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General Repression of RNA Polymerase III Transcription Is Triggered by Protein Phosphatase Type 2A-Mediated Dephosphorylation of Maf1

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Summary

We report genome-wide analyses that establish Maf1 as a general and direct repressor of yeast RNA polymerase (Pol) III transcription. Chromatin immunoprecipitation (ChIP) coupled to microarray hybridization experiments showed an increased association of Maf1 to Pol III-transcribed genes under repressing condition (rapamycin treatment) correlated with a dissociation of Brf1 and Pol III. Maf1 can exist in various phosphorylation states and interacts with Pol III in a dephosphorylated state. The largest subunit of Pol III, C160, was identified as a target of Maf1. Under repressing conditions, Maf1 is dephosphorylated and accumulates in the nucleus, and Pol III-Maf1 interaction increases. Mutations in protein phosphatase type 2A (PP2A) catalytic subunit-encoding genes prevented rapamycin-induced Maf1 dephosphorylation, its nuclear accumulation, and repression of Pol III transcription. The results indicate that Pol III transcription can be globally and rapidly downregulated via dephosphorylation and relocation of a general negative cofactor.

Introduction

In eukaryotic cells, RNA polymerases (Pol) I and III are responsible for the synthesis of the RNA species involved in ribosome biogenesis and the process of translation. RNA synthesis by Pol I and Pol III represents more than 80% of all yeast nuclear transcription and is controlled in a coordinated way in response to various cellular and environmental conditions (reviewed in Warner [1999] and Willis et al. [2004]).

Pol III is responsible for the transcription of some 300 different genes in yeast (class III genes), mostly tRNA genes (Harismendy et al., 2003; Roberts et al., 2003; Moqtaderi and Struhl, 2004). In-depth analyses of the yeast Pol III transcription system have revealed a cascade of protein-DNA and protein-protein interactions leading to the recruitment of Pol III to its target tRNA genes: the binding of the 6-subunit TFIIC factor to the intragenic promoter, the TFIIC-directed recruitment and assembly of the three components of TFIIB (TBP, Brf1, and Bdp1), and the subsequent recruitment of the 17-subunit Pol III enzyme (reviewed in Geiduschek and Kassavetis [2001]). Whereas the essential factors and the basal mechanisms of class III gene transcription are well identified, much less is known about the molecular mechanisms of Pol III regulation.

In mammalian cells, there is biochemical evidence that RB and p53 repress Pol III transcription. Both RB and p53 bind to TFIIB and sequester it in an inactive complex that is not recruited to the promoter (Sutcliffe et al., 2000; Crighton et al., 2003; Hirsch et al., 2004), probably contributing in this way to the control of cell growth rate (reviewed in White [2005]). In yeast, Maf1 appears to be a central actor of Pol III regulation. Highly conserved from yeast to man, Maf1 was identified as a negative regulator of Pol III transcription. The genetic and physical interaction between Maf1 and Pol III suggested a direct role for Maf1 in Pol III downregulation (Boguta et al., 1997; Pluta et al., 2001). Based on Northern blot analysis of selected precursor tRNAs, Maf1 was found to mediate several signaling pathways that repress Pol III transcription in response to rapamycin-induced nutrient limitation, growth to stationary phase, DNA damage, secretory pathway defects, oxidative stress, and carbon starvation (Upadhyaya et al., 2002). In vitro studies identified Brf1 as a putative target of chlorpromazine-induced Maf1-dependent repression, in addition to Pol III, revealing a defect in TFIIB-DNA complex assembly and a reduced occupancy of tRNA genes by Pol III (Upadhyaya et al., 2002; Desai et al., 2005). Unexpectedly, Maf1 did not seem to differentially sequester Brf1 or Pol III under normal or repressing conditions, suggesting a nonstoichiometric mechanism of action (Desai et al., 2005). These observations raise a number of questions. First, it remains to be established on a genome-wide basis whether Maf1 is or is not a global repressor for all class III genes. Second, the molecular mechanisms that trigger Maf1 activity in response to different signaling pathways remain obscure. Third, the target(s) and the mode of action of Maf1 also need to be clarified.

In the present work, we describe genome-wide studies that confirm Maf1 as a global and direct repressor of Pol III transcription. Moreover, we show that Maf1 activity depends on its phosphorylation state, the regulation of which involves protein phosphatase type 2A. Finally, we identify the N-terminal region of the largest subunit of Pol III, C160, as a target of Maf1.

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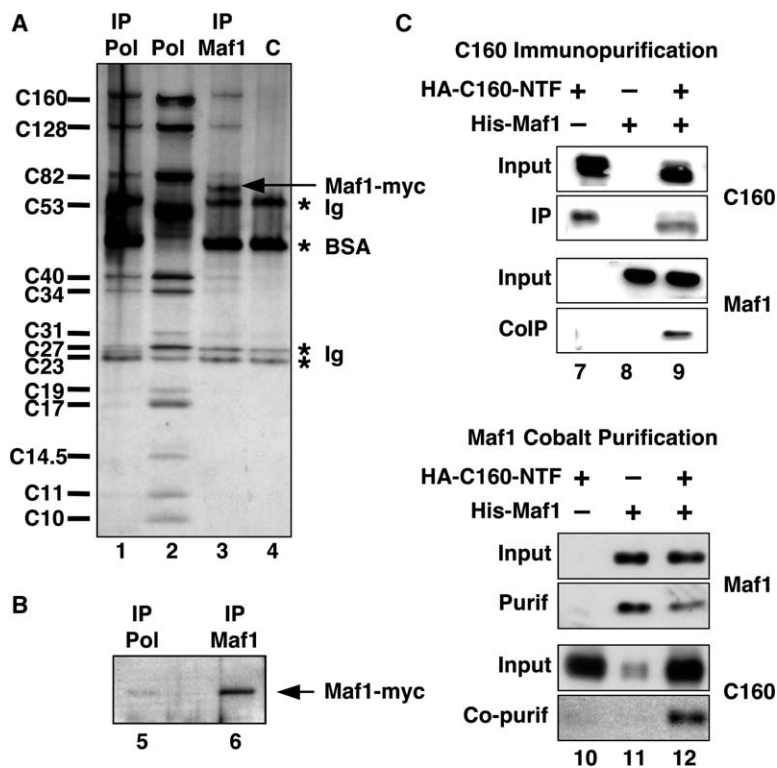


Figure 1. Maf1 Interacts Mainly with Pol III via the N-Terminal Domain of C160 Subunit

Protein extracts from cells expressing HA-tagged C160 and myc-tagged Maf1 were incubated with magnetic beads coated with anti-HA (IP Pol) or anti-myc (IP Maf1) antibodies. The beads were washed, and bound polypeptides were eluted and analyzed by SDS-PAGE.

(A) The proteins were silver stained; purified Pol III subunits (lane 2) are indicated on the left, myc-tagged Maf1 on the right. Mock control immunopurification (lane 4) allows the identification of immunoglobulins and BSA (stars).

