

PP4 dephosphorylates Maf1 to couple multiple stress conditions to RNA polymerase III repression

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Maf1 is the ‘master’ repressor of RNA polymerase III (Pol III) transcription in yeast, and is conserved in eukaryotes. Maf1 is a phospho-integrator, with unfavourable growth conditions leading to rapid Maf1 dephosphorylation, nuclear accumulation, binding to RNA Pol III at Pol III genes and transcriptional repression. Here, we establish the protein phosphatase 4 (PP4) complex as the main Maf1 phosphatase, and define the involved catalytic (Pph3), scaffold (Psy2) and regulatory subunits (Rrd1, Tip41), as well as uninvolved subunits (Psy4, Rrd2). Multiple approaches support a central role for PP4 in Maf1 dephosphorylation, Maf1 nuclear localization and the rapid repression of Pol III in the nucleus. PP4 action is likely direct, as a portion of PP4 co-precipitates with Maf1, and purified PP4 dephosphorylates Maf1 *in vitro*. Furthermore, Pph3 mediates (either largely or fully) rapid Maf1 dephosphorylation in response to diverse stresses, suggesting PP4 plays a key role in the integration of cell nutrition and stress conditions by Maf1 to enable Pol III regulation.

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Introduction

The protein synthesis machinery (PSM) is derived from both protein-coding and noncoding RNAs, and their synthesis is regulated by multiple growth conditions. Transcription of the PSM involves regulation of all three RNA polymerases: ribosomal RNAs are mostly transcribed by polymerase I (Pol I) (5S rRNA excepted, which is transcribed by Pol III), ribosomal protein-coding mRNAs are transcribed by Pol II, and tRNAs are transcribed by Pol III. Together, this PSM-related transcription accounts for ~80% of nuclear transcription in proliferating cells (Moss and Stefanovsky, 2002). Pol III transcribes short (<550 bp) noncoding RNAs involved in translation (e.g., tRNAs, SCR1), splicing (e.g., U6), and a variety of other functions (Dieci *et al*, 2007). For tRNAs, the most common Pol III gene class by far, important regulatory elements include intragenic A- and B-box DNA consensus elements, as well as a stretch of at least four thymidine

residues, which terminates transcription (Orioli *et al*, 2011). Three subcomplexes constitute the basic Pol III machinery, each performing essential functions. In simplified terms, TFIIC complex recognizes the A- and B-boxes and recruits TFIIB complex; TFIIB is the initiation complex, containing Brf1, Bdp1 and TBP, and together with TFIIC recruits Pol III; and Pol III produces the RNA (Schramm and Hernandez, 2002).

In response to various unfavourable growth conditions and stress, repression of virtually all components of the PSM is observed—and is conveyed to Pol III via multiple signalling pathways, including those mediated by Target of Rapamycin (TOR) (Willis *et al*, 2004; Wullschleger *et al*, 2006). Our work here focusses on Pol III targets, which undergo rapid (<30 min) repression during nutrient deprivation (ND) and other stress conditions, which is likely important for resource conservation. Furthermore, many cancer types overexpress Pol III target RNAs, due in part to regulation by certain tumour suppressors/oncogenes (Felton-Edkins *et al*, 2003; Gomez-Roman *et al*, 2003). While yeast do not possess the tumour suppressor counterparts, all eukaryotes share a conserved central regulator of Pol III, termed Maf1 (Pluta *et al*, 2001).

