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Cell, Vol. 102, 303–314, August 4, 2000, Copyright ©2000 by Cell Press

Regulation of Transcription by Ubiquitination without Proteolysis: Cdc34/SCF^{Met30}-Mediated Inactivation of the Transcription Factor Met4

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Summary

Polyubiquitination of proteins by Cdc34/SCF complexes targets them for degradation by the 26S proteasome. The essential F-box protein Met30 is the substrate recognition subunit of the ubiquitin ligase SCF^{Met30}. The critical target of SCF^{Met30} is the transcription factor Met4, as deletion of *MET4* suppresses the lethality of *met30* mutants. Surprisingly, Met4 is a relatively stable protein and its abundance is not influenced by Met30. However, transcriptional repression of Met4 target genes correlates with Cdc34 /SCF^{Met30}-dependent ubiquitination of Met4. Functionally, ubiquitinated Met4 associates with target promoters but fails to form functional transcription complexes. Our data reveal a novel proteolysis-independent function for Cdc34/SCF and indicate that ubiquitination of transcription factors can be utilized to directly regulate their activities.

Introduction

Ubiquitination of proteins plays a major role in regulation of cellular processes. The best studied function of ubiquitination is its role in protein degradation, where polyubiquitinated proteins are recognized by the 26S proteasome and rapidly degraded. However, ubiquitin is also attached to stable proteins, most notably to a number of cell surface receptors, where ubiquitination in response to ligand binding serves as an internalization signal. In either case, ubiquitination of target proteins is catalyzed by a cascade of enzymatic reactions. Ubiquitin is first activated in an ATP dependent step by E1 enzymes, and the activated ubiquitin is then transferred to a specific cysteine residue on one of a family of ubiquitin-conjugating enzymes (E2). E2 enzymes can directly ubiquitinate target proteins but often require factors (E3 enzymes or ubiquitin–protein ligases) that confer substrate specificity. The SCF complexes, probably the best studied class of E3 enzymes, consist of the three core components Skp1, Cdc53 (in yeast), or Cul-1 (in mammalian cells) and one member of the F-box protein family. The F-box protein subunit of SCF directly binds substrates (Ciechanover, 1998; Hershko and Ciechanover, 1998; Koepp et al., 1999).

Three other proteins that are important for SCF function have been shown to directly associate with SCF complexes, namely Cdc34, Rbx1/Hrt1 and Sgt1 (Kamura et al., 1999; Kitagawa et al., 1999; Seol et al., 1999; Skowyra et al., 1999). Cdc34 is the ubiquitin-conjugating enzyme that is responsible for ubiquitination of most SCF substrates identified, and Rbx1/Hrt1 recruits Cdc34 to SCF complexes by bridging the Cdc34/Cdc53 interaction. Among the SCF components and their associated proteins, it is the F-box protein subunit that confers substrate specificity. Database searches predict that there are hundreds of F-box proteins (at least 15 in yeast alone). Different F-box proteins define distinct SCF complexes. In budding yeast, three different SCF complexes have been studied in some detail, namely SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30} (Deshaies, 1999). Whereas most of the morphological and cell proliferation defects associated with loss of Cdc4 or Grr1 function can be explained by inappropriate accumulation of their known target proteins, the essential function of SCF^{Met30} is not known. Temperature sensitive *met30* mutants arrest with both 1N and 2N DNA content. However, the cell cycle arrest is not due to the known defect in Swe1 degradation, because deletion of *SWE1* does not suppress the lethality or cell cycle arrest associated with *met30* mutation (Kaiser et al., 1998).

MET30 was initially identified in a screen for *S. cerevisiae* mutants defective in the regulation of genes involved in sulfur amino acid metabolism (Thomas et al., 1995). Specifically, yeast strains harboring a semi dominant-negative allele of *MET30* failed to repress *MET25* transcription in the presence of high levels of methionine. Subsequently, it has been demonstrated that this failure to respond to repressing levels of methionine is due to a defect of Met30 in the context of SCF^{Met30} because *cdc34*, *skp1*, and *cdc53* mutants also fail to repress *MET25* transcription (Patton et al., 1998). Transcription of *MET25* and a number of other genes involved in sulfur amino acid metabolism (*MET* genes) depends on the transcriptional activator Met4.

We show here that inability to downregulate Met4 activity accounts for the cell cycle arrest phenotype associated with *met30* mutants. Mechanistically, Cdc34/SCF^{Met30}-mediated ubiquitination of Met4 leads to its inactivation. Met4 ubiquitination, however, does not induce its degradation by the 26S proteasome, but rather results in direct inhibition of its activity as a transcriptional activator.

Results

Deregulation of *MET4* Causes Cell Cycle Arrest in *met30* Mutants

MET30 is an essential gene in *S. cerevisiae*. When temperature sensitive *met30* mutants are shifted to the restrictive temperature, they arrest as both unbudded and budded cells with 1N and 2N DNA content, respectively (Kaiser et al., 1998). It has been reported that deletion of *MET4* bypasses the cell cycle arrest associated with *met30* mutations (Patton et al., 1998). We obtained similar results in our strain background. Tetrad analysis revealed that the double mutant *met4 met30* is viable (Figure 1A) whereas the *met30* single mutant germinated but arrested in the first cell cycle as an unbudded (about 70%) or large budded cell (data not shown). Deletion of *MET4* also suppressed the lethality of temperature

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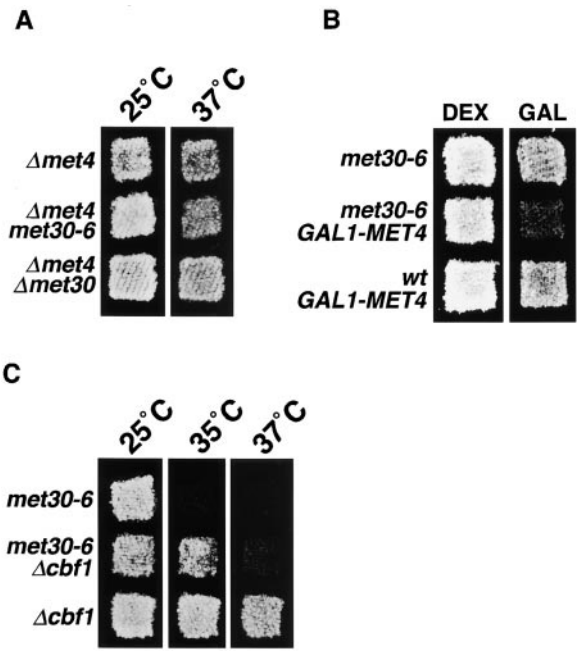

Figure 1. Genetic Interaction between *MET4*, *CBF1*, and *MET30*

(A) The mutant strains indicated *(Δmet4* (PY518), Δ*met4 met30-6*(PY723), and Δ*met4* Δ*met30* (PY596)) were grown on YEPD plates at 25°C, replica-plated to fresh plates and the plates were incubated at the temperatures indicated.

(B) Wild-type cells and temperature sensitive *met30-6* mutants expressing *MET4* under control of the *GAL1* promoter (strains PY657 and PY656, respectively) and *met30-6* mutants (PY283) were grown on YEPD plates at 25°C, replica-plated to galactose containing plates to induce overexpression of *MET4* and incubated at 25°C.

(C) Same as in (A) but with the strains indicated *(met30-6*(PY283), Δ*cbf1* (PY640), and Δ*cbf1 met30-6*(PY667)).

sensitive *met30-6* mutants at all temperatures tested (Figure 1A). These genetic results suggest that *met30* mutants arrest due to hyperactive Met4. Accordingly, overexpression of Met4 in temperature sensitive *met30-6* mutants, but not in wild-type cells, resulted in a growth defect even at permissive temperature (25°C) (Figure 1B), suggesting that *met30* mutants are particularly sensitive to Met4 levels.

Met4 is a transcriptional activator that is required for expression of many genes involved in sulfur amino acid metabolism. The basic helix-loop-helix protein Cbf1 is another important transcription factor that acts in concert with Met4 in activation of *MET* genes. Deletion of *CBF1* results in a phenotype of methionine auxotrophy, slow growth, and chromosome instability (Cai and Davis, 1990). Deletion of *CBF1* partially suppressed loss of Met30 function, as indicated by a higher restrictive temperature for *cbf1 met30-6* double mutants compared to *met30-6* single mutants (Figure 1C), indicating that the lethality of *met30* mutants is related to the transcriptional activation function of Met4. However, deletion of *CBF1* could not bypass the requirement for Met30 function because the double deletion strain *cbf1 met30* was inviable (data not shown).

These genetic interactions imply that Met30 is required to downregulate Met4 activity and that Met4 induces transcription of a gene, that if hyperinduced, leads to cell cycle arrest.

Met30- and Cdc34-Dependent Modification of Met4 Correlates with Met4 Activity

The genetic analysis suggested that Met4 is hyperactive in *met30* mutants. Since Met30 is a component of SCF, which is thought to function exclusively in ubiquitin-mediated protein degradation, we asked whether Met4 levels were increased in *met30* mutants. Met30 was inactivated in a temperature sensitive *met30-6* mutant by shifting to 37°C and we compared levels of myc-epitope tagged Met4 expressed from the endogenous promoter in wild-type and *met30* mutant cells at 25°C and 37°C (Figure 2A). No increase in the level of Met4 was observed in the *met30* mutant strain at the restrictive temperature (Figure 2A). However, we observed a dramatic difference in Met4 modification in wild-type cells and *met30* mutants (Figure 2A). In wild-type cells, Met4 existed as at least four forms of different mobility (Met4-A, -B, -C, and -D, respectively). *met30* mutants had only small amounts of Met4-A and -B, but had an additional distinct Met4 species (Met4-E). The differences in Met4 modification were not caused by the cell cycle arrest induced in *met30* mutants; there was no cell cycle dependent change in Met4 modification during the cell cycle in a synchronized population of wild-type cells (data not shown).