(B) Immunopurified samples (IP) were analyzed by Western blot for myc-tagged Maf1 using anti-myc antibody.

(C) Crude extracts from *E. coli* (lanes 7–9) or baculovirus-infected insect cells (lanes 10–12) expressing HA-tagged C160 N-terminal fragment (HA-C160-NTF, lanes 7 and 10), 6 His-tagged Maf1 (His-Maf1, lanes 8 and 11), or both (lanes 9 and 12) were incubated with magnetic beads coated with anti-HA (lanes 7–9) or with a cobalt resin (lanes 10–12). The beads were washed, and bound polypeptides were eluted and analyzed by SDS-PAGE followed by immunoblotting using anti-HA or anti-His antibodies to detect HA-C160-NTF or His-Maf1, respectively. The polypeptides revealed by the tag-specific antibodies are identified on the right of each

panel. Protein input analysis indicated that all experiments were conducted with equal quantities of HA-C160-NTF or His-Maf1 (see Input). An unspecific band migrating at the level of HA-C160-NTF was revealed by anti-HA antibodies in lane 11 input. The beads coated with anti-HA antibody retained specifically HA-C160-NTF (lanes 7 and 9, IP). His-Maf1 coimmunopurified with HA-C160-NTF (lane 9, CoIP). Reciprocally, HA-C160-NTF was specifically retained on the cobalt resin only when coexpressed with His-Maf1 (lane 12, Co-purif).

Results

Maf1 Interacts with the N-Terminal Domain of Pol III Subunit C160

Previous immunopurification of Maf1 revealed its association with Pol III by Western blot analysis (Pluta et al., 2001). A weak interaction between Maf1 and Brf1 was also observed in pull-down experiments (Desai et al., 2005). To identify more exhaustively the interaction partners of Maf1, proteins coimmunopurifying with Maf1 were isolated and identified by mass spectrometry.

Maf1 or Pol III was immunopurified from crude extracts of double-tagged yeast cells expressing myc-tagged Maf1 and HA-tagged C160, the largest Pol III subunit. Coimmunopurified proteins were analyzed by SDS-PAGE and silver staining. Patterns of proteins coimmunopurified with Maf1 and with C160 were almost identical (Figure 1A, lanes 1 and 3). Five Pol III subunits coimmunopurifying with Maf1 were clearly identified in this way: C160, C128, C82, C40, and C34 (Figure 1A, compare Maf1 immunopurification in lane 3 with the subunits of purified Pol III in lane 2). A control experiment using an untagged strain revealed only the immunoglobulin chains and the BSA carrier (Figure 1A, lane 4). Mass spectrometry analysis of the tryptic peptides derived from the protein mixture immunopurified with Maf1 essentially confirmed the presence of peptides derived from Pol III subunits (C160, C128, C82, AC40, C34, AC19, C17, and ABC14.5; altogether 33 peptides) together with six peptides from a few proteins unrelated

to transcription, including Ded1 (see Discussion). Interestingly, when compared with a direct Pol III immunopurification experiment (Figure 1A, lane 1), a significant fraction (about 10%) of Pol III was found to coimmunopurify with Maf1 (Figure 1A, lane 3). Reciprocally, about 20% of the Maf1 present in the extract copurified with Pol III, as can be observed by comparing the relative amounts of Maf1 immunopurified directly or coimmunopurified with Pol III (Figure 1B, compare lanes 6 and 5, respectively). These values obtained with cells exponentially growing in a rich medium are possibly underestimated, as one cannot exclude a partial dissociation of the Pol III-Maf1 complex during the extraction and purification steps.

To further characterize Pol III-Maf1 association, we sought to identify the Pol III-subunit interacting physically with Maf1. DNA plasmids encoding N-terminal domains of C160 (the Pol III largest subunit) were previously found to suppress partially the phenotype of antisuppression and temperature-sensitive respiratory growth of the *maf1-1* mutation (Boguta et al., 1997). This genetic interaction could suggest a physical interaction between Maf1 and C160. This possibility was investigated by pull-down experiments using recombinant proteins. The N-terminal domain of HA-tagged C160 (235 residues, named HA-C160-NTF) and His-tagged Maf1 was coexpressed in *E. coli* or in insect cells (baculovirus system). Maf1 was selectively purified from crude extracts on cobalt resin and HA-C160-NTF on anti-HA magnetic beads. Western blot analysis of

E. coli (Figure 1C, lanes 7–9, Input) or insect cells (Figure 1C, lanes 10–12, Input) crude extracts revealed that HA-C160-NTF and His-Maf1 were present at about the same level. After immunopurification using anti-HA monoclonal antibody, Maf1 coimmunopurified specifically with HA-C160-NTF (Figure 1C, CoIP, compare lanes 9 and 8). Similarly, cobalt purification of Maf1 revealed that HA-C160-NTF copurified specifically with Maf1 (Figure 1C, Co-Purif, compare lanes 12 and 10). We thus conclude that the N terminus of C160 is a site of interaction with Maf1, although it cannot be excluded that Maf1 interacts with other Pol III subunits.

Pol III and TFIIB Recruitment to Class III Genes Is Downregulated by Maf1

Maf1-dependent repression of Pol III transcription has been observed in response to various drugs or stress conditions (Upadhyaya et al., 2002; Desai et al., 2005). To appreciate the physiological importance of such a regulatory event and the significance of *MAF1* under stress conditions, we plated wild-type and *maf1*Δ cells at various dilutions on drug-supplemented YPD media and allowed the cells to grow at 30°C for several days. *maf1*Δ cells were not more sensitive than wild-type cells to hydrogen peroxide, UV, or γ irradiation (data not shown). However, growth of *maf1*Δ strains on rapamycin plates was markedly impaired in comparison to the corresponding wild-type strains in two different genetic backgrounds (data not shown). Thus, upon rapamycin treatment, which mimics nutrient limitation, Maf1-mediated Pol III transcription repression appears to be important for cell survival. Furthermore, rapamycin was found to inhibit Pol III-directed transcription by affecting Pol III as well as the transcription initiation factor TFIIB (Zaragoza et al., 1998). As a consequence, we have focused our study on Pol III repression induced upon rapamycin treatment or in stationary growth phase.

