbf1 requires its own motif to bind promoters and cannot be recruited indirectly to promoters through interactions with Met4 tethered via a Met31/Met32 motif. In addition, Cbf1 promoter binding was unexpectedly maintained at high levels under both inducing and repressing conditions (Figure 8A, fifth panel; Supplemental Figure S8). Cbf1 promoter binding is independent of transcriptional activation, as demonstrated by the presence of TFIIB[Myc] in the absence of methionine and the dramatic decrease in TFIIB[Myc] occupancy upon methionine addition at the promoters in the identical samples. In addition to its role in *MET* gene transcription, Cbf1 assists in proper centromere function by binding to its consensus element at centromeres (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990). To determine if modulation of sulfur metabolism affects the ability of Cbf1 to bind these non-Met4 targets, we investigated Cbf1 binding at centromeric regions,

CEN3 and *CEN6*. As expected, binding to *CEN3* and *CEN6* was maintained independent of sulfur status (Supplemental Figure S8).

Although both Met31 and Met32 exhibited similar profiles of promoter binding, the absolute percent capture values for Met32[Myc] were approximately 10-fold higher than those for Met31[Myc] (Figure 8B). Because anti-Myc immunoblots indicate similar protein levels for Met32[Myc] and Met31[Myc] (Figure 7, A and B), this ChIP result suggests Met32 interacts with promoters more avidly than Met31, despite no obvious differences in the profile of promoters that are bound by each cofactor. Cells lacking Met32 have a large cell phenotype and bypass *met30Δ* lethality, whereas *met31Δ* cells lack these features (Patton *et al.*, 2000; Jorgensen *et al.*, 2002). In light of these data, the phenotypic distinctions between *met31Δ* and *met32Δ* cells may be due to Met32 comprising the vast majority of Met4-bound promoter complexes. If this
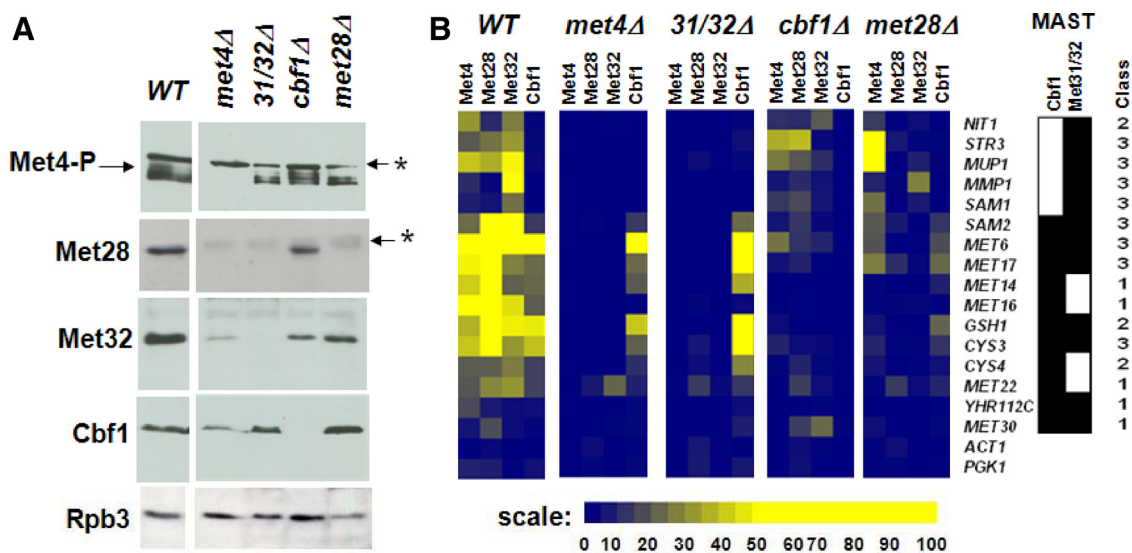
Figure 9. (A) Western analysis of untagged Met4, Cbf1, Met28, Met32, and Rpb3 from wild-type and deletion strains grown in minimal media at 1 h after methionine removal. Asterisk (*) indicates a nonspecific cross-reactive protein. (B) ChIP promoter binding properties for Met4, Met28, Met32, and Cbf1 in minimal media at 1 h after methionine removal for wild-type and deletion strains. Promoter binding is represented on a yellow-blue color scale relative to the highest percent capture detected for each antibody, which was arbitrarily set at 100. The MAST panel uses black boxes to identify promoters that were detected by MAST to contain Cbf1 and/or Met/Met32 motifs. Column labeled class indicates category of Cbf1/Met28 dependency.

was the case, Met31 would be unable to bind promoters to the same extent as Met32, even in the absence of Met32. Also, the high level of variability within the Met31/Met32 motif may reflect promiscuous motif selection by Met32 (and Met31), rather than distinct motif preferences between Met31 and Met32.