These results suggested that Met4 degradation was not defective in *met30* mutants, and that the cell cycle arrest of *met30* mutants is not due to accumulation of Met4. This was further supported by our observation that cells expressing GAL-MET4 did not undergo cell cycle arrest (Figure1B) despite having a 10-fold higher level of Met4 than wild type cells or *met30* mutants (Figure 2A).

We next asked whether Met4 transactivating activity is upregulated in *met30-6* mutants. We analyzed the mRNA levels of three different genes (*MET16*, *MET25*, and *MET28*) that are known to be controlled by Met4 activity (Thomas and Surdin, 1997). In methionine-containing medium, expression of *MET16*, *MET25*, and *MET28* is repressed (Thomas and Surdin, 1997). Accordingly, we detected only very low levels of these mRNAs in wild-type cells at both 25°C and 37°C (Figure 2A). In contrast, *MET16*, *25*, and *28* expression was derepressed in *met30-6* mutants, even in methionine-containing medium (Figure 2A). Overexpression of Met4 in wild-type cells only modestly derepressed *MET16*, *25*, and *28* expression (Figure 2A), suggesting their expression is not regulated at the level of Met4 abundance.

We then analyzed Met4 modification during methionine starvation. Expression of *MET* genes is repressed in medium containing methionine. However, when methionine is withdrawn, expression is rapidly induced (Thomas and Surdin, 1997). When we shifted wild-type cells to growth medium without methionine we observed a rapid change in Met4 modification (Figure 2B). The Met4-A and Met4-B forms decreased, whereas Met4-E accumulated (Figure 2B). This change correlated with Met4 activity as indicated by induction of *MET16*, *MET25*, and *MET28* (Figure 2B). Furthermore, Met4 modification in conditions that induce *MET* gene transcription was very similar to Met4 modification in cells lacking Met30 function (compare Figures 2A and 2B), suggesting that Met30 is required to downregulate the transactivating activity of Met4.

It has recently been suggested that components of Cdc34/SCF^{Met30} are required for proper regulation of *MET*

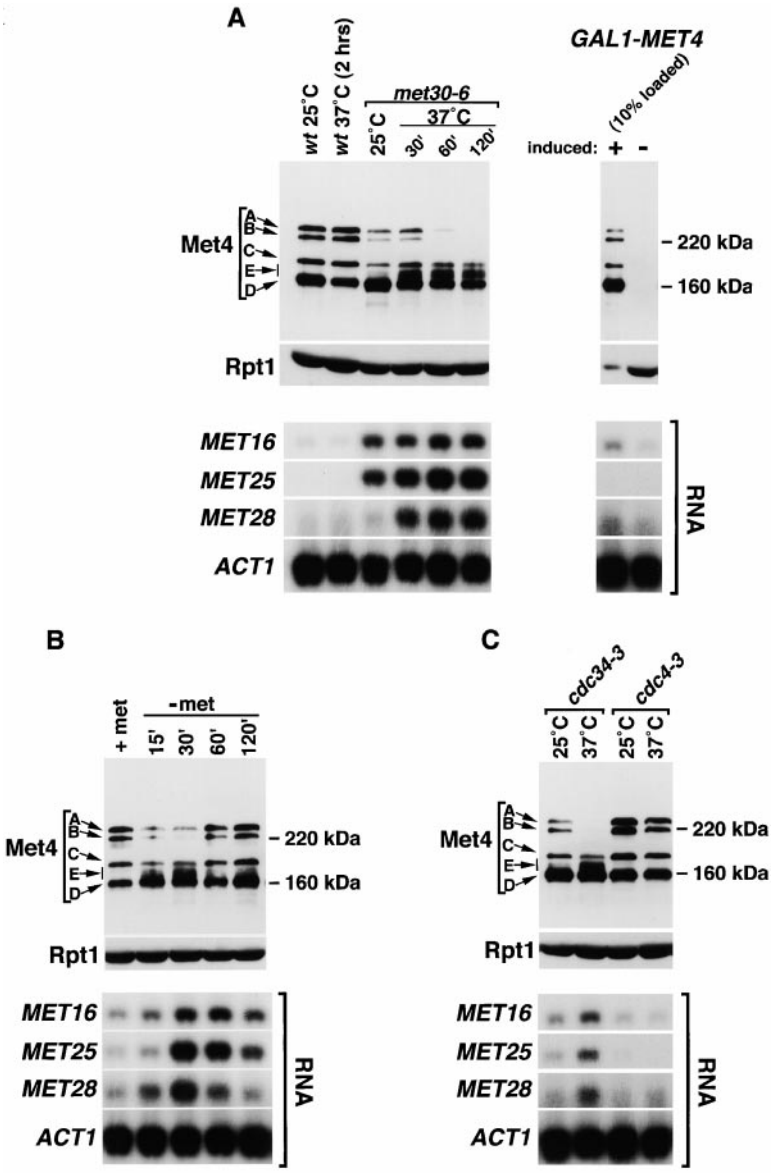

Figure 2. Met4 Modification Depends on Met30 Activity and Correlates with Met4 Transactivation Activity

(A) Left panel: Wild type cells and *met30* mutants expressing *MET4* tagged with multiple copies of the myc-epitope from the endogenous promoter (strains PY725 and PY743) were shifted to 37°C for the time indicated. Met4-myc was detected by Western blotting (7.5% gel; loading control: Rpt1). The different forms of Met4 are indicated by arrows and letters. Transcript levels of *MET16*, *MET25*, and *MET28* were analyzed by Northern blotting (loading control: *ACT1*). Right panel: A wild-type strain expressing myc-epitope tagged *MET4* under control of the inducible *GAL1* promoter (PY742) was grown to mid-log phase at 30°C either under inducing conditions (YEPG) or repressive conditions (YEPD) and cell lysates were analyzed as described above. For better comparison of Met4 levels, ten times less protein was loaded from the induced sample. Note that equal amounts of RNA were loaded for Northern blot analysis.

(B) Wild-type cells expressing endogenous *MET4-myc* (PY725) were grown at 30°C to an OD_{600} = 0.3 in minimal medium (SD + 5 mM methionine) (+met). Cells were washed twice in SD −met and incubation at 30°C was continued in SD −met. Samples were taken after the time indicated and analyzed as described in (A).

(C) Wild-type cells and *cdc34-3* mutants expressing endogenous *MET4-myc* (PY725, PY754) were grown in YEPD at 25°C to an OD_{600} = 0.3. The cultures were split into two and incubation was continued for 2 hr at 25°C and 37°C, respectively. Protein and RNA analyses were performed as described in (A).

gene expression (Patton et al., 1998; Seol et al., 1999). Specifically, *cdc34*, *cdc53*, *MET30–1*, and *rbx1/hrt1* mutants are deficient in repression of *MET25* expression (Patton et al., 1998; Seol et al., 1999). Since Met4 modification was dependent on Met30 and regulation of *MET* gene expression correlated with Met4 modification, we analyzed Met4 modification in *cdc34* mutants. We detected a dramatic change in Met4 modification after inactivation of Cdc34 (Figure 2C). In contrast, we saw no such change in Met4 mobility in *cdc4* mutants defective in a heterologous SCF complex (Figure 2C), although they arrested at the same cell cycle position as *cdc34* mutants. Consistent with the strong correlation between Met4 modification and Met4 activity (Figures 2A and B), *MET16*, *25*, and *28* expression was deregulated in *cdc34* mutants but not in *cdc4* mutants (Figure 2C). Taken together, these results suggest that Cdc34/SCF^{Met30} activity is required for modification and inactivation of Met4 but not for Met4 degradation.

Met4 Is a Relatively Stable Protein and Its Abundance Is Independent of Met30

Met30 is a component of SCF, which together with Cdc34 plays an important role in processes that lead to ubiquitin-mediated protein degradation. Our results that Met4 was hyperactive but did not accumulate in *cdc34* or *met30* mutants were, therefore, unexpected (Figure 2). For that reason, we compared the half-life of Met4 in wild-type cells and *met30* mutants. Fully functional C-terminal myc-epitope-tagged Met4 (Figures 2 and 6) was expressed for 30 min from the *GAL1* promoter in either wild-type cells or *met30* mutants following a shift to 37°C to inactivate the temperature sensitive *met30-6* allele. Samples were taken over a period of 100 min after repression of the *GAL1* promoter. Western blot analysis revealed that Met4 degradation was very slow and not impaired in *met30* mutants (Figure 3A). However, we noticed a striking change in Met4 modification in *met30* mutants as compared to wild-type cells (Figure