ChIP on chip analysis of class III gene occupancy by the Pol III machinery revealed a decrease in the recruitment of Pol III and Bdp1 in stationary-phase cultures (Harismendy et al., 2003). We investigated whether this decrease in gene occupancy was linked to the presence of Maf1. *maf1*Δ and wild-type cells were harvested in exponential or stationary growth phase or after rapamycin treatment. Epitope-tagged chromatin proteins Brf1-3HA and Bdp1-3HA (TFIIB subunits), τ95-13myc (TFIIIC subunit), and 3HA-C160 (pol III subunit) were crosslinked to their targets in vivo. Each strain harbored one epitope-tagged bait: 3HA-C160 (Figure 2A, 1–3), Brf1-3HA (Figure 2A, 4–6), Bdp1-3HA (Figure 2A, 7–9), or τ95-13myc (Figure 2A, 10–12). Protein-DNA interactions were fixed in exponential growth phase (Figure 2A, 1, 4, 7, and 10), after 30 min of treatment with rapamycin (Figure 2A, 2, 5, 8, and 11), or in stationary growth phase (Figure 2A, 3, 6, 9, and 12). Genome-wide analysis of the enrichment of DNA fragments immunopurified with antibodies directed to epitope tags was performed. The fold enrichment for each DNA locus was normalized to the fold enrichment obtained for the wild-type strain in exponential growth phase (Figure 2A, 1, 4, 7, and 10). Each line inside each panel represents a Locus Enriched by the Pol III Transcription Machinery (LEPTM); they are color coded and ranked by increasing levels of occupancy changes in the wild-type strain in stationary growth phase

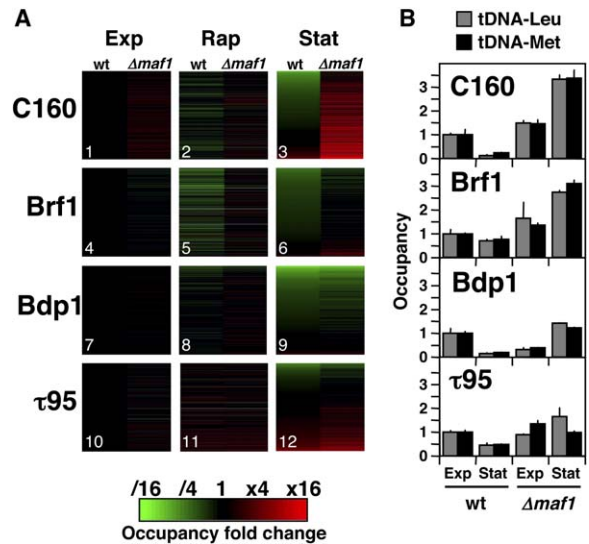


Figure 2. Genome-Wide Effect of Maf1 on Class III Gene Occupancy by the Transcription Machinery under Repressing Conditions

(A) Chromatin was immunopurified via different tagged proteins: C160 (1, 2, and 3), Brf1 (4, 5, and 6), Bdp1 (7, 8, and 9), or τ95 (10, 11, and 12) from wild-type (wt) or *maf1*Δ strains, as indicated. The cells were fixed in formaldehyde either during exponential growth phase in rich medium (1, 4, 7, and 10), after 30 min treatment with rapamycin 0.2 ng/μl (2, 5, 8, and 11), or in stationary phase (3, 6, 9, and 12). The color scale diagrams show the changes of occupancy of the transcription machinery on its target loci (LEPTM), relative to the wild-type cells in exponential growth phase.

(B) Enrichment of the tRNA-Leu and tRNA-Met genes by chromatin immunopurification of Brf1, Bdp1, C160, or τ95, in exponential or stationary growth phase in *MAF1* or *maf1*Δ strains. Enrichment is normalized to that obtained with the wt strain in exponential growth phase. Error bars represent the standard deviation.

(Figure 2A, 3, 6, 9, and 12). The following results were derived from 36 independent experiments. Although some genes may display variable responses, we have been unable to statistically identify some subclasses of class III genes from these occupancy studies.

In exponential growth phase, very minor differences in occupancy were observed between wild-type and *maf1*Δ strains (Figure 2A, compare the right and left sides of 1, 4, 7, and 10). After 30 min rapamycin treatment (Figure 2A, 2, 5, and 8), the occupancy of class III genes by C160, Brf1, and Bdp1 dropped in the wild-type strain while it increased slightly or did not change in the *maf1*Δ strain. Note that Bdp1 occupancy appeared to decrease less than that of Brf1. However, two genome-wide experiments using different baits and antibodies cannot be directly compared on quantitative grounds. Class III gene occupancy by TFIIC remained essentially identical in wild-type and *maf1*Δ strains (Figure 2A, 11, right and left parts slightly red). In stationary growth phase, as previously observed (Harismendy et al., 2003), Pol III (C160) and TFIIB (Bdp1 and Brf1) tended to dissociate from class III genes in the wild-type strain, whereas TFIIC (τ95) mainly remained bound (Figure 2A, 3, 6, 9, and 12, left parts). In the *maf1*Δ context, TFIIC and Bdp1 behaved the same as in the wild-type (Figure 2A, 9 and 12, compare right and left sides). TFIIC binding was influenced relatively little by the growth phase or the presence of Maf1. In

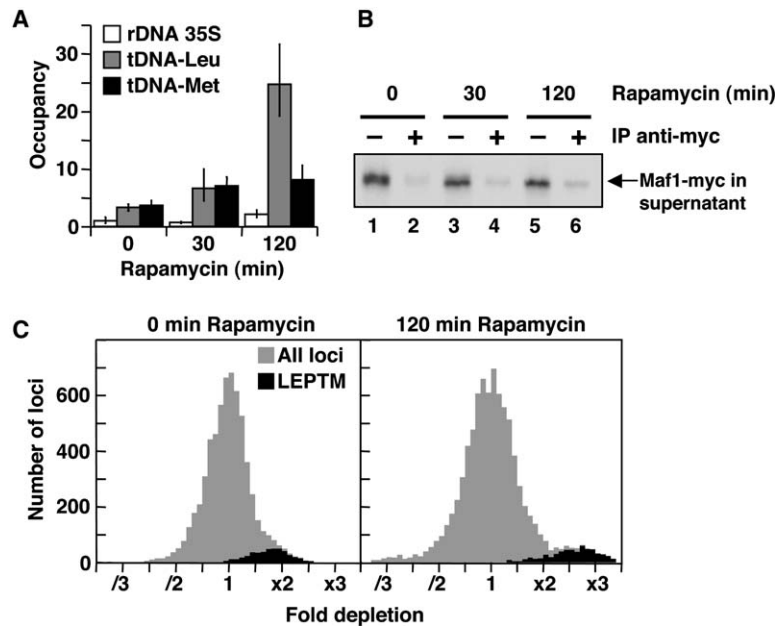


Figure 3. Recruitment of Maf1 to Class III Genes

Cells were incubated with rapamycin (0.2 ng/ μ l) for 0, 30, and 120 min then protein-DNA interactions were fixed by formaldehyde and whole-cells extracts were prepared as described.

(A) Chromatin was immunoprecipitated via the epitope-tagged Maf1, and DNA enrichment was measured by real-time quantitative PCR analysis of genes encoding 35S rRNA, tRNA-Leu, and tRNA-Met. Enrichment is normalized to that obtained for 35S rRNA in exponentially growing cells (0 min sample). Error bars represent the standard deviation. (B) Immunodepletion of Maf1 from whole-cell extracts was evaluated by Western blotting before (–) or after (+) the immunopurification of epitope-tagged Maf1.