Consistent with its role as an accessory factor, Met28 promoter occupancy correlates with the promoter presence of Cbf1, Met31, or Met32 (Figure 8A, third and fourth panels; Supplemental Figure S10). Although Met4, Met28, Met31, and Met32 exhibited reduced binding under repressing conditions, residual promoter binding was detected at some promoters. Interestingly, these promoters corresponded to ones that displayed high levels of Cbf1 (Figure 8A, fifth panel). Cooperative interactions may thus allow residual binding by Met31 or Met32 at promoters where Cbf1 is present. In support of this idea, target promoters that only contain Met31/Met32 sites generally exhibited lower recruitment for Met28, Met4, and general transcription factors upon sulfur starvation compared to other target promoters (Figure 8A). Consistent with our transcriptional analyses (Figure 3E) and previous studies (Kuras *et al.*, 1997; Blaiseau and Thomas, 1998; Chiang *et al.*, 2006), promoters containing both Cbf1 and Met31 or Met32 constitute higher affinity platforms for Met4 and, therefore, the general transcription machinery. Also, strong Cbf1 binding was detected at the two class 3 promoters that contain Cbf1 motifs (*MET6* and *MET17*). These class 3 promoters are bound more by Met4 and its cofactors than the other tested class 3 promoters.

Of promoters that contain Cbf1 sites, only Cbf1-dependent targets include Met31/Met32 motifs that failed MAST detection. In contrast, all categories of Met4 targets include promoters with both MAST-identified Cbf1 and Met31/Met32 motifs. These promoters can be assisted by Cbf1 to achieve stronger (or more extended) Met32 binding. Stronger binding by Met32 under inducing conditions may allow higher Met4 recruitment and expression. Alternatively, this extended, residual binding by Met32 under repressing conditions could allow faster loading of Met4 upon activation,

and hence allow earlier expression. Consistent with this hypothesis, *MET6* and *MET17* are induced very early upon sulfur limitation (Figure 4).

Components of the Met4 Transcriptional System Are Interdependent

To further examine cooperative interactions between Met4 cofactors, we determined how loss of each cofactor affects the remaining components. We first ascertained steady-state protein levels of Met4, Met28, Met32, and Cbf1 in *met4Δ*, *met31Δmet32Δ*, *cbf1Δ*, and *met28Δ* cells under inducing conditions (Figure 9A). Given that *MET28* is a class 2 Met4 target gene, Met28 levels were decreased in the *cbf1Δ* strain and were not detectable in both *met4Δ* and *met31Δmet32Δ* strains. Conversely, *MET28* deletion had no effect on the protein levels of the remaining transcription factors. Both Cbf1 and Met32 protein levels were decreased in *met4Δ* cells. Because microarray data indicates that *CBF1* and *MET32* transcript levels remain the same upon sulfur limitation and Met4 hyperactivation (Figure 1B and data not shown), these decreases may be due to destabilization of Cbf1 and Met32 in the absence of Met4 or Met4-dependent factor(s). In contrast, Met4 levels were unaffected by loss of its cofactors. Intriguingly, phosphorylation of Met4 occurs independently of its cofactors (Figure 9A). Because the phosphorylated state of Met4 correlates strongly with the transcriptionally active form of Met4 under conditions of sulfur limitation, Met4 hyperactivation, and cadmium exposure (Kaiser *et al.*, 2000; Barbey *et al.*, 2005), this finding demonstrates that this activating step is not dependent on Met4 cofactors.