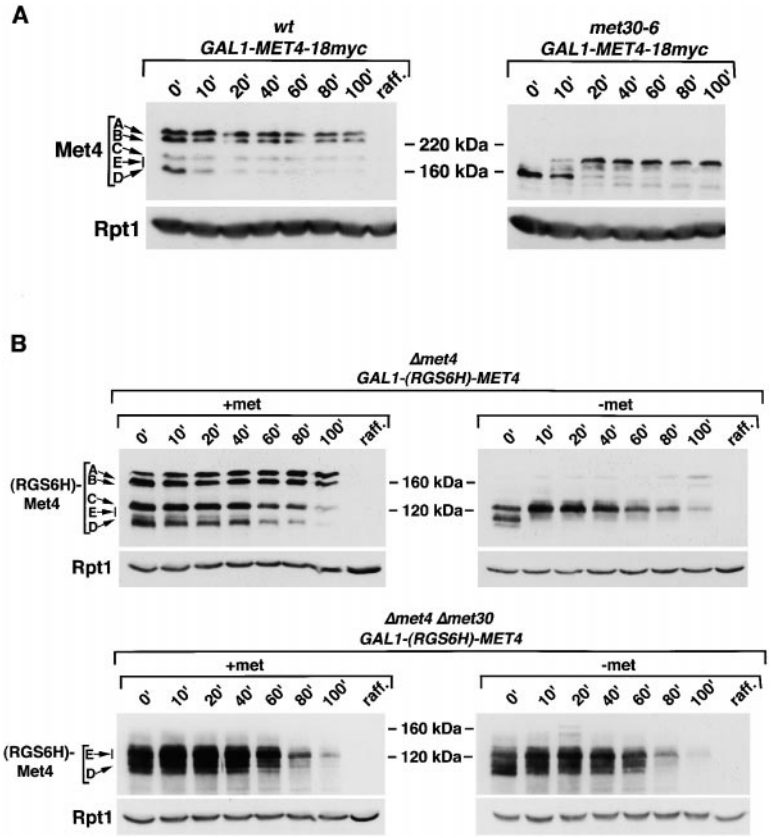

Figure 3. Met4 Degradation Is Not Affected by *met30* Mutation

(A) Wild-type cells and *met30-6* mutants expressing C-terminal myc-epitope-tagged Met4 under control of the *GAL1* promoter (PY742, PY760) were grown in raffinose media supplemented with 5 mM methionine to an OD_{600} = 0.3 at 25°C, shifted to 37°C for 15 min, and Met4-myc expression was induced by addition of galactose to a final concentration of 2%. After 30 min, cells were transferred to prewarmed medium (supplemented with 5 mM methionine) containing 2% dextrose to repress Met4-myc expression. Samples were taken at times indicated and analyzed for Met4-myc levels by Western blotting (7.5% gel).

(B) Δ*met4* (PY793) and Δ*met4* Δ*met30* (PY794) mutant cells expressing N-terminal (RGS6H)-epitope- tagged Met4 under control of the *GAL1* promoter were grown in raffinose media to an OD_{600}= 0.3 at 30°C. The cultures were split into two, collected on filter and washed with SD media containing 5 mM methionine ("+met") or SD media without methionine ("−met"). Cells were transferred to galactose (2%) containing media supplemented with methionine ("+met") or without methionine ("−met") to induce expression of (RGS6H)-Met4. After 30min (RGS6H)-Met4 expression was repressed by addition of glucose (2% final) and samples were analyzed as described in (A).

3A). Rouillon et al. (2000) reported that Met4 is rapidly degraded specifically under repressive conditions (+met) but stable under nonrepressive conditions (−met). We had analyzed a full-length Met4 construct containing a C-terminal myc epitope tag under repressive conditions (Figure 3A), whereas the construct analyzed by Rouillon et al. (2000) had short N-terminal and C-terminal truncations and was tagged at the N terminus with three HA epitopes. To eliminate the possibility that the difference arose from the difference in the two constructs, we analyzed the degradation of Met4 tagged on its N terminus with the short (RGS6H)-epitope under repressive and nonrepressive conditions (Figure 3B). Similar to the C terminally myc-tagged Met4, degradation of (RGS6H)-Met4 was slow, and we found no difference in Met4 half-life under repressive (+met) as compared to nonrepressive conditions (−met) (Figure 3B). Consistent with our previous results (Figure 2B), Met4 modification was dramatically different under repressive conditions as compared to nonrepressive conditions (Figure 3B). We also tested degradation of *GAL1* expressed (RGS6H)-Met4 in *met30* deletion strains that were kept alive by deletion of the endogenous *MET4* and observed no differences under repressive or nonrepressive conditions as compared to wild-type cells (Figure 3B). Similar to the temperature sensitive *met30-6* mutant (Figures 2A and 3A), Met4-A and Met4-B were absent in these mutants (Figure 3B). We specifically failed to extract Met4-A and Met4-B using the TCA/SDS procedure employed by Rouillon et al. (2000). Since Met4 gets modified to forms A and B only in wild-type cells under repressive conditions (Figure 3B), a failure to extract the slower mobility forms might lead to the conclusion that Met4 becomes unstable under these conditions.

Met4 Is Phosphorylated and Ubiquitinated In Vivo

The results described above suggest that Met4 modification is intimately connected to both Met30 function and Met4 activity. To establish the nature of the modified species, we first asked whether Met4 was phosphorylated. Myc-epitope-tagged endogenous Met4 was immunoprecipitated from wild-type cells and *met30* mutants and the immune complexes were treated with lambda phosphatase. Phosphatase treatment did not result in any obvious mobility change in Met4 species isolated from wild-type cells (Figure 4A). However, Met4-E, the modification specifically observed in *met30* mutants, collapsed into form Met4-D upon phosphatase treatment (Figure 4A), suggesting that Met4-E is a phosphorylated form of Met4.

Met30 is a component of the ubiquitin ligase SCF^{Met30}. Therefore, we asked whether any of the different Met4 species corresponded to ubiquitin conjugates. 6× His-tagged ubiquitin was expressed in cells expressing Met4-myc from the endogenous promoter. Whole cell lysates were prepared in 6M guanidinium-HCl and ubiquitinated proteins were purified on Ni^{2+} chelate resin. Lysate preparation in 6M guanidinium-HCl resulted in a much better preservation of the modified Met4 species as compared with conventional lysis procedures. The majority of Met4 was preserved as slowly migrating

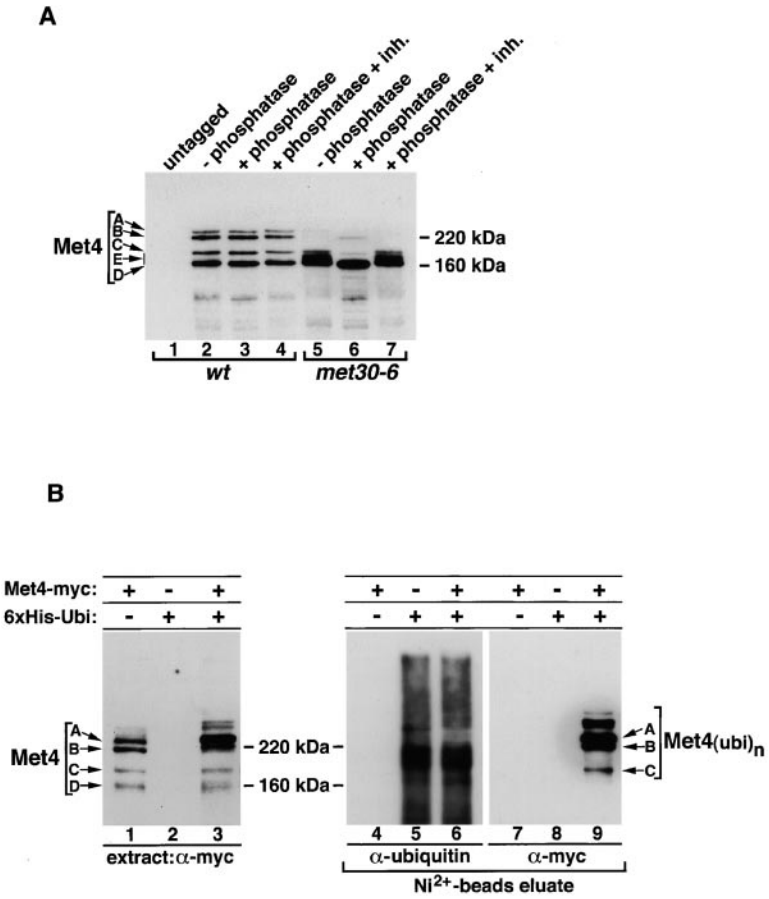

Figure 4. Met4 Is Phosphorylated and Ubiquitinated

(A) Wild-type cells and *met30* mutants expressing endogenous *MET4*-myc (strains PY725 and PY743) were grown in YEPD at 30°C to an $OD_{600} = 0.3$ and shifted to 37°C for 2 hr. Immunopurified Met4 was split into three equal parts. One part was incubated in phosphatase reaction mix without phosphatase (lanes 2 and 5), the second part was incubated with lambda-phosphatase (lanes 3 and 6), and the remaining part was incubated with lambda-phosphatase and a phosphatase inhibitor cocktail (lanes 4 and 7). As a control sample, immune complexes from wild-type cells expressing untagged *MET4* were treated with phosphatase (lane 1). Met4 was detected by Western blotting (7.5% gel).

(B) Wild-type cells expressing untagged or myc-tagged endogenous MET4 and overexpressing 6× His-tagged ubiquitin were lysed in a buffer containing 6 M guanidinium-HCl and part of the lysates was separated by SDS-PAGE (7.5% gel) and Met4 was detected by Western blotting (lanes 1, 2, and 3). The remaining lysate was incubated with Ni-NTA-agarose to purify proteins covalently bound to 6× His-ubiquitin. Ubiquitinated proteins were detected with a monoclonal antibody directed against ubiquitin (Kaiser et al., 1999; lanes 4, 5, and 6) and ubiquitinated Met4 was detected with 9E10 antibodies.

Lanes 1, 4, and 7: strain PY725; lanes 2, 5, and 8: PY780; lanes 3, 6, and 9: PY781.