(C) Selective depletion of intergenic regions adjacent to class III genes upon Maf1 immunodepletion before or after rapamycin addition. The target loci of the Pol III machinery (LEPTM) are highlighted in black.

contrast, in absence of Maf1 and in stationary growth phase, there was a strong increase in occupancy of Pol III (Figure 2A, 3, right) and Brf1 occupied class III genes as in the exponential growth phase situation (Figure 2A, 6, right). The Maf1-dependent Brf1 and Pol III decreased occupancies observed here in vivo are in keeping with the observation that Maf1 inhibits the recruitment of Brf1 and Pol III in vitro (Desai et al., 2005).

Using real-time PCR amplification (Figure 2B), we confirmed and quantified the occupancy of the Pol III transcription machinery for two tRNA gene species encoding tRNA-Leu and tRNA-Met (also referred to as tL[CAA] and tM[CAU] in the *Saccharomyces* Genome Database). In exponentially growing cells, the deletion of *MAF1* slightly increased or did not influence substantially the levels of tRNA-Leu and tRNA-Met genes occupancy by the components of the Pol III machinery (except for an intriguing decrease in Bdp1 occupancy that was not observed in the genome-wide analysis). In contrast, instead of the marked decrease in gene occupancy observed in the wild-type, stationary-phase *maf1* Δ cells retained a high level of occupancy similar to that of wild-type cells in the exponential growth phase (Figure 2B, Bdp1 and τ 95) or even 3-fold higher (Figure 2B, C160 and Brf1). Altogether, these results clearly correlated the genome-wide repression of TFIIIB and Pol III recruitment with the presence of Maf1.

Increased Genome-Wide Recruitment of Maf1 to Class III Genes under Repressing Conditions

The Maf1-mediated modification of the occupancy of class III genes by the Pol III transcription machinery under conditions of repression (stationary phase cells or rapamycin) raised the question of the mode of action of Maf1. Although previous attempts to chromatin immunoprecipitate Maf1 were unsuccessful (Desai et al., 2005), we wondered whether Maf1 is recruited (possibly as a complex with Pol III) to class III genes and, if this were the case, whether the level of Maf1 recruitment varies upon rapamycin treatment.

ChIP of Maf1 analyzed by real-time PCR showed a clear enrichment of DNA fragments of two tRNA genes when cells were harvested in the exponential growth phase (Figure 3A, time 0). This enrichment was small but specific to the tRNA genes when compared to the background level obtained with a Pol I promoter that is present in 100–150 copies in the yeast genome. By comparison, we observed a 10-fold higher enrichment of the same tRNA species by chromatin immunopurification of Pol III (data not shown). The presence of Maf1 on the two tRNA species increased markedly after a 30 or 120 min treatment with rapamycin (Figure 3A, compare times 30 and 120 min with 0 min), indicating an enhanced recruitment of Maf1 under repressing conditions.

A genome-wide study of class III gene occupancy by Maf1 was undertaken to confirm and extend the above results to the whole genome as well as to establish the postulated role of Maf1 as a general regulator of Pol III transcription. We took advantage of the observation that immunopurification of myc-tagged Maf1 was very efficient. Incubation of crude extracts with antibodies directed against the myc epitope resulted in a near complete depletion of Maf1 from the supernatant (Figure 3B, compare lanes 2, 4, and 6 with lanes 1, 3 and 5). We compared the DNA fragments associated with the cross-linked chromatin before and after Maf1 immunopurification by hybridization to intergenic DNA microarrays (depletion analysis). This procedure was more sensitive and more precise than the classical ChIP on chip approach where the immunopurified DNA and DNA from whole-cell extracts are competitively hybridized. Figure 3C shows the histograms of fold depletion of the Maf1-depleted loci before and after rapamycin treatment. Loci enriched by the Pol III transcription machinery (LEPTM; [Harismendy et al., 2003]) were significantly more depleted after the immunopurification of Maf1 in exponentially growing cells, compared to the distribution of all the loci. The depletion of the LEPTM was further increased after 120 min of rapamycin treatment (Figure 3C, compare the distribution of black bars and

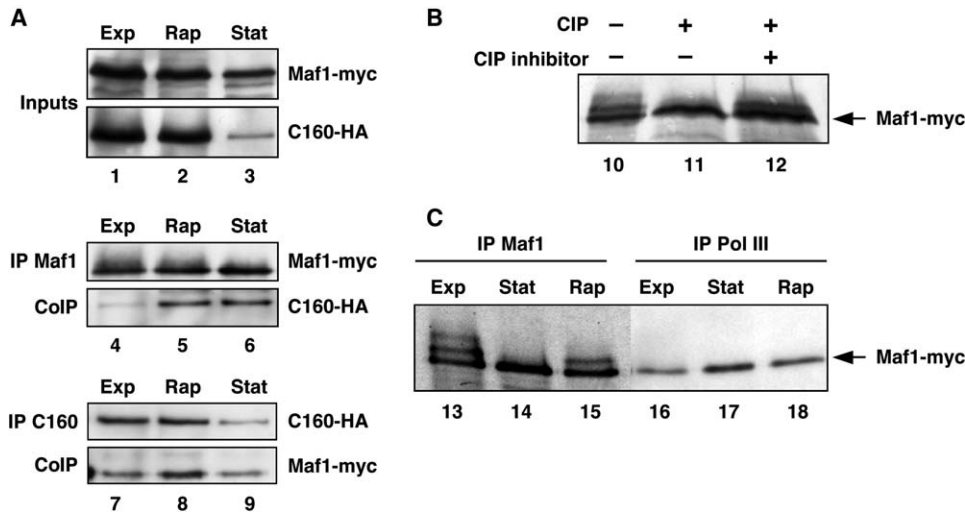


Figure 4. Pol III-Maf1 Interaction Increases in Stress Conditions. Maf1 Is Phosphorylated, but Only the Dephosphorylated Form of Maf1 Interacts with Pol III

Cells expressing HA-tagged C160 and myc-tagged Maf1 were collected either in exponential growth phase in rich medium (lanes 1, 4, 7, 10–13, and 16), after 60 min treatment with rapamycin 0.1 ng/ μ l (lanes 2, 5, 8, 15, and 18), or in stationary phase of growth (lanes 3, 6, 9, 14, and 17). Protein extracts were incubated with magnetic beads coated with anti-myc (IP Maf1, lanes 4–6 and 10–15) or anti-HA (IP C160, lanes 7–9 and 16–18) antibodies. The beads were washed, and the bound polypeptides were eluted and analyzed by SDS-PAGE, followed by immunoblotting using specific anti-HA or anti-myc antibodies. For clarity, the polypeptides revealed by the specific antibodies are identified on the right.