We next assessed how in vivo promoter binding is affected by loss of Met4 or its cofactors. In *met4Δ* or *met31Δmet32Δ* cells, Cbf1 was the only factor to remain strongly associated to target promoters (Figure 9B); this result coincides with the constitutive binding profile of Cbf1 in wild-type cells (Figure 8A, fifth panel). Although Cbf1 can bind promoters in the absence of Met4, Met31, and Met32, *met28Δ* cells exhibit reduced Cbf1 promoter binding. This reduced occupancy is consistent with the role of Met28 as a

stabilizer of Met4-containing complexes on DNA (Kuras *et al.,* 1997; Blaiseau and Thomas, 1998). However, the low Cbf1 occupancy levels that were measured in *met28Δ* cells differed from the high and constitutive Cbf1 occupancy levels observed in *met4Δ* and *met31Δmet32Δ* cells, which do not express detectable levels of Met28 (Figure 9A). This finding suggests that deletion of *MET28* creates an environment (unique from that of *met4Δ* and *met31Δmet32Δ* cells) that interferes with Cbf1 promoter binding.

Met4 and Met32 exhibited similar reduced promoter binding patterns in both *cbf1Δ* and *met28Δ* strains (Figure 9B), extending the microarray similarities observed between these strains. Met4 bound class 3 promoters in *cbf1Δ* and *met28Δ* cells, consistent with the induction of class 3 genes. The absence of Met4 at class 1 and 2 promoters is likely due to the absence or decreased levels of Cbf1 found at these promoters in *cbf1Δ* and *met28Δ* cells, respectively. In contrast, Met4 was not associated with any of the 16 tested promoters in *met31Δmet32Δ* cells, confirming and extending findings from previous in vitro single promoter studies (Kuras *et al.,* 1996; Blaiseau and Thomas, 1998). Indeed, phosphorylated, and thus transcriptionally competent, Met4 is not recruited to Cbf1-bound promoters in *met31Δmet32Δ* cells.

Likewise, Met32 was not detected at promoters in *met4Δ* cells. This loss of promoter binding may due to the fact that Met32 protein levels are greatly reduced in *met4Δ* cells. Alternatively, loss of Met4 may interfere with the ability of Met31 and Met32 to bind DNA. Decreased Met31/Met32 recruitment in cells exposed to high methionine (which decreases Met4 levels but maintains Met32 levels; Figures 7 and 8) suggests that although Met32 is capable of binding DNA by itself in vitro (Blaiseau *et al.,* 1997), high-affinity DNA binding in vivo is posttranslationally regulated and may require functional Met4. This sharp Met4–Met32 interdependence explains the strong similarities observed between microarray profiles of *met4Δ* and *met31Δmet32Δ* cells. Finally, Met32 protein levels were decreased in *cbf1Δ* and *met28Δ* cells; this effect may be due to a destabilization of Met32 protein that was not promoter-bound due to the loss of cooperative interactions with Cbf1 and Met28.

DISCUSSION

The transcriptional regulation of sulfur metabolism in *S. cerevisiae* depends on the single activator Met4, whose function requires different combinations of the cofactors Cbf1, Met28, Met31, and Met32. In contrast to other yeast activators such as Gal4, which regulates a single-branched metabolic pathway or Gcn4, which is regulated by a single signaling pathway, Met4 faces the double challenge of regulating a multibranched metabolic network that furnishes different essential metabolites while responding to several distinct signaling cues. These signals include sulfur-containing compounds and amino acids, cadmium, arsenite, zinc, and potentially diauxic shift (Kent *et al.,* 1994; Kuras *et al.,* 2002; Barbey *et al.,* 2005; Yen *et al.,* 2005; Chandrasekaran *et al.,* 2006; Menant *et al.,* 2006; Thorsen *et al.,* 2007; Wu *et al.,* 2009). Although the mechanisms by which some of these inputs and outputs affect Met4 and its cofactors remain to be determined, our studies provide important insights into how Met4 and their cofactors collaborate in vivo.

Our microarray analyses establish regulatory boundaries between sulfate assimilation and other sulfur metabolic processes via transcriptional dependency on Cbf1 (Figure 5). This bifurcation in the sulfur metabolic network may be explained by differences in energy consumption. Reductive

sulfate assimilation requires extensive use of NADPH (Thomas and Surdin-Kerjan, 1997), as demonstrated by sulfur auxotrophy in yeast lacking glucose-6-phosphate dehydrogenase, the main enzyme of the pentose phosphate shunt that provides NADPH (Thomas *et al.,* 1991; Slekar *et al.,* 1996). Because uptake of methionine and AdoMet, the methyl cycle, and the transulfuration pathway do not require NADPH, cells may conserve reducing power by repressing sulfate assimilation when this process is not required. In addition, failure to repress sulfate assimilation genes upon zinc deficiency results in increased oxidative stress, presumably due to a decrease in NADPH-dependent antioxidant activities (Wu *et al.,* 2009). Therefore, transcriptional control of sulfate assimilation by Cbf1 can allow cells to adapt to a variety of conditions.