Met4-A and Met4-B under these lysis conditions (Figure 4B, lanes 1 and 3). We also detected additional, lower mobility forms of Met4, particularly in lysates from cells overexpressing ubiquitin (Figure 4B, lane 3). When we analyzed the total pool of ubiquitinated proteins purified by Ni^{2+} chelate chromatography by immunoblotting with anti-myc antibody, we detected several forms of Met4 (Figure 4B, lane 9). Among them were Met4-A, -B, and -C and several more slowly migrating Met4 species (Figure 4B, lane 9). Met4-D was absent from the pool of ubiquitinated proteins (compare lanes 3 and 9 in Figure 4B), suggesting that it corresponds to the unmodified form of Met4, and that Met4-A, -B, and -C and the lower mobility forms represent Met4 ubiquitin conjugates. These experiments demonstrate that Met4 is ubiquitinated in vivo in a Met30-dependent manner. The phosphorylated forms of Met4 migrate with approximately the same mobility as the ubiquitinated form Met4-C (Figures 4A and 4B).

Met30 Binds Specifically to Met4 and Is Required for Met4 Ubiquitination In Vitro

F-box proteins confer substrate specificity to SCF complexes by directly interacting with substrate proteins and enabling their ubiquitination (Koepp et al., 1999). A physical interaction between Met4 and Met30 has been suggested based on a positive two-hybrid interaction (Thomas et al., 1995). To confirm by biochemical criteria that Met30 and Met4 interact, we expressed Gst-tagged Met30 in cells expressing endogenous myc-tagged Met4. As controls, we analyzed cells expressing either Gst alone or Gst-tagged Cdc4. We found a small fraction of Met4 (less than 5%) specifically in protein complexes bound to Gst-Met30 (Figure 5A). Interestingly, Met30 associated only with the nonubiquitinated form of Met4 (Figure 5A).

The specific physical interaction between Met30 with Met4 and the loss of ubiquitinated Met4 species in *cdc34* and *met30* mutants suggested that Cdc34/SCF^{Met30} directly ubiquitinates Met4. We therefore analyzed whether Met30 was required for Met4 ubiquitination in vitro. As substrate for the ubiquitination reaction, we used immunopurified Met4 from *met30* mutants. Only weak ubiquitination of Met4 was detected using partially purified SCF^{Met30}, purified E1, and Cdc34. SCF^{Met30} was purified using Ni-NTA-agarose from yeast cells expressing 6× His-tagged Met30 (data not shown). However, when we combined whole cell extract prepared from *met30* mutants with partially purified SCF^{Met30}, Met4 was efficiently ubiquitinated in an ATP-dependent manner (Figure 5B, lanes 4, 5, and 6), whereas the extract alone was inactive (Figure 5B, lanes 1, 2, and 3). Furthermore, the pattern of in vitro ubiquitination closely resembled that observed in vivo (Figure 3; Figure 5, lanes 4, 5, and 13). Addition of "mock-purified" SCF, prepared from cells expressing untagged Met30, to *met30* mutant extract did not promote Met4 ubiquitination (Figure 5B, lanes 7 and 8). Taken together, we conclude that Met30 is required for Met4 ubiquitination in vitro.

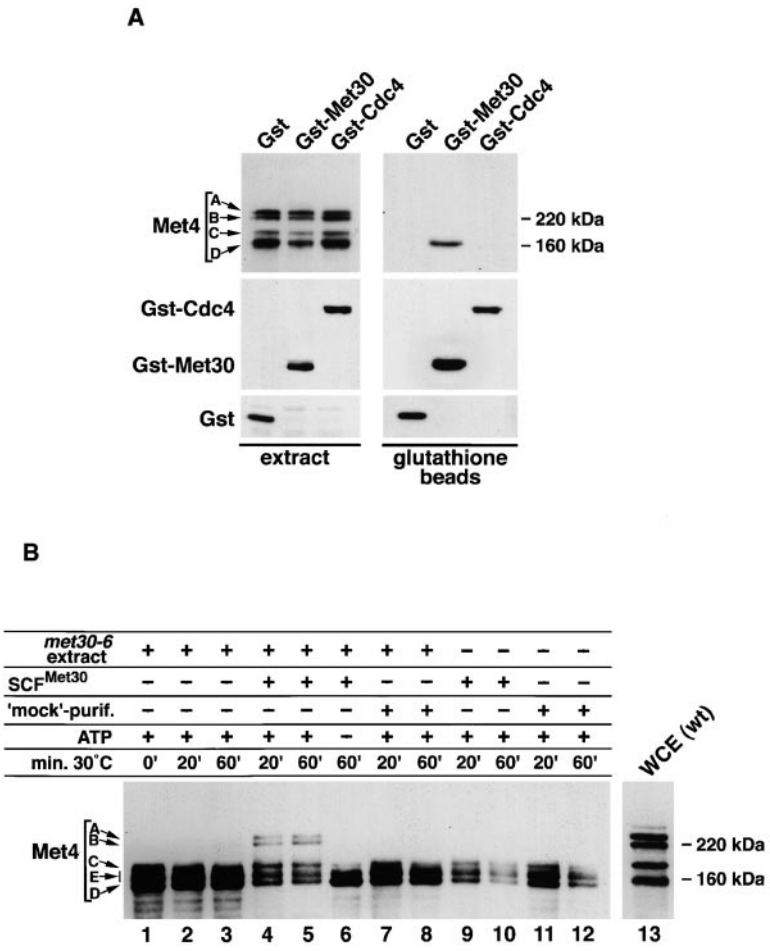

Figure 5. Met30 Binds and Ubiquitinates Met4

(A) Cells harboring endogenous Met4-myc and expressing Gst (PY768), Gst-Cdc4 (PY770), or Gst-Met30 (PY769) from the *GAL1* promoter were grown in raffinose medium at 30°C to an OD_{600} = 0.3. Expression from the *GAL1* promoter was induced for 40 min by addition of galactose to a final concentration of 1%. Proteins were purified on glutathione beads and analyzed by Western blotting. The Western blot showing myc-tagged Met4 co-purifying with Gst-Met30 on glutathione beads was exposed for a longer period as compared to the other blots.

(B) Extracts prepared from *met30-6* mutants (PY286) fortified with yeast E1, Cdc34, ubiquitin, and an ATP regenerating system (except lane 6) were incubated at 30°C for the period indicated (lanes 1, 2, and 3). Partially purified SCF^{Met30} (lanes 4, 5, and 6) or "mock purified extract" (lanes 7 and 8) were added to the reaction mix. In lanes 9–12, reactions including yeast E1, Cdc34, ubiquitin, an ATP regenerating system, and either partially purified SCF^{Met30} (lanes 9 and 10) or "mock purified extract" (lanes 11 and12) are shown. As a reference, Met4-myc detected in a wild-type cell extract is shown (lane 13). All reactions were analyzed by Western blotting (7.5% gel).

Met4 Ubiquitination Prevents Cbf1 Accumulation at *MET* Promoters

To gain insight into the mechanism by which Met4 ubiquitination regulates *MET*-gene transcription, we analyzed binding of Met4 and Cbf1 to several *MET*-promoters in vivo. Specifically, we evaluated the extent of Met4 and Cbf1 binding to these promoters during a time course of transcriptional activation and repression. We grew cells expressing epitope-tagged chromosomal versions of *MET4* or *CBF1* in medium supplemented with repressing concentrations of methionine. Cells were then shifted to medium lacking methionine, and samples were withdrawn after 15 and 60 min. The final sample was taken 30 min after re-repression of *MET* gene expression by addition of methionine. The samples were analyzed by chromatin immunoprecipitation assays (ChIP-assays) (Aparicio et al., 1997; Strahl-Bolsinger et al., 1997; Tanaka et al., 1997) to assess Met4 and Cbf1 association with the *MET* promoters in vivo and by Northern blotting to examine the extent of transcription from these promoters.

As expected, *MET16* and *MET25* transcription was tightly regulated in response to methionine levels in wild-type cells and constitutively derepressed in *met30* mutants (Figure 6A). Deletion of *MET4* abolished expression of both *MET16* and *MET25*, whereas Cbf1 was strictly required for *MET16* expression but less important for *MET25* expression (Figure 6A). Interestingly, *MET16* and *MET25* responded to withdrawal of methionine with slightly different kinetics (Figure 6A).

Surprisingly, Met4 binding to the *MET16* and *MET25* promoters was constant during the entire time course, whereas binding of Cbf1 to the same promoter regions was tightly regulated (Figure 6B). Low levels of Cbf1 were bound under transcriptionally inactive conditions whereas high levels were bound under conditions of active transcription (Figures 6A and 6B). As predicted by the lack of transcriptional repression of *MET* genes in *met30* mutants (Figure 6A), we observed constant levels of Met4 and, more importantly, Cbf1 associated with *MET* promoters throughout the time course, irrespective of methionine levels in the growth medium (Figure 6C). During the entire time course, we found no increase in the nuclear fractions of Met4 or Cbf1 in *met30* mutants as compared to wild-type cells (data not shown).

The results described above suggest that methionine levels in the growth medium regulate the ability of Met4 to recruit Cbf1 to *MET* promoters and consequently activate *MET* gene expression. Consistent with this hypothesis, we detected a low and constant level of Cbf1 binding to the *MET16* promoter and the *MET25* promoter in *met4* mutants during the activation/rerepression time course (Figure 6C).