As the Pol III repertoire is largely (though not solely) dedicated to the PSM, Pol III regulation is less complex than Pol II. Notably, and in contrast to the Pol II system, Pol III promoters and the basal machinery in yeast largely conform to a ‘default on’ state, and rely on a ‘master’ Pol III repressor, Maf1, which is required for the attenuation of the Pol III system in all tested unfavourable growth conditions: ND, DNA damage, oxidative stress and cell wall stress (Upadhyaya *et al*, 2002; Boissnard *et al*, 2009; Nguyen *et al*, 2010). Maf1 interacts *in vivo* (directly or indirectly) with the Pol III components Rpc160, Rpc34 and Rpc82, as well as the TFIIB component Brf1 (Pluta *et al*, 2001; Desai *et al*, 2005; Roberts *et al*, 2006). Best characterized is the direct interaction of Maf1 with the N-terminus of Rpc160, shown first *in vitro* (Oficjalska-Pham *et al*, 2006) and greatly enhanced by the recent crystal structure of Maf1 and cryo-electron microscopy structure of Maf1 bound to Pol III (Vannini *et al*, 2010). Maf1 is not involved in the repression of ribosomal protein genes or Pol I-encoded ribosomal RNAs, suggesting that Maf1 is specifically dedicated to repression of Pol III (Upadhyaya *et al*, 2002). In summary, Maf1 functions as a master regulator/integrator that specifically represses Pol III transcription in response to multiple stresses by direct interaction with the Pol III machinery.

Mechanistically, yeast Maf1 is a phosphoprotein, and is phosphorylated and mostly cytoplasmic during favourable growth conditions (Roberts *et al*, 2006), allowing robust Pol III transcription. Phosphorylation by Sch9 and protein kinase A (PKA), and nuclear export by Msn5, are important for maintaining its cytoplasmic localization in yeast (Moir *et al*, 2006; Towpik *et al*, 2008; Huber *et al*, 2009; Lee *et al*, 2009). Maf1 phosphorylation by casein kinase 2 or TOR complex 1

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(TORC1) also antagonizes Maf1 repression of Pol III, in part by inhibiting its association with Pol III at Pol III-transcribed genes, a property necessary to execute repression in the nucleus (Wei *et al*, 2009; Graczyk *et al*, 2011). Upon stress, Maf1 is rapidly dephosphorylated, accumulates in the nucleus, and becomes highly enriched at Pol III target genes, as shown by whole-genome chromatin immunoprecipitation (ChIP)-on-chip studies (Oficjalska-Pham *et al*, 2006; Roberts *et al*, 2006). Importantly, Maf1 dephosphorylation is a required step in Pol III repression conserved from yeast to human (Reina *et al*, 2006; Goodfellow *et al*, 2008; Kantidakis *et al*, 2010; Michels *et al*, 2010). Furthermore, *in vitro* systems have demonstrated that Maf1 blocks recruitment of TFIIB to preformed TFIIC–DNA complexes or recruitment of Pol III to TFIIB–TFIIC–DNA complexes (Desai *et al*, 2005). However, *in vivo*, Maf1 can repress Pol III while all three subcomplexes remain on DNA (Pol III, TFIIB and TFIIC). The data can be reconciled by postulating two phases of repression: (1) the acute phase (within 30 min of stress/ND), during which Maf1 associates with Pol III on DNA and represses transcription and (2) the prolonged phase, during which TFIIB and Pol III are displaced from DNA, the recruitment of new active Pol III complexes is inhibited, and repression is maintained. (Note: models for kinase/phosphatase interplay are addressed in the Discussion). As Pol III specifically interacts with the unphosphorylated form of Maf1, Maf1 dephosphorylation likely impacts the acute establishment phase of repression (Roberts *et al*, 2006). Movement to the nucleus itself is insufficient to impose Pol III repression, as Pol III remains fully active when the Maf1 exportin Msn5 is deleted, even though *msn5Δ* mutants have constitutively nuclear Maf1 (Towpik *et al*, 2008). This strongly suggests that dephosphorylation of Maf1 is needed in the nucleus to execute repression, not simply to antagonize nuclear export. Therefore, it is important to be able to separate the effect of phosphorylation on nuclear localization of Maf1 from that of execution of Pol III repression. To this end, we have created a Maf1–Pol III fusion, in attempt to bypass the regulation of nuclear localization and focus on the critical nuclear stage of repression by Maf1.