Our extensive microarray analyses also revealed a large core regulon for Met4 and allowed a statistical reformulation of the Cbf1 and Met31/Met32 consensus binding sequences. However, only 49% of promoters containing Cbf1 and Met31/Met32 sites, as determined by the MAST algorithm, were induced upon Met4 hyperactivation (Supplemental Figure S4). Moreover, of the ~400 transcripts induced by Met4 hyperactivation, only 84 induced genes contained promoters with Cbf1 sites, and only 90 induced genes contained Met31/Met32 promoter sites (Supplemental Figure S3). Some of this disparity is due to the false positives associated with a broad definition of a promoter region (-950 to $+50$) by the MAST program (Supplemental Figure S5). With respect to the false negatives associated with the highly variability of the Met31/Met32 consensus motif, a recent study on synthetic promoters indicates that weak transcription factor binding sites that are not detectable by motif programs can have very strong transcriptional effects (Gertz *et al.,* 2009). The identification and characterization of these presumptive cryptic sites will be critical for a full understanding of Met4-dependent transcription.

Figure 10 depicts a general model for how Met4 cofactors allow different gene expression patterns based on promoter composition, promoter binding behavior, and gene expression data. Based on core regulon features, all Met4 target promoters are predicted to contain some version of a Met31/Met32 motif. The high variability of the Met31/Met32 motif is due to the wide range of acceptable binding sequences for both Met31 and Met32 (with Met32 forming the main platform for Met4) as opposed to Met31 and Met32, each targeting distinct motifs. Binding by Met31 and Met32 is regulated at the posttranslational level, whereas Cbf1 remains promoter-bound under both conditions. Previous analyses of *MET16* and *MET28* promoters suggested that cooperative interactions exist between Met31/Met32 and Cbf1 sites, with Met28 acting as a link between the two DNA-binding complexes (Kuras *et al.,* 1997; Blaiseau and Thomas, 1998). Consistent with this idea, we find that Cbf1 contributes to different expression outcomes when paired with different Met31/Met32 motifs. Promoters that combine Cbf1 sites with Met31/Met32 sites that failed detection by MAST are strictly Cbf1-dependent. In contrast, promoters with both MAST-identified Cbf1 and Met31/Met32 motifs can be found in all three classes of Met4 targets. Based on ChIP studies, Cbf1 stabilizes Met31 and Met32 at promoters to allow either earlier, higher, and/or more extended expression of targets. A better understanding this dynamic will require correlating sequence determinants within the Met31/Met32 motif to specific Cbf1 effects; however, other transcriptional regulators converging on these targets will likely make this determination difficult.
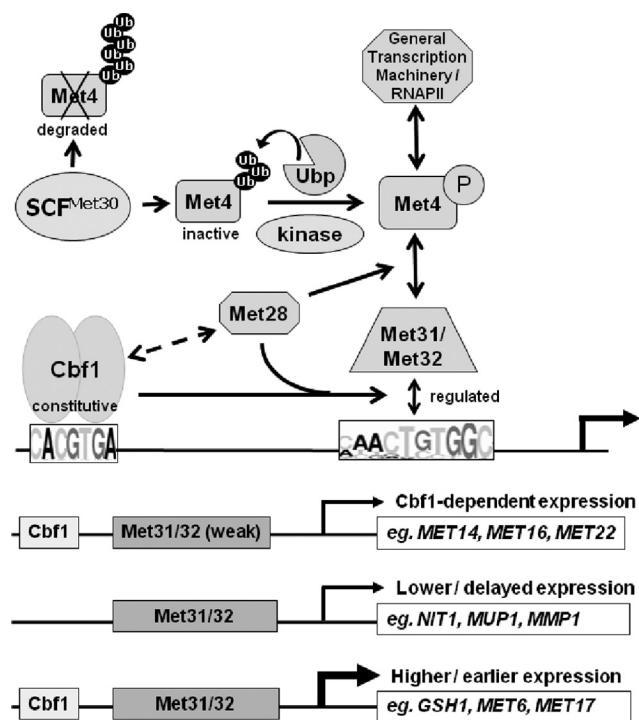
Figure 10. Model for target gene activation by Met4 and its cofactors based on promoter composition, promoter binding behavior, and gene expression data. Met4 activity is inhibited by the SCF^Met30 ubiquitin ligase, which targets Met4 for ubiquitylation. Poly-ubiquitylated Met4 is degraded while oligo-ubiquitylated Met4 is inactive. Deubiquitylation by an uncharacterized ubiquitin protease (Ubp) and phosphorylation by an uncharacterized kinase convert Met4 to its active form. Activated Met4 interacts with Met31, Met32, Met28, and Cbf1 at promoters to recruit RNA polymerase II (RNA-PII) and the general transcriptional machinery to Met4 target genes. See text for details.