To evaluate the extent of Met4 ubiquitination during the activation/rerepression time course, we analyzed

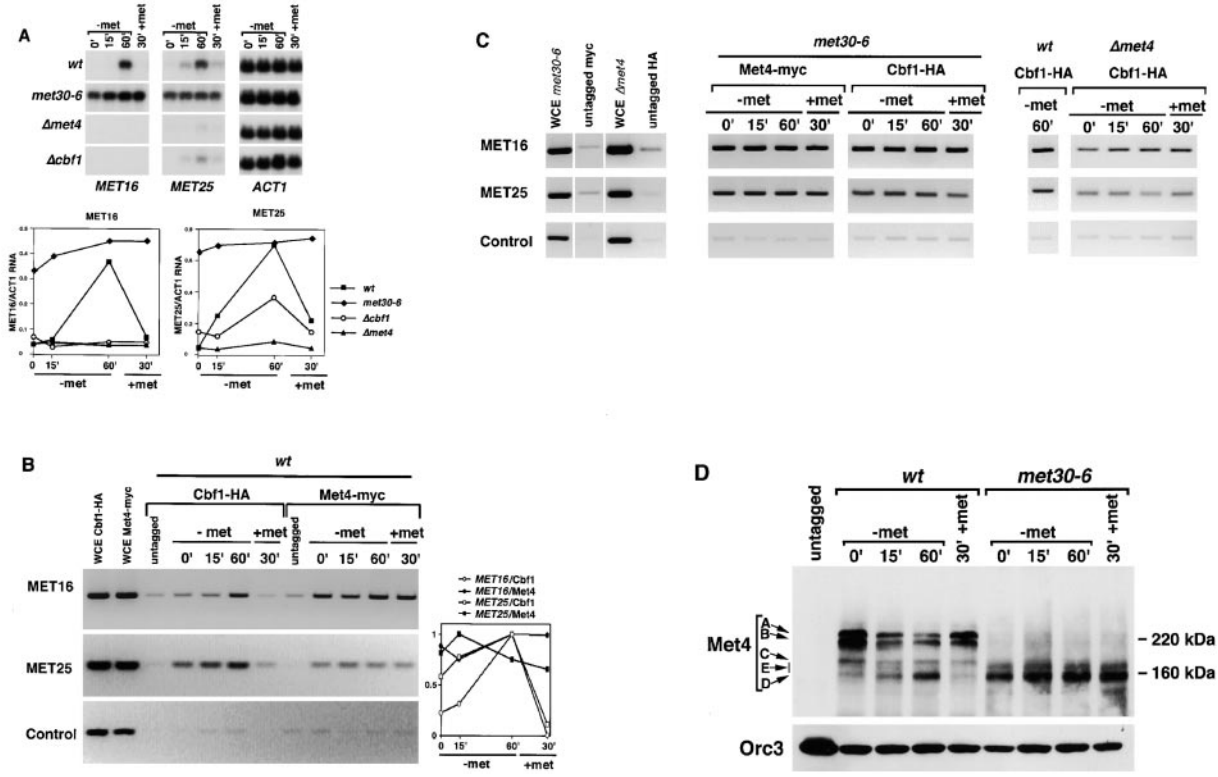

Figure 6. Met4 Ubiquitination Regulates Cbf1 Recruitment to *MET* Promoters

Cells were grown in SD medium supplemented with 5 mM methionine at 25°C to an $OD_{600} = 1$, washed in SD −met and incubated (in SD −met) at 30°C. Samples were taken at 0, 15, and 60 min after cells were shifted to SD −met. After the 60 min sample was taken, *MET* gene expression was repressed by addition of methionine to a final concentration of 5 mM and incubation was continued for 30 min. Strains: wild type: Met4-myc (PY725) Cbf1-HA (PY753); *met30-6*: Met4-myc (PY743) Cbf1-HA (PY775); Δ*cbf1* Met4-myc (PY771); Δ*met4* Cbf1-HA (PY773).
(A) Northern blot analyses of *MET16* and *MET25* expression. *MET16* and *MET25* levels were quantitated by PhosphoImager and ImageQuant v1.2 software, normalized to *ACT1*. (B and C) CHIP analyses of Met4 and Cbf1 binding to *MET* promoters. Met4-myc and Cbf1-HA immune complexes were washed, eluted, and DNA/protein cross-linking was reversed. PCR was performed on purified DNA with primers amplifying ~250 bp of the *MET16* promoter, *MET25* promoter, and a control fragment on chromosome XVI. Control PCR reactions were performed on DNA from cell lysates before immunoprecipitation (WCE) and immune complexes purified from strains with untagged Met4 or Cbf1 ("untagged"). PCR products were quantitated with NIH-Image software normalized to the untagged control.
(D) Regulation of Met4 ubiquitination. Culture conditions were as described above, but cells were shifted to 37°C instead of 30°C (PY752 (wt), PY751 (*met30–6*)). The chromatin fraction of the formaldehyde cross-linked samples was sonicated, after reversion of the cross-linking analyzed by Western blotting (7.5% gel; loading control: Orc3).

the samples by Western blotting. Serendipitously, the protein extraction conditions used in the ChIP assay procedure resulted in excellent preservation of ubiquitinated forms of Met4. Presumably, deubiquitination during extraction was blocked, allowing a better assessment of the Met4 ubiquitination state in vivo. Whereas the majority of Met4 is ubiquitinated under repressive conditions (+met), a shift toward unubiquitinated forms occurred in response to methionine withdrawal in wild-type cells (Figure 6D). In *met30* mutants, where *MET* gene transcription is constitutively active, the entire pool of Met4 was in the unubiquitinated form. This suggested that the nonubiquitinated forms of Met4 (Met4-D and -E) are transcriptionally active whereas the ubiquitinated forms are inactive, as predicted by earlier experiments (Figure 2).

Taken together, these results suggest that ubiquitination of Met4 prevents Cbf1 association with the promoter regions at levels sufficient to promote transcription.

Ubiquitinated Met4 Binds to the *MET25* Promoter but Shows Reduced Binding to Cbf1 In Vitro

Although the amount of Met4 bound to *MET* promoters was constant (Figure 6), it was possible that the various forms of Met4 have different affinities for the promoters. We therefore directly tested binding of Met4 to a *MET25* promoter fragment. Cell lysates prepared from wild-type cells expressing endogenous Met4-myc and Cbf1-HA were incubated with a biotinylated *MET25* promoter fragment. We then purified the promoter fragment on strepavidin beads and analyzed associated proteins by Western blotting. Cbf1 and all forms of Met4 bound to the *MET25* promoter (Figure 7A). We detected no bias for any of the various forms of Met4, suggesting that Met4 association with *MET* promoters is not regulated by ubiquitination.

Since the results obtained in the ChIP assays strongly suggested that ubiquitination of Met4 prevents Cbf1 association with *MET* promoters, we directly analyzed Cbf1/Met4 protein interaction. We incubated recombi-

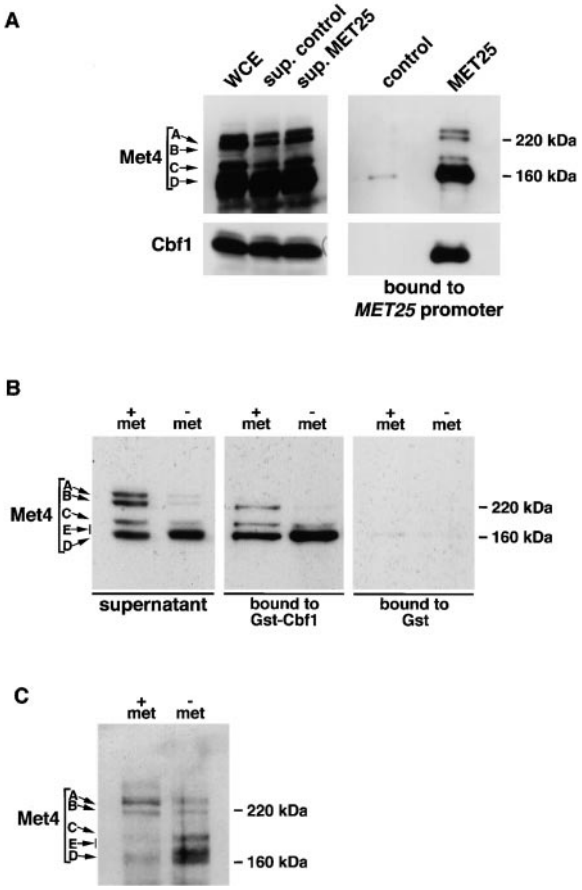

Figure 7. In Vitro Binding of Met4 to the *MET25* Promoter and to Cbf1

(A) All forms of Met4 bind to the *MET25* promoter: Extracts prepared from cells expressing endogenous Met4-myc and Cbf1-HA (PY752) were incubated with biotinylated *MET25* promotor fragments or a biotinylated control DNA. The biotinylated DNA fragments were purified on strepavidin beads and associated proteins were analyzed by Western blotting (7.5% gel).

(B) Two micrograms of purified recombinant Gst-Cbf1 bound to glutathione beads was used in the binding studies. Yeast cells expressing endogenous Met4-myc and Cbf1-HA (PY752) were grown in SD medium (+5 mM methionine) to an $OD_{600} = 0.4$, washed with SD −met and incubated 60min in SD −met ("−met" sample). *MET* gene expression was repressed by addition of methionine to a final concentration of 5 mM and incubation was continued for 30 min ("+met" sample). Cell lysates were incubated with Gst-Cbf1 or as a control, Gst, and bound proteins were analyzed by Western blotting as described in (A). To account for any nonspecific deubiquitination of Met4 during the incubation periods, the supernatant of the binding experiment rather than whole cell lysates were compared to the Gst-Cbf1 bound fractions.