Since Maf1 dephosphorylation is a common mechanism in all reports of rapid Maf1-dependent repression of Pol III to date, the precise identity of the phosphatase(s) and how it/they are regulated is a central question. Indeed, the rapid repression of Pol III via this mechanism is likely important for conserving protein synthesis resources when unfavourable conditions are encountered. In principle, Maf1 dephosphorylation could be conducted by many phosphatases, or instead by a ‘master’ phosphatase. Here, one report implicated the PP2A phosphatase complex and a set of alternative PP2A catalytic subunits in Maf1 dephosphorylation (Oficjalska-Pham *et al*, 2006). These phosphatases are all regulated by TOR kinase, and rapamycin induces Maf1 dephosphorylation and rapid inhibition of Pol III transcription (Zaragoza *et al*, 1998; Roberts *et al*, 2006). Pph21, Pph22 and Pph3 have frequently been reported as alternative catalytic subunits of PP2A. As previewed above, a triple catalytic mutant combination (*pph21Δ, pph22-ts pph3Δ*) is defective for Maf1 dephosphorylation and Pol III repression (Oficjalska-Pham *et al*, 2006), but whether one or all of these proteins is involved was not determined. However, other work questioned PP2A

involvement, as the PP2A scaffold mutant (*tpd3Δ*) displayed clear Maf1 dephosphorylation (Roberts *et al*, 2006)—a result that requires the speculative release of rogue catalytic subunits to reconcile. Furthermore, Pph3 has known roles in an evolutionarily conserved phosphatase complex, protein phosphatase 4 (PP4), with conserved roles in the DNA damage response (Gingras *et al*, 2005; Kim *et al*, 2007; Mendoza *et al*, 2007), complicating previous work on Maf1 involving Pph3.

In this study, we provide multiple lines of evidence that the PP4 complex, with Pph3 as the catalytic subunit, is the major and direct phosphatase of Maf1. We also identify both the core and regulatory subunits of PP4 involved in Maf1 dephosphorylation. Importantly, PP4/Pph3 is needed for acute repression in response to diverse stresses, suggesting that Maf1 and PP4 work together to integrate cell nutrition, stress and integrity. The work was initiated by employing a Maf1–Pol III fusion protein, which has proven useful in characterizing functional PP4 components for this process, and which may also be useful for revealing and characterizing other components involved in Pol III repression.

Results

A Maf1–Rpc160 fusion protein recapitulates Maf1-dependent Pol III transcriptional repression

As nuclear localization of Maf1 is not sufficient for repression of Pol III, our initial goal was to determine the factors required for Maf1-dependent execution of Pol III transcriptional repression in the nucleus. To this end, we created a genetic tool to screen candidate factors: a fusion protein involving Maf1 fused to the amino-terminus of the large subunit of RNA Pol III (Rpc160), which directly binds Maf1 during repression (Boguta *et al*, 1997; Oficjalska-Pham *et al*, 2006) (see Introduction). We expected this Maf1–Rpc160 protein to integrate into Pol III in the nucleus (bypassing nuclear/cytoplasmic shuttling), attenuate Pol III activity and reduce cell/colony growth (addressed below)—enabling us to screen for Maf1 interactors that would relieve this repression and restore growth.

Maf1–Rpc160 contains the entire open reading frame (ORF) of *MAF1*, a linker of 10 amino acids (aa), the entire ORF of *RPC160*, and a 3 × HA tag (Figure 1A). Here, we utilized the *GAL1* promoter, which enables galactose-inducible expression of Maf1–Rpc160. A control construct expressed tagged Rpc160, lacking Maf1. In galactose-containing medium, the Maf1–Rpc160 fusion and Rpc160 constructs expressed proteins of the expected ~212 kDa and ~167 kDa, respectively (Supplementary Figure S1A). Co-immunoprecipitation (co-IP) experiments between myc-tagged Rpc82 and HA-tagged Maf1–Rpc160 fusion or HA-tagged Rpc160 confirmed that the fusion protein incorporates into Pol III, although at a slightly reduced level compared with Rpc160 (Supplementary Figure S1B). To assess Pol III transcription, northern blot was performed using probes complementary to U4 (a Pol II target gene, used as an internal control) and pre-tRNA^{Leu3}, a Pol III target. Here, pre-tRNAs are examined to distinguish new transcription from the existing, highly stable spliced tRNAs. Notably, with fusion expression, new tRNA^{Leu3} production was dramatically reduced (Figure 1B and C), showing that the Maf1–Rpc160 protein confers Pol III transcription repression. We note a reduction in tRNA^{Leu3} expression with overexpression of Rpc160 alone (Figure 1B),