(A) Analysis of Maf1 and C160 levels in protein extracts (Inputs, lanes 1–3), immunopurification of Maf1 (IP Maf1, lanes 4–6) and coimmunopurification of C160 (CoIP, lanes 4–6), and immunopurification of C160 (IP Pol III, lanes 7–9) and coimmunopurification of Maf1 (CoIP, lanes 7–9). (B) Western blot analysis using a modified acrylamide:bisacrylamide ratio SDS-PAGE of immunopurified Maf1-myc incubated without (lane 10) or with Calf intestinal phosphatase (CIP, lane 11) or with CIP and phosphatase inhibitors (lane 12).

(C) Western blot analysis using a modified acrylamide:bisacrylamide ratio SDS-PAGE of Maf1-myc immunopurified from exponential phase, stationary phase, or rapamycin-treated cells (IP Maf1, lanes 13–15) and of Maf1-myc coimmunopurified with C160 from the same cells (IP Pol III, lanes 16–18).

gray bars). These results confirmed the selective recruitment of Maf1 to all class III genes, even, though to a lesser extent, in conditions of active growth. The general and enhanced recruitment of Maf1 upon rapamycin treatment confirmed Maf1 as a general regulator of Pol III transcription.

Increased Pol III-Maf1 Interaction under Repressing Conditions

We explored the possibility that Maf1 repressional activity could be regulated by a modification of the level of interaction between Maf1 and Pol III. To immunopurify Maf1 and Pol III independently, crude extracts were prepared from double-tagged cells expressing Maf1-myc and HA-C160. Western blot analysis of equal amounts of crude extracts showed that a rapamycin treatment did not affect the level of Maf1-myc or HA-C160 (Figure 4A, compare lanes 1 and 2). In contrast, less Maf1 and markedly lower amounts of HA-C160 were recovered from stationary phase cells (Figure 4A, compare lanes 1 and 3). To determine the amount of Maf1 interacting with Pol III, myc-tagged Maf1 was immunopurified from the same crude extracts and HA-C160 subunit copurifying with Maf1 was analyzed by Western blot. Despite the presence of equal amounts of immunopurified Maf1 (Figure 4A, IP Maf1, compare lanes 4–6), we observed a drastic increase in coimmunopurified HA-C160 upon rapamycin treatment (Figure 4A, CoIP, compare lanes 5 and 4), in sharp contrast with a recent report concluding that the mechanism of repression by Maf1 does not involve a quantitative change in its inter-

action with Pol III (Desai et al., 2005). Remarkably, the level of Maf1 bound Pol III was the same in stationary phase and rapamycin-treated cells (Figure 4A, CoIP, compare lanes 6 and 5), although the Pol III:Maf1 ratios in crude extracts were different (Figure 4A, compare lanes 2 and 3). In a reciprocal experiment, HA-tagged C160 was immunopurified from the same crude extracts to analyze Maf1-myc copurifying with Pol III (Figure 4A, IP C160 and CoIP, lanes 7–9). Again, more Maf1 was coimmunopurified with Pol III after rapamycin treatment. The proportion of Pol III complexed to Maf1 was also similarly increased in stationary phase cells. The ratio of C160 to copurified Maf1 signals was 0.6, 1.2, and 1.2 in the exponential, rapamycin, and stationary phase situation, respectively. In conclusion, the repression of Pol III transcription upon rapamycin treatment does not seem to be correlated to a change in Maf1 or Pol III levels but to an increase in Pol III-Maf1 interaction. In stationary phase, both a lower level of Pol III and an increase in Pol III-Maf1 interaction probably contribute to transcriptional repression.

Nuclear Accumulation of Maf1 in Response to a Rapamycin Treatment or in Stationary Growth Phase

We considered the possibility that Maf1 activity might be regulated by posttranslational modifications and/or at the level of its intracellular localization. We used polyclonal antibodies to localize endogenous Maf1 (i.e., not overexpressed) in yeast cells by immunofluorescence. These antibodies were highly specific, as control cells

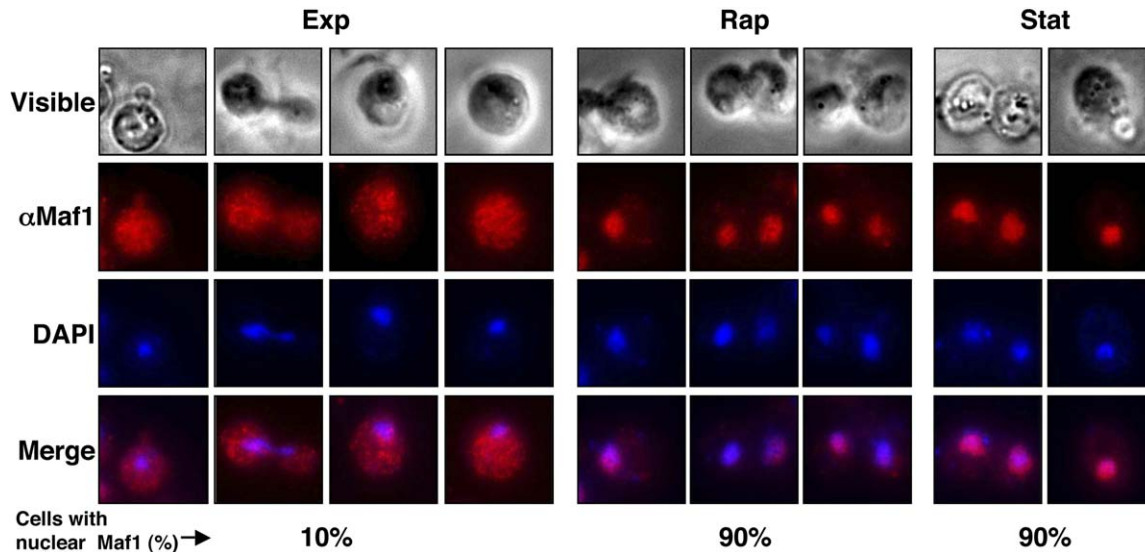


Figure 5. Maf1 Accumulates in the Nucleus upon Rapamycin Treatment or in Stationary Phase

Maf1 localization by immunofluorescence microscopy in exponentially growing wild-type cells taken before (Exp) or 60 min after addition of 0.2 ng/ μ l rapamycin (Rap) or in stationary phase cells (Stat). The cells were analyzed by phase-contrast light microscopy (Visible), by immunofluorescence (α Maf1), and by DAPI staining (DAPI). Maf1 signal (red) was essentially in the cytoplasm of exponentially growing wild-type cells and nuclear in stationary phase cells or in rapamycin-treated cells (see row Merge). The percentage of cells with the nuclear Maf1 signal clearly above the cytoplasmic signal is indicated.

deleted for the *MAF1* gene did not show any labeling (data not shown). In exponentially growing cells, the fluorescence signal appeared uniformly distributed throughout most of the cells (Figure 5, Exp). We next investigated whether the intracellular distribution of Maf1 was affected by rapamycin or stationary phase. Remarkably, in both cases, the fluorescence signal was concentrated in the nuclear compartment and overlapped the DAPI staining (Figure 5, Rap and Stat). This nuclear signal accumulation was observed in the vast majority of cells (90%), consistent with the interpretation that Maf1 intracellular distribution is regulated and under the control of the TOR pathway. There are previous examples of Pol II transcription factors that translocate to the nucleus after addition of rapamycin (Beck and Hall, 1999; Komeili et al., 2000).