(C) Cells expressing endogenous Met4-myc (PY725) were grown as described in (B). Cells were spun down and immediately resuspended in 20% TCA to minimize nonspecific deubiquitination. Cells were broken in 20% TCA, proteins resolubilized by boiling in 8 M urea/4% SDS, and analyzed by Western blotting as described in (A).

nant Gst-Cbf1 bound to glutathione beads with cell lysates prepared from wild-type cells expressing endogenous Met4-myc and Cbf1-HA. A small fraction of Met4-myc (1%–5%) was specifically retained on Gst-Cbf1 beads (Figure 7B). Interestingly, proportionally much less Met4-A, the most ubiquitinated form of Met4, interacted with Gst-Cbf1 (Figure 7B). We also detected a specific interaction of Cbf1-HA with Gst-Cbf1 (data not shown).

This experiment demonstrated that interaction of Met4-A with Cbf1 was dramatically and specifically reduced, whereas the other ubiquitinated forms of Met4 (Met4-B and C) associated with Cbf1. These results are consistent with our findings that ubiquitination of Met4 correlates with low Cbf1 binding at *MET* promoters in vivo (Figure 6). However, since the Cbf1/Met4-A interaction was specifically impaired, we would expect that Met4-A is the inactive form of Met4 and that the majority of Met4 should be fully ubiquitinated under fully repressed conditions. Although it is very difficult to preserve the ubiquitinated forms of Met4 during cell lysis and extract preparation due to nonspecific deubiquitination, using protein extraction methods that minimize nonspecific loss of ubiquitination, we find that the majority of Met4 is indeed in the inactive Met4-A form (Figure 7C). This validates the biological significance of the Cbf1/Met4 binding studies described above.

Discussion

A Proteolysis-Independent Function for Cdc34/SCF

The results presented in this report suggest a degradation-independent, regulatory function for Cdc34/ SCF^{Met30}. Our data indicate that the transcriptional activator Met4 is ubiquitinated in a Cdc34/ SCF^{Met30}-dependent manner and that ubiquitination of Met4 results in its inactivation. Analysis of events at Met4-regulated promoters during activation and rerepression of transcription indicated that Met4 ubiquitination inhibits recruitment of heterologous transcription factors that are required for *MET* gene expression. Thus, Cdc34/ SCF^{Met30}-dependent ubiquitination of Met4 appears to regulate the assembly of active transcriptional complexes at *MET* promoters.

These results were quite unexpected, as Cdc34/SCF function has been exclusively implicated in ubiquitination of proteins that are subsequently degraded by the 26S proteasome. However, regulatory proteolysis-independent functions of ubiquitination have been previously described (Hicke, 1999). The results strongly suggest that regulation of Met4 by Cdc34/ SCF^{Met30}-mediated ubiquitination is independent of proteolysis. Protein half-life experiments showed that Met4 is a stable protein and Met4 was not stabilized in *met30* mutants although Met4 ubiquitination was impaired (Figure 3). Similarly, Met4 ubiquitination was inhibited when Met30 or Cdc34 function was inactivated, yet endogenous Met4 levels did not increase, suggesting that Cdc34/Met30-dependent ubiquitination does not contribute to Met4 proteolysis (Figures 2A and 2C). Increasing Met4 levels by Met4 overexpression did not induce transcription of *MET* genes, thus *MET* gene expression was unaffected by Met4 abundance (Figure 2A).

It was possible that only a small, but relevant pool of Met4 was controlled by proteolysis, which would not have been reflected in changes in total Met4 levels. Therefore, we asked whether Met4 proteolysis might be restricted to *MET* promoters and, if so, whether local regulation of Met4 abundance at *MET* promoters regulated *MET* gene transcription. ChIP assays demonstrated that Met4 association with *MET* promoters was constant regardless of the regulatory status of the promoters (Figures 6B and 6C). Hence, proteolysis of the *MET* promoter-associated Met4 pool was not responsible for transcriptional regulation. Taken together, these

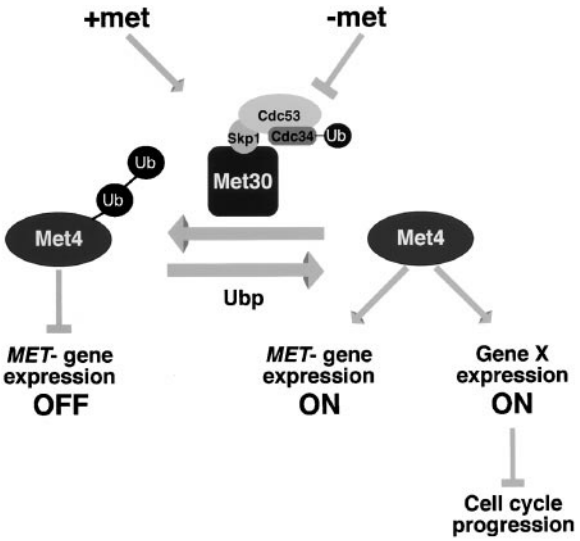

Figure 8. Model for Met4 Regulation: Cdc34/SCF^{Met30}-Mediated Ubiquitination of Met4 Leads to Its Inactivation and Repression of *MET* Gene Expression.

Ubiquitin hydrolases (Ubp) deubiquitinate and activate Met4 which results in expression of *MET* genes and a proposed inhibitor of cell cycle progression (gene X).

results support the idea that Cdc34/ SCF^{Met30}-mediated ubiquitination regulates Met4 function in a proteolysis-independent manner.

Our data do not exclude a nonproteolytic function of the 26S or 19S proteasome in regulation of *MET* gene transcription. It is possible that ubiquitinated Met4 recruits 26S/19S proteasomes and that some as of yet uncharacterized nonproteolytic function of the proteasome is important for regulation of *MET* gene expression. Such a proteolysis-independent function for the 19S proteasome has been postulated in the regulation of excision repair in budding yeast (Russell et al., 1999).

It is reasonable to assume that other cellular processes regulated by Cdc34/SCF-dependent ubiquitination are independent of proteolysis and that ubiquitination directly regulates protein function or association with other proteins. Interestingly, the yeast kinetochore component Cbf2 has been shown to be ubiquitinated in a Cdc34 dependent manner (Yoon and Carbon, 1995), yet Cbf2 is a stable protein (Kaplan et al., 1997). Furthermore, mutations in the SCF component *SKP1* or inactivation of the SCF associated protein Sgt1 result in high levels of chromosome loss, a phenotype that is consistent with misregulation of Cbf2 function. It is noteworthy that the Met4 coactivator Cbf1 is also a component of the kinetochore (see below).

Regulation of Transcription Factors by Ubiquitination

Our analyses of Cdc34/SCF^{Met30}-mediated ubiquitination of the transcription factor Met4 demonstrated that transcriptional activation and repression of *MET* genes are regulated by ubiquitination. Met4 activity correlated with its ubiquitination status (Figures 2 and 6), suggesting that ubiquitination inhibited Met4 activity but did not regulate its proteolysis. Consequently, defects in the ubiquitination complex required for Met4 ubiquitination (Cdc34/SCF^{Met30}) result in constitutively active Met4 leading to constitutive expression of *MET* genes (Figures 2A and 2C; Figure 6A) (Patton et al., 1998, Seol et al., 1999).

In an attempt to understand how Met4 ubiquitination regulates *MET* gene transcription, we analyzed the events at two distinct *MET* promoters (*MET16* and *MET25*) during activation and rerepression. Surprisingly, Met4 binding at both promoters was unchanged during transcriptional induction and rerepression (Figure 6B). However, Cbf1 association was tightly correlated with transcriptional activity and, in an inverse manner, with Met4 ubiquitination (Figure 6). This suggests that Cbf1 recruitment to *MET* promoters leads to their activation

Our data are consistent with Met4 acting either to promote or, in its ubiquitinated form, to inhibit association of Cbf1 with the *MET* promoters. To decide whether ubiquitinated Met4 functions as an active repressor or is simply inactive as a transcription factor will require further investigation.

As pointed out above, Cbf1 is also a component of the yeast kinetochore. As with *MET* gene transcription, kinetochore function appears to be controlled by ubiquitination, in this case, of the kinetochore protein Cbf2. It is therefore possible that recruitment of Cbf1 to the kinetochore is regulated by ubiquitination of Cbf2, analogous to the relationship between Cbf1 and Met4 at *MET* promoters. In this regard, it is interesting to note that Met4 and Cbf2 share significant primary structure homology over their respective lengths (15% identity) and, therefore, are likely to have evolved from a common ancestral protein.

Met4 activation and concomitant *MET* gene expression appear to be initiated by a rapid loss of ubiquitinated forms of Met4 (Figure 2B and Figure 6) suggesting that deubiquitination plays an important role. It will be interesting to learn whether deubiquitination of Met4 is regulated or constitutively active. If the latter is the case, regulation of Cdc34/SCF^{Met30} activity is likely to be a key event in *MET* gene activation. Such regulation might occur via direct inhibition of Cdc34/SCF^{Met30} or by inhibition of the as yet unknown protein kinase that targets Met4 for ubiquitination.