Finally, our studies indicate a high level of interdependency among Met4 and its cofactors in which there is a reciprocal requirement between Cbf1 and Met28 and between Met4 and Met32. The requirement of Met4 for efficient promoter binding by Met32 was unexpected. Met4 may directly or indirectly stabilize Met32 interactions with DNA. Alternatively, we can manually detect a Cbf1 motif upstream of a Met31/Met32 motif in the *MET32* promoter (-100 to -450). Therefore, even though our microarray studies did not show a Met4-dependent induction of *MET32*, *MET32* may be a Met4 target. Regardless of the exact mechanism, these multiple dependencies are a key feature of the Met4 system and are likely to contribute to the optimization of sulfur metabolic processes under a wide range of environmental conditions.

Previous genome-wide studies characterized promoter binding for Met4, Cbf1, Met28, Met31, and Met32 in rich media or in response to the branched amino acid inhibitor sulfometuron methyl (SM) in synthetic complete media (Lee *et al.,* 2002; Harbison *et al.,* 2004). Met4-dependent transcription is inhibited on a genome-wide level in the presence of excess sulfur containing compounds and amino acids (as is found in both synthetic complete and rich media) even when *MET4* is overexpressed (Figure 1A), and previous microarray studies indicate SM addition causes severe repression of *MET* genes (Jia *et al.,* 2000). Our data indicates that Met4 target genes are not bound by Met4, Met28, Met31, and Met32 under repressive conditions. Previously published genome-wide ChIP datasets show that most of the genes that we identified as Met4 targets (by microarray and individual promoter ChIP studies) are not bound by Met31, Met32, and Met4 in both rich and SM media and none are bound by Met28 (Supplemental Figure S13; Lee *et al.,* 2002; Harbison *et al.,* 2004). The regulation of Cbf1 is somewhat more contentious. Harbison *et al.* observed that Cbf1 binds promoters that contain Cbf1 sites upon repressive SM treatment but not in repressive rich medium (Supplemental Figure S13). Previous individual promoter ChIP studies suggested that Cbf1 is ejected from target promoters, whereas Met4 remains promoter-bound upon methionine addition in synthetic dropout media (Kaiser *et al.,* 2000). In contrast, our current study indicates that Cbf1 remains bound to its target promoters regardless of induction or repression, whereas Met4 is no longer bound to promoters upon repression; this pattern is consistent with other earlier published studies with minimal media (Kuras *et al.,* 2002). Because we have characterized promoter binding in minimal media and not synthetic dropout media, it is plausible that manipulation of methionine levels in synthetic dropout media cause Met4 and Cbf1 to be regulated in an entirely different manner.

Chromatin structure is likely to play a role in the transcriptional outcome of Met4 targets. Previous studies have shown that Met4 recruits the mediator and SAGA complexes to various target promoters (Leroy *et al.,* 2006). However, inspection of high-resolution nucleosome positioning data from cells grown in rich media (Lee *et al.,* 2007) revealed no significant differences in nucleosome position between induced and uninduced genes identified by MAST (Supplemental Figures S14, S15). Likewise, no obvious differences were detected for various post-translational modifications on histones when we compared induced and uninduced genes identified by MAST with previously published histone modification databases (Pokholok *et al.,* 2005; Supplemetal Figures S16, S17). While it is clear that many aspects of this combinatorial system are unknown, our studies provide new insights into how Met4 cofactors mediate differential expression of Met4 targets.

ACKNOWLEDGMENTS

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