The Dephosphorylated Form of Maf1 Is Associated with Pol III

We explored the possibility that posttranslational modifications could affect Maf1 intracellular distribution and its interaction with Pol III. Maf1-myc immunopurified from exponentially growing cells migrated as at least three bands by using a modified acrylamide:bisacrylamide ratio SDS-PAGE (Figure 4B, lane 10). The upper, slow-migrating band was present in variable amounts. Calf intestinal phosphatase treatment of immunopurified Maf1 caused the loss of the two upper bands and a proportional increase in the fast-migrating band (Figure 4B, lane 11). Phosphatase inhibitors counteracted this change, confirming that the slow-migrating forms of Maf1-myc are modified by phosphorylation (Figure 4B, compare lane 12 with lanes 10 and 11).

Because we showed that the amount of Pol III-Maf1 complex changed after nutrient limitation (see above), it was interesting to investigate which form(s) of Maf1 in-

teracts with the enzyme. Maf1 was immunopurified from exponential growing cells (double-tagged cells expressing Maf1-myc and HA-C160), treated or not with rapamycin, and from stationary phase cells and analyzed by SDS-PAGE to resolve the phosphorylated forms (Figure 4C, lanes 13–15). Western blot analysis showed that Maf1 migrated as three electrophoretic-mobility forms in the exponential growth phase (Figure 4C, lane 13). Maf1 from stationary phase cells migrated as a single band, corresponding to the fast-migrating dephosphorylated form (Figure 4C, lane 14). Rapamycin treatment also affected the phosphorylation pattern of Maf1, causing the disappearance of the slowest-migrating phosphorylated form (Figure 4C, lane 15). Thus, Maf1 can be differently modified under different physiological conditions. To investigate which forms of Maf1 interact with Pol III, epitope-tagged C160 was immunopurified from the same crude extracts and Maf1 copurifying with Pol III was analyzed on the same gel (Figure 4C, lanes 16–18) alongside immunopurified Maf1 (Figure 4C, lanes 13–15). Remarkably, only the fast, dephosphorylated form of Maf1 coimmunopurified with Pol III in all the conditions tested: exponential or stationary growth phase cells and rapamycin-treated cells. Note that the yield of Pol III bound Maf1 was lower in rapidly dividing cells than in stationary-phase cells or rapamycin-treated cells (Figure 4C, compare lane 16 with lanes 17 and 18). The phosphorylation state of Maf1 therefore appears to be a key factor in the modulation of Pol III-Maf1 interaction.

PP2A Controls the Maf1-Mediated Pol III Transcription Repression via Maf1

Dephosphorylation and Nuclear Localization

Defective Pol III transcription has been observed in a mutant of *TPD3*, the gene encoding the scaffold

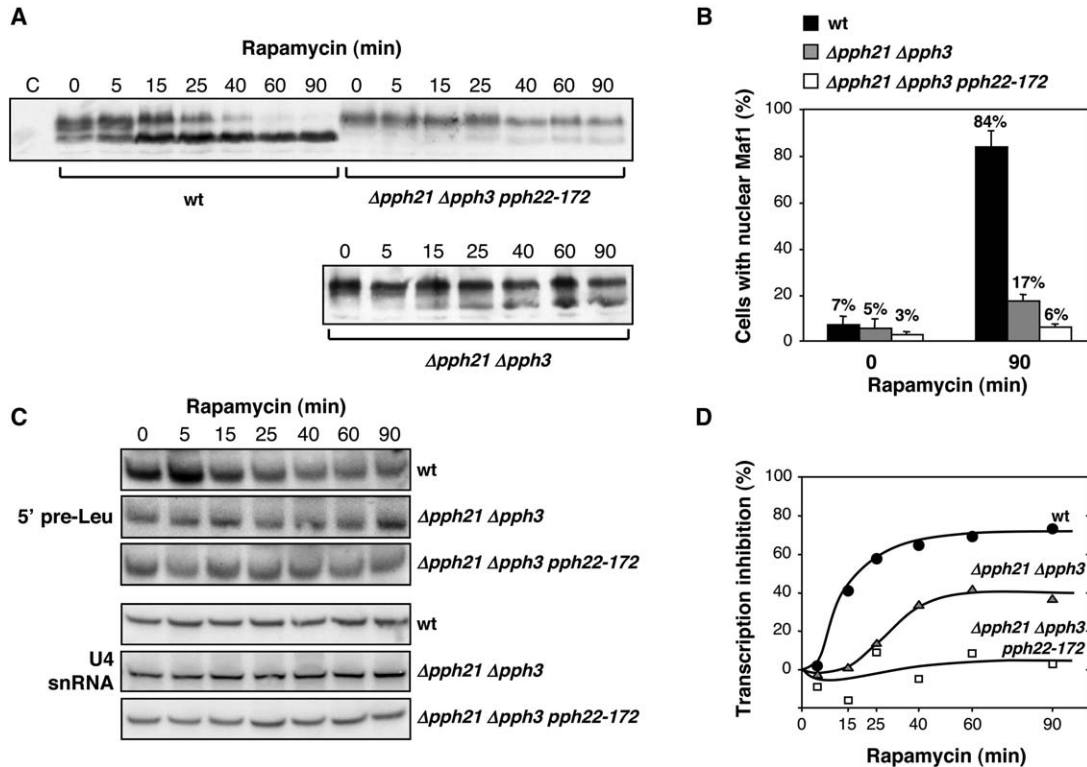


Figure 6. PP2A Controls Maf1-Mediated Pol III Transcription Regulation via Maf1 Dephosphorylation and Nuclear Localization

Wild-type strain (wt), $pph21\Delta pph3\Delta$, and $pph21\Delta pph3\Delta pph22-172$ mutant strains derivative were grown to midlog phase at 23°C and harvested at the indicated time after rapamycin (0.2 ng/ μ l) addition. To stop Maf1 neosynthesis during by the rapamycin treatment, cycloheximide (10 μ g/ml) was added 10 min before rapamycin addition.

(A) Crude extracts from TCA-precipitated cells were analyzed by using a modified acrylamide:bisacrylamide ratio SDS-PAGE followed by immunoblotting using polyclonal anti-Maf1 antibodies. Specific Maf1 bands were identified by comparison with a crude extract from *maf1* Δ cells that contained only a faintly immunoreactive protein band (lane C).