The Cell Cycle Arrest Phenotype of *met30* Mutants

We initiated the experiments on Met4 and Cdc34/SCF^{Met30} in an attempt to elucidate the molecular mechanism that leads to cell cycle arrest in *met30* mutants. Although our experiments led to the identification of a novel mode of transcriptional regulation and a previously unknown function for Cdc34/SCF, we learned little about the basis for the cell cycle arrest phenotype conferred by *met30* mutants. However, the genetic interactions and the biochemical studies described in this report clearly demonstrated that hyperactivation of Met4 is an essential event for the cell cycle arrest observed in *met30* mutants. An attractive hypothesis is that Met4 controls transcription of an unknown gene (gene X) that induces cell cycle arrest. Loss of Met30 function leads to hyperactivation of Met4 and would consequently induce constitutive expression of gene X, causing cell cycle arrest. During the unperturbed cell cycle, Met4 is in an inactive state. However, when methionine is withdrawn from the growth medium, Met4 is transiently activated until *MET* genes are expressed and methionine biosynthesis restores adequate concentrations of important methionine metabolites in the cell, notably S-adenosyl methionine (Figure 2B). It is reasonable to propose that under conditions where these central metabolites have not yet reached sufficiently high

Table 1. Yeast Strains Used in This Study

Strain	Relevant genotype	Source
15Daub	a *bar1Δ ura3Δns ade1 his2 leu2-3,112 trp1-1*[a]	(Reed et al. 1985)
PY236	a *bar1 pep4::URA3*	(Kaiser et al. 1999)
PY283	a *met30-6::KAN*^{R}	This study
PY401	a *GAL1-MET30(RGS6H)::LEU2::KAN*^{R} *met30::KAN*^{R} *pep4::URA3*	This study
PY518	a *met4::KAN*^{R}	This study
PY589	a *met30::KAN*^{R} *met4::KAN*^{R}	This study
PY640	a *cbf1::ZEO*^{R}	This study
PY667	a *cbf1::ZEO*^{R} *met30-6::KAN*^{R}	This study
PY656	a *met30-6::KAN*^{R} *GAL1-(RGS6H)-MET4::LEU2*	This study
PY657	a *GAL1-(RGS6H)-MET4::LEU2*	This study
PY723	a *met30-6::KAN*^{R} *met4::KAN*^{R}	This study
PY725	a *MET4-(18myc)::TRP1 pep4::URA3*	This study
PY742	a *GAL1-MET4(18myc)::LEU2::TRP1 pep4::URA3*	This study
PY743	a *met30-6::KAN*^{R} *MET4-(18myc)::TRP1 pep4::URA3*	This study
PY751	a *met30-6::KAN*^{R} *MET4(18myc)::TRP1 CBF(HA)3::LEU2 pep4::URA3*	This study
PY752	a *MET4(18myc)::TRP1 CBF1(HA)3::LEU2 pep4::URA3*	This study
PY753	a *CBF1(HA)3::LEU2 pep4::URA3*	This study
PY754	α *cdc34-3 MET4(18myc)::TRP1 pep4::URA3*	This study
PY760	a *met30-6::KAN*^{R} *GAL1-MET4(18myc)::LEU2::TRP1 pep4::URA3*	This study
PY768	a *2μGAL1-GST::LEU2 MET4(18myc)::TRP1 pep4::URA3*	This study
PY769	a *2μGAL1-GST-MET30::LEU2 MET4(18myc)::TRP pep4::URA3*	This study
PY770	a *2μGAL1-GST-CDC4::LEU2 MET4(18myc)::TRP pep4::URA3*	This study
PY771	a *cbf1::ZEO*^{R} *MET4(18myc)::TRP1 pep4::URA3*	This study
PY773	a *met4::KAN*^{R} *CBF1(HA)3::LEU2 pep4::URA3*	This study
PY775	a *met30-6::KAN*^{R} *CBF1(HA)3::LEU2 pep4::URA3*	This study
PY780	a *2μGAL1-6×His-UBI::LEU2 pep4::URA3*	This study
PY781	a *2μGAL1-6×His-UBI::LEU2 MET4(18myc)::TRP1 pep4::URA3*	This study
PY793	a *Δmet4::KAN*^{R} *GAL1-RGS6H-MET4::LEU2*	This study
PY794	a *Δmet30::KAN*^{R} *Δmet4::KAN*^{R}*GAL1-RGS6H-MET4::LEU*	This study

levels to sustain cell cycle progression, a transient cell cycle arrest is induced to safeguard cellular integrity. We suggest that such a cell cycle arrest would be linked to methionine levels by Met4-dependent expression of a cell cycle inhibitor (encoded by gene X), and hence that expression of gene X is constitutive in *met30* mutants, resulting in permanent cell cycle arrest (Figure 8). Microarray analyses of gene expression in *met30* mutants should help to identify gene X and elucidate the molecular mechanism of cell cycle arrest in *met30* mutants, and by inference, in response to methionine starvation.

Experimental Procedures

Yeast Strains and Methods

The relevant genotypes of the yeast strains used in this study are listed in Table 1. All strains are isogenic to 15DaubΔ, a *bar1Δ ura3Δns*, derivative of BF264-15D (Reed et al., 1985). All strains were grown in standard culture media and standard yeast genetic methods were used (Guthrie and Fink, 1991).

In most experiments involving protein analyses, strains harboring a deletion of *PEP4* were used. Deletion of *PEP4* did not alter Met4 modification or change any other result, however the experiments were more reliable because nonspecific proteolysis during extract preparation and immunoprecipitation was decreased in *pep4* mutants.

All epitope-tagged proteins were fully functional because the strains were indistinguishable from their parental strains in all the parameters tested. Details of the construction of plasmids and strains and primer sequences are available upon request (peterka@scripps.edu).

Protein and RNA analyses

For Western blot analysis, protein extracts were prepared in RIPA-buffer (1% deoxycholic acid, 1% Triton-X-100, 0.1% SDS, 250 mM NaCl, 50mM Tris-HCl [pH.7.5], α-Ph [10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, and 0.1 mM orthovanadate], and α-Pr [1 mM PMSF, 2μg/ml aprotinin, leupeptin, and pepstatin A] if not otherwise stated. Cells were broken with glass beads 3 × 40 s in a FastPrep FP120 (BIO 101/ Savant).

Total RNA isolation and Northern blotting was performed as described (Stuart and Wittenberg, 1994). The membrane was hybridized with radiolabeled probes (HiPrime, Boehringer Mannheim) in buffer H (100 mM sodium phosphate buffer [pH 7.0], 400 mM NaCl, 5 mM EDTA, 1% SDS, 10% dextran sulfate, and 0.1 mg/ml denatured salmon sperm DNA). Northern blots were analyzed by PhosphoImaging and ImageQuant software.

Analysis of Met4 Turnover

Met4-myc:wild-type cells and *met30-6* mutants containing an integrated *GAL1-MET4(myc18)* allele (PY742, PY760) were grown in raff/-leu medium (supplemented with 5mM methionine) at 25°C to an OD_{600} = 0.3. Cells were then shifted to 37°C and after 15 min galactose was added to a final concentration of 2% to induce the *GAL1* promoter. To terminate Met4 expression after 30 min, cells were collected on filters and transferred to dex/-leu medium (supplemented with 5mM methionine, prewarmed to 37°C) and incubation was continued at 37°C. Extracts were prepared in 50 mM Tris-HCl (pH.7.5), 150 mM NaCl, 0.2% NP_4O, α-Ph, and α-Pr and analyzed for the amount of Met4-myc by Western blotting (9E10 antibodies).

(RGS6H)-Met4: Δ*met4* (PY793) and Δ*met4* Δ*met30* (PY794) mutant cells expressing N-terminal (RGS6H)-epitope-tagged Met4 under control of the *GAL1* promoter were grown in raff/-leu medium to an OD_{600}= 0.3 at 30°C. The cultures were divided into two, collected

on filter and washed with SD media containing 5mM methionine ("+met") or SD media without methionine ("−met"). Cells were transferred to galactose (2%) containing media supplemented with 5mM methionine ("+met") or without methionine ("−met") to induce expression of (RGS6H)-Met4. After 30 min (RGS6H)-Met4 expression was repressed by addition of glucose (2% final) and samples were analyzed as described above with anti-(RGS4H) antibodies (Qiagen).

Analyses of Met4 Phosphorylation and Ubiquitination

To analyze the phosphorylation status of Met4, wild type cells and *met30-6*mutant cells expressing endogenous *MET4*-myc (strains PY725 and PY743), and a untagged control strain (PY236) were grown in YEPD at 30°C to an $OD_{600} = 0.3$. Cultures were shifted to 37°C for 2 hr, protein extracts were prepared in RIPA-buffer plus 5 mM N-ethylmaleimide and Met4-myc was immunoprecipitated from 2.5 mg protein with polyclonal antibodies directed against the myc-epitope (Santa Cruz Biotechnology). Immune complexes were washed twice with 1 ml RIPA buffer, twice with 1 ml RIPA buffer without phosphatase inhibitors (α-Ph), and once in 1 ml 50 mM Tris-HCl (pH 7.5), 5 mM DTT, and 0.1 mg/ml BSA. Immunopurified Met4 was split into three equal parts. One part was incubated in 100 μl phosphatase reaction mix (50 mM Tris-HCl [pH 7.5], 5 mM DTT, 0.1 mM EDTA, 0.01% Brij35, and 2 mM $MnCl_2$) without phosphatase. The two other parts were incubated with phosphatase reaction mix and 1200 units lambda-phosphatase (NEB), but to one of them a phosphatase inhibitor cocktail (α-Ph) was added. The reactions were incubated at 30°C for 60 min, and the immunocomplexes were washed with 300 μl RIPA buffer and analyzed by Western blotting (9E10 antibodies).