(B) Maf1 localization in wild-type, $pph21\Delta pph3\Delta$, and $pph21\Delta pph3\Delta pph22-172$ cells was analyzed by immunofluorescence microscopy using anti-Maf1 polyclonal antibodies before and 90 min after rapamycin addition. Nuclei were stained with DAPI. The results from three independent experiments are plotted as percent of cells with nuclear Maf1 immunofluorescence intensity clearly above the cytoplasmic signal. Error bars represent the standard deviation.

(C) Total RNA was extracted from wild-type, $pph21\Delta pph3\Delta$, and $pph21\Delta pph3\Delta pph22-172$ mutant cells at different times after rapamycin addition and analyzed by Northern blot hybridization (25 μ g/lane) with precursor tRNA^{Leu} (5' pre-Leu) and U4 snRNA-specific probes.

(D) Northern blot experiment (as above) was quantified by PhosphorImager analysis. RNA values were normalized to those for U4 RNA and plotted as percent of inhibition of transcription upon rapamycin addition.

subunit of protein phosphatase 2A (PP2A). Deregulated PP2A was proposed to dephosphorylate and activate a hypothetical inhibitor of transcription acting on TFIIIB and Pol III (Van Zyl et al., 1992). These old observations prompted us to investigate a putative effect of PP2A phosphatase activity on Maf1 phosphorylation state, localization, and repressor activity. Three redundant genes, *PPH21*, *PPH22*, and *PPH3*, encode the canonical catalytic subunit of PP2A (Ronne et al., 1991; Düvel et al., 2003). The inactivation of all three genes is lethal. We used a mutant strain defective in PP2A catalytic activity as a result of deletions of *PPH21* and *PPH3* in combination with the thermosensitive allele *pph22-172*. This mutant strain exhibits a near complete reduction in rapamycin-induced transcriptional changes (Düvel et al., 2003). Because we observed a marked increase of the level of Maf1 in Western blots during rapamycin treatment, the following experiments were carried out in the presence of cycloheximide to prevent Maf1 neosynthesis. In control experiments, cycloheximide alone, unlike rapamycin, did not cause the dephosphorylation

of Maf1, did not lead to its nuclear accumulation, and did not reduce the level of pre-tRNA^{Leu} (data not shown). In addition, as the cell growth rate of the triple mutant was already affected at permissive temperature (23°C), we chose not to further perturb the cells by superimposing a temperature stress. Maf1 level and its electrophoretic mobility were monitored by immunoblotting (Figure 6A). In exponentially growing cells, the fast-migrating, dephosphorylated form of Maf1 became predominant soon after rapamycin addition and represented 90% of total Maf1 after 90 min. In contrast, in $pph21\Delta pph3\Delta pph22-172$ triple mutant, the phosphorylated species remained largely predominant up to 90 min treatment, whereas the dephosphorylated form of Maf1 increased slowly and represented at most 20% of total Maf1 at 90 min. This small level of residual dephosphorylation was likely due to the leakage of the *pph22-172* mutation at 23°C. Next, we examined the cellular localization of Maf1 in the mutant cells. Compared to the situation in wild-type cells, the nuclear accumulation of Maf1 in $pph21\Delta pph3\Delta pph22-172$ cells was

slightly lower during active growth and was dramatically reduced to about 10% of the cells instead of 80% of wild-type cells upon rapamycin treatment (Figure 6B). We examined by Northern blotting the level of rapamycin-induced repression of tRNA synthesis in PP2A-defective cells (Figure 6C). The results were corrected for variations in the recovery of the internal control U4 snRNA (Figure 6C). After rapamycin addition, transcription of the tRNA^{Leu} gene was rapidly and drastically repressed in wild-type strain (70% inhibition at 90 min, Figure 6D). In contrast, *pph21Δ pph3Δ pph22-172* cells showed no detectable repression (0%–5% inhibition at 90 min). In similar experiments, we also used a double mutant deleted for *PPH21* and *PPH3* that grows like the wild-type. The inhibition of Maf1 dephosphorylation, of Maf1 nuclear localization, and of transcription repression was more limited than with the triple mutant, but the three effects were remarkably correlated both quantitatively and kinetically (Figure 6 and the Supplemental Data experiments performed without cycloheximide, available with this article online). These observations indicated that the three events are tightly linked and that Maf1 activation is sensitive to PP2A levels. In conclusion, upon rapamycin treatment, the dephosphorylation of Maf1, its accumulation in the nucleus, and the repression of the expression of a class III gene were inhibited in PP2A-defective cells, which argues in favor of PP2A-mediated activation of Maf1.

Discussion

In this work, we present evidence that Maf1 is a general negative cofactor of the Pol III transcription system. Maf1 activity is modulated by a phosphorylation-dephosphorylation mechanism involving PP2A that affects its nuclear localization and interaction with its predominant target, Pol III.

The two different genome-wide approaches presented here strongly support the hypothesis of Maf1 acting as a master switch of Pol III transcription: (1) the general reduced occupancy of Pol III and Brf1 on class III genes in stationary phase or after rapamycin treatment was found to be Maf1-dependent, and (2) Maf1 was selectively recruited on the vast majority of class III genes, and the occupancy of Maf1 on class III genes was enhanced upon rapamycin treatment (see also the accompanying article by Roberts et al. [2006]). Therefore, it can be concluded that Maf1 is required for repression of the whole Pol III transcriptome. The recruitment of Maf1 on all class III genes, its physical and genetic interaction with Pol III (Boguta et al. [1997], Pluta et al. [2001], and this work), and its interaction with Brf1 (Desai et al., 2005) added to its essential role in mediating repression (Upadhyaya et al., 2002) qualify Maf1 as a general negative cofactor of the Pol III transcription system. *MAF1* gene is not essential for cell viability. However, the growth of *maf1Δ* strains on rapamycin plates was markedly impaired compared to *MAF1* cells. Repression of Pol III transcription is therefore physiologically important to cope with adverse conditions. Remarkably, even in exponentially growing cells, there was a low but significant level of Maf1 occupancy on class III genes. It is therefore likely that Pol III transcription is finely tuned all along the culture growth phases.

Along the same line of thought, it would be reasonable to predict the existence of a general positive cofactor of Pol III transcription as antagonist to Maf1.