To analyze Met4 ubiquitination in vivo, a strain expressing endogenous Met4-myc and 6× His-tagged ubiquitin under control of the inducible *GAL1* promoter from a high-copy plasmid (PY781) was grown at 30°C to an $OD_{600} = 0.3$ in raffinose media. As a control, strains expressing untagged Met4 and 6× His ubiquitin (PY780) or tagged Met4 but no tagged ubiquitin (PY725) were processed in parallel. 6× His-tagged ubiquitin was expressed for 4 hr and cells were broken in buffer C (6 M guanidinium-HCl, 50 mM sodium phosphate buffer [pH 8.0], 10 mM Tris-HCl [pH 8.0], 300 mM NaCl, α-Pr, and 5 mM N-ethylmaleimide). 2.75 mg protein extract was incubated with 50 μl Ni-NTA-agarose and imidazole (final concentration 10 mM) for 4 hr at 4°C. The beads were successively washed with 1 ml each of buffer C + 20 mM imidazole, buffer D (same as buffer C but 8 M urea instead of 6 M guanidinium-HCl) + 20mM imidazole and twice with buffer D adjusted to pH 6.0 + 20 mM imidazole. Bound proteins were eluted in 200 μl 2× SDS sample buffer and analyzed by Western blotting with the 9E10 antibody to detect Met4 or antibodies directed against ubiquitin (Kaiser et al., 1999).

Analysis of Met30/Met4 Interaction

Strains expressing endogenous Met4-myc and harboring either a YEp-*GAL1-GST* (PY768), YEp-*GAL1-GST-CDC4* (PY770), or YEp-*GAL1-GST-MET30* (PY769) construct were grown to an $OD_{600} = 0.3$ in raffinose medium at 30°C. Galactose was added to a final concentration of 1% and incubation was continued at 30°C for 40 min. Protein extracts were prepared in buffer B150 (50 mM HEPES-KOH [pH 7.3], 150 mM NaCl, 10 mM magnesium acetate, 10% glycerol, 1% Triton X-100, 20 mM β-glycerol phosphate, 1 mM DTT, α-Ph, and α-Pr). DTT concentration was adjusted to a final concentration of 5 mM and 1 mg protein was incubated for 2 hr at 4°C with 50 μl glutathione-Sepharose. The beads were washed 4 times with 1 ml B150 + 5 mM DTT, bound proteins were eluted in 150 μl 2× SDS sample buffer and analyzed by Western blotting. Met4 was detected with 9E10 antibodies, and Gst or Gst-fusion proteins were detected with polyclonal antibodies directed against Gst.

Met4 In Vitro Ubiquitination

Extracts for in vitro ubiquitination were prepared from *met30-6* mutants (PY286) as described (Kaiser et al., 1998). SCF^{Met30} was partially purified from a Δ*met30* deletion strain expressing RGS6H-tagged Met30 from the *GAL1* promoter (PY401). An isogenic wild-type strain expressing untagged Met30 (PY236) was used for the "mock-purification". Cells were grown at 30°C in YEPGal to an $OD_{600} = 1.5$. To 7.5 ml cell pellet, 12.5 ml 2× buffer NM (1× NM: 50 mM sodium phosphate buffer [pH 7.5], 10 mM HEPES [pH 7.5], 10% glycerol, 250 mM NaCl, 10 mM β-mercaptoethanol, and α-Pr), and 5 ml H_2O were added and cells were broken by 4 passages through a French Pressure Cell. Cell debris was spun down for 20 min at 17,000 rpm (JA20). After filtration through a 0.45 μm filter, the pH was adjusted to 7.5, imidazole (final concentration 10 mM) was added, and 125 mg protein was incubated with 200μl Ni-NTA-agarose for 1 hr at 4°C. Proteins bound to Ni-NTA-agarose were washed 5 times with 1 ml NM buffer plus 10 mM imidazole. Bound proteins were eluted 4 times with 200 μl NM buffer plus 250 mM imidazole. The buffer was changed to YEB buffer (Verma et al., 1997) by 5 cycles of 4-fold concentration and 4-fold dilution in centricon-50. The final protein concentration was 2 μg/μl for both SCF^{Met30} and the "mock-purification", indicating that the majority of proteins were nonspecifically bound to Ni-NTA-agarose. To prepare Met4 substrate for in vitro ubiquitination assays, *met30* mutants expressing Met4-myc (PY743) were grown at 37°C for 3.5 hr. Cells were broken in buffer B150, 15 mg of protein was incubated with polyclonal antibodies directed against the myc epitope (Santa Cruz Biotechnology) and proteinA beads for 2 hr at 4°C. Immune complexes were washed 2 times with 2 ml buffer B150 and 2 times with 1 ml YEB. Met4-substrate bound to beads was equally distributed between the in vitro ubiquitination reactions. In vitro ubiquitination reactions contained 5 mM Mg acetate, 1 μg/ml aprotinin, leupeptin and pepstatin A, 1mM DTT, an ATP regenerating system (Kaiser et al., 1999), 2 μg/ml bovine ubiquitin (Sigma), recombinant Cdc34 (40 μg/ml), purified yeast E1 (15 μg/ml) and, as indicated, 5 mg/ml whole cell extract prepared from *met30-6* mutants, and/or 1 mg/ml partially purified SCF^{Met30}or "mock-purification", respectively. Ubiquitination reactions were incubated at 30°C for 0 to 60 min, washed with 300 μl buffer NM and Met4-substrate was eluted from the proteinA beads by boiling for 5 min in 2× SDS sample buffer. The reactions were analyzed by Western blotting (9E10 antibodies).

Chromatin Immunoprecipitation (ChIP) Assay

Cells were grown in SD medium supplemented with 5 mM methionine at 25°C to an $OD_{600} = 1$, washed with prewarmed (30°C) SD medium lacking methionine and incubated in SD (−met) at 30°C. Samples were taken at 0, 15, and 60 min. After the 60 min sample was taken, *MET* gene expression was repressed by addition of methionine to a final concentration of 5 mM and incubation was continued for 30 min. ChIP assays were performed by a protocol based on Tanaka et al., 1997 (see http://www.fhcrc.org/labs/breeden/Methods/chromatinIP.html) except that after the cells were broken and centrifuged, the supernatant was discarded and the pellet (chromatin fraction) was resuspended in the initial volume of Lysis buffer. Met4-myc and Cbf1-HA were immunoprecipitated from an equivalent of 6.75×10^8 cells with polyclonal anti-myc antibodies (Santa Cruz Biotechnology) or 12CA5 antibodies, respectively. PCR reactions (15 min 94°C, 27×[50 s 94°C, 1 min 30 s 50°C, and 2 min 72°C], 10 min 72°C) were performed using HotStartTaq Master Mix Kit (Qiagen) on 1/6000 of the input (WCE) and 1/60 of the immunoprecipitation. PCR primers were used at a concentration of 0.2 μM. The primers were designed to amplify a fragment of 266bp [(−370) − (−125)] of the *MET25* promoter, 257 bp [(−278) − (−22)] and 370bp of a control fragment on chromosome XVI (within the promoter region of *HFI1*). Sequences of the primers are available at request from K. F. (karin@scripps.edu). PCR fragments were separated on a 2.5% agarose gel and visualized by ethidium bromide.

DNA-Affinity Purification

Cells were grown in YEPD to an $OD_{600} = 0.5$, shifted to 37°C for 2 hr, and extracts were prepared from approximately 3×10^7 cells in 2× buffer D1 (buffer D1: 50 mM HEPES [pH 7.5], 0.5 M NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100, α-Pr, 0.1mM orthovanadate, and 10mM pyrophosphate). The cleared lysate was diluted 1:10 with buffer D2 (50 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, α-Pr, 0.1 mM orthovanadate, and 10 mM pyrophosphate). Five milligrams of total protein was incubated with 1 μg/ml either biotinylated PCR MET25 promoter fragment or a biotinylated PCR control fragment. The PCR fragments were obtained with the same primer sequences as used for the ChIP assay, but the 5′ primer was biotinylated. After incubation for 2 hr at 4°C, 100 μl Strepavidin MagneSphere beads

(Promega, 1 mg/ml) were added and incubated for 1 hr at 4°C. The beads were washed 3 times in Buffer D3 (50mM HEPES [pH 7.5], 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 0.05% Triton X-100, α-Pr, 0.1 mM orthovanadate, and 10 mM pyrophosphate), eluted in 2× SDS sample buffer and analyzed by Western blotting.

Met4/Gst-Cbf1 Interaction

To express Gst-Cbf1 in *E. coli*, the *CBF1* orf was amplified by PCR and cloned into the BamH1 site of pGEX-4T2 (Pharmacia). Yeast cell lysates were prepared in buffer D3 supplemented with 5 mM EDTA, 5 mM EGTA, and 50 mM NaF. Eight-hundred micrograms total protein was incubated with 2 μg purified Gst or Gst-Cbf1 bound to glutathione beads at 4°C for 2 hr. Beads were washed 5 times with 1 ml buffer D3, proteins eluted in 2× SDS sample buffer, and analyzed by Western blotting.

Acknowledgments

We are grateful to C. Mann (Rpt1 antibody), S. Sadis and D. Finley (Gst-CDC4 and Gst-Met30 expression plasmids, purified E1 and Cdc34), B. Stillman (Orc3 antibody), and M. Tyers and D. Thomas (GAL-HA3-MET4 plasmid). We thank S. Haase and M. N. Boddy for helpful suggestions on the manuscript, Tapio Kesti for helpful suggestions on the DNA binding experiment and all members of the Scripps cell cycle group in the McGowan, Reed, Russell, and Wittenberg labs for a stimulating environment and many constructive discussions. P. K. acknowledges generous support from the Austrian Academy of Science (APART-fellowship). This work was supported by a grant from Hoffmann-LaRoche to S. I. R. and NIH grants GM38328 to S. I. R. and GM43487 and GM46006 to C. W.

Received December 27, 1999; revised June 5, 2000.

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