Although Maf1 appears to effect repression directly at the level of the transcriptional units, its mode of action is still obscure. TFIIB is the likely target of Maf1-mediated repression (Van Zyl et al., 1992; Ghavidel and Schultz, 1997; Zaragoza et al., 1998; Upadhyaya et al., 2002; Desai et al., 2005). We sought to identify the predominant targets of Maf1 by mass spectrometry after immunopurification. Pol III and TFIIB components were anticipated from previous work (Pluta et al., 2001; Desai et al., 2005). Whereas Pol III subunits were predominantly coimmunopurified with Maf1, TFIIB components were not found, suggesting a much weaker or transient interaction. Among the few additional copurified proteins, the finding of Ded1, identified by two peptides, was interesting because *DED1* gene was isolated as a multicopy suppressor of mutations affecting two Pol III subunits and one TFIIC subunit (Lefebvre et al., 1994; Thuillier et al., 1995; Rubbi et al., 1999). Ded1 is an ATP-dependent RNA helicase of the DEAD family, cytoplasmic, essential for growth, and required for translation, at the initiation step (Chuang et al., 1997). The suppression of Pol III transcription defects, therefore, was supposed to be indirect: Ded1 overexpression might overcome a translation defect that may arise from low levels of tRNAs and particularly of initiator tRNA in the mutants (Conesa et al., 2005). One interesting hypothesis could be that Ded1 might contribute to stimulate Pol III transcription by sequestering Maf1 in the cytoplasm.

The interaction of Maf1 with the N-terminal region of C160 was another interesting observation. Overexpression of this domain was originally found to suppress the *maf1-1* mutation (i.e., to reduce Pol III transcription [Boguta et al., 1997]). We checked that cells overexpressing this small part of C160 had normal levels of Pol III subunits (data not shown). Therefore, one hypothesis is that excess of this domain titrates a putative activator or an essential component of the transcription system (that could be TFIIB). Maf1 could repress transcription in part by blocking this critical interaction site in the enzyme.

Maf1 effects Pol III repression under conditions that activate at least three signaling pathways (secretory pathway defect, target of rapamycin [TOR], and DNA damage response [Upadhyaya et al., 2002]). How Maf1 is activated by these pathways remains obscure. We found that Maf1 purified from actively growing cells migrated electrophoretically as multiple bands corresponding to different phosphorylation states. Only the dephosphorylated form of Maf1 (which is largely predominant in stationary phase or rapamycin-treated cells) was present in isolated Pol III-Maf1 complexes. These observations strongly suggested a regulation of Maf1 activity by a phosphorylation-dephosphorylation mechanism. In addition, we observed that, well correlated with the level of the dephosphorylated form, Maf1 accumulates in the nucleus under repressing conditions (rapamycin or stationary phase). Similar observations are presented by Cairns and collaborators in the accompanying article (Roberts et al., 2006). A simplified scenario emerges from these data. Under repressing conditions, Maf1 is rapidly dephosphorylated and

accumulates in the nucleus, causing an increase in Pol III-Maf1 complexes that bring Maf1 physically to all the class III transcription units where it opposes TFIIB and Pol III recruitment, as suggested by Desai et al. (2005), leading to transcriptional repression. The mode of action of Maf1 is still obscure: in a variation of this scheme, Roberts et al. (2006) suggest that the reduced occupancy of Pol III at the onset of repression may reflect a Maf1-induced transition of transcription complexes into a nonproductive recruitment state in which Pol III becomes less accessible to DNA cross-linking. The agent(s) that dephosphorylates Maf1 remained to be identified. Protein phosphatase 2A, a member of the TOR pathway (see review of Düvel and Broach [2004]) was a good candidate based on the old observation that *tpd3* null mutants show defective Pol III transcription (Van Zyl et al., 1992). Tpd3 is the PP2A A subunit that provides a scaffold between the catalytic subunit and the type B regulatory subunits (Groves et al., 1999). The deregulated PP2A activity was hypothesized to dephosphorylate an unidentified protein that, in its unphosphorylated state, would interfere with Pol III transcription. Because addition of TFIIB, and to a lesser extent Pol III, restored transcription activity of *tpd3* extracts, these components were proposed to be targets of PP2A or of the postulated inhibitor (Van Zyl et al., 1992). Maf1 displayed all the characteristics of this postulated, PP2A-activated inhibitor. Indeed, in a reciprocal experiment, we found that mutation of the genes encoding the catalytic subunit of PP2A reduced dramatically rapamycin-induced Maf1 dephosphorylation as well as its nuclear accumulation and Pol III repression. We conclude that PP2A activity, required for the activation of Maf1, is part of the Pol III repression pathway. As Maf1-mediated repression responds to at least three signaling pathways (Upadhyaya et al., 2002), other proteins phosphatases may activate Maf1 under different stress conditions. Regarding the TOR pathway, Sit4, the catalytic subunit of the other type 2A protein phosphatase (Sutton et al., 1991), does not seem to be involved in rapamycin-induced Pol III repression (Willis et al., 2004).

Experimental Procedures

Yeast Strains

All strains used in this study are described in the Supplemental Data available with this article online.

Cell Growth Conditions, Rapamycin Treatment, and Formaldehyde Fixation

For standard growth condition, strains were cultivated in YPD and collected at 0.6–1 OD_{600nm}. Cells were incubated with or without rapamycin (0.2 ng/μl), and protein-DNA interactions were fixed with formaldehyde as described previously (Harismendy et al., 2003). Cells in stationary growth phase were collected 48 hr after having reached 0.6 OD_{600nm} (at an OD_{600nm} varying between 8 and 12, depending on the strains) and fixed. The fixed and washed cells were fractionated in 30 OD_{600nm} aliquots stored at –80°C before extraction.

Dephosphorylation of Immunoprecipitated Proteins

To check Maf1 phosphorylation state, Maf1-immunoprecipitated protein was dephosphorylated with 10 U of Calf intestinal phosphatase (CIP) for 30 min at 37°C in a 20 μl total volume of reaction buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM ZnCl₂, and 1 mM MgCl₂). Maf1 phosphorylation state was analyzed by a modified acrylamide:bisacrylamide ratio (33.5:0.3) SDS-PAGE and immunoblotting.

Phosphatase inhibitors (5 mM β-glycerophosphate, 1.5 mM p-NO₂-phenylphosphate, and 1 mM sodium orthovanadate) were added when indicated.

Protein Extraction and Immunoblotting

To ensure that kinases or phosphatases were not functional during cell harvesting and protein extraction, yeast cells were rapidly harvested in the cold by centrifugation (3000 × g, 4°C, 1 min), and 20% trichloroacetic acid (TCA) was added to the cell pellet (Dubacq et al., 2004). Cells were broken with acid-washed glass beads, the supernatant was retained, and the glass beads were washed in 5% TCA. The supernatant and wash were combined, and precipitated proteins were pelleted by centrifugation. The pellet was resuspended in sample buffer (pH 8.8) and boiled for 5 min. Proteins (10–20 μg) were analyzed by a modified acrylamide:bisacrylamide ratio (33.5:0.3) SDS-PAGE and Western blotting using anti-Maf1 polyclonal antibody.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and one figure and can be found with this article online at <http://www.molecule.org/cgi/content/full/22/5/623/DC1/>.

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Accession Numbers

Microarray data are available at <http://ncbi.nih.gov/geo/> under the following accession numbers: GSE3468 and GSE3469.