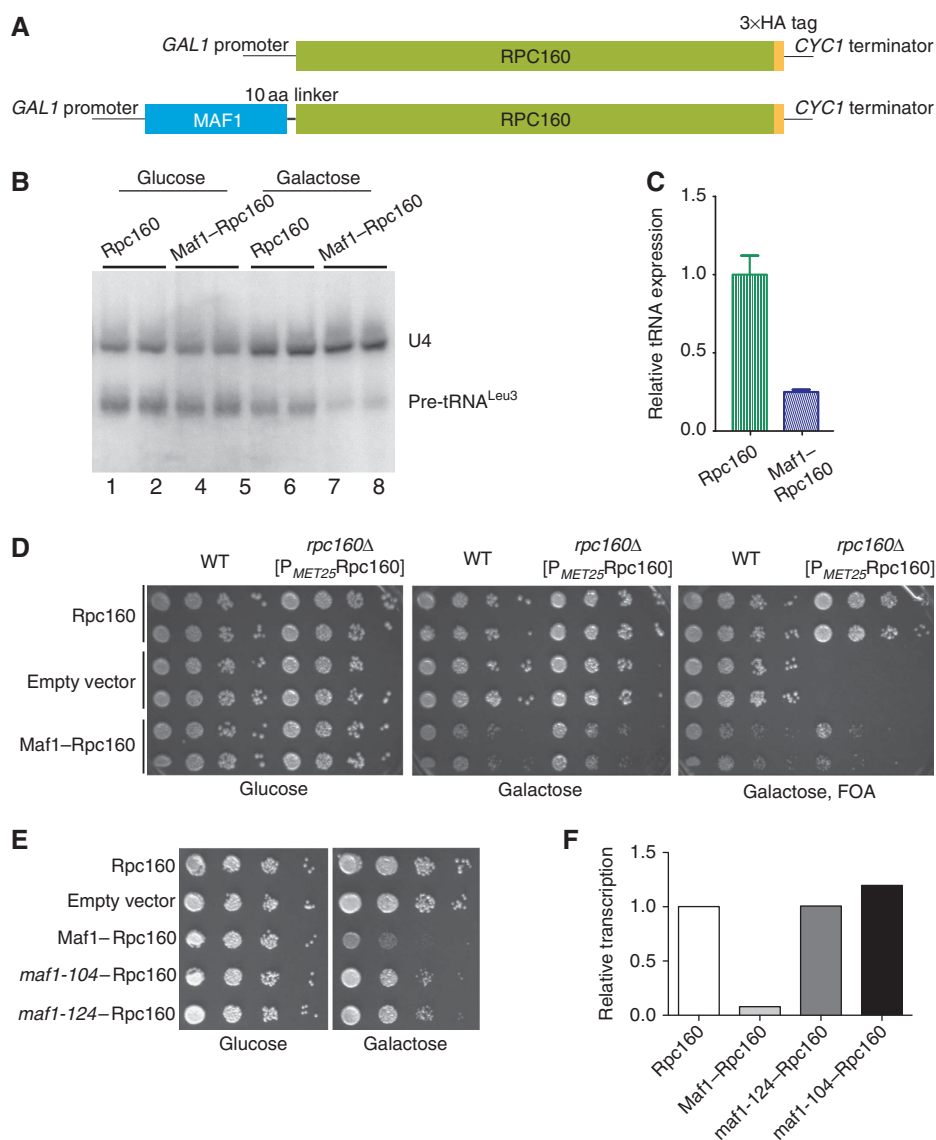


Figure 1 A Maf1-Rpc160 fusion functionally represses Pol III transcription. **(A)** Constructs of galactose-inducible Rpc160 or Maf1-Rpc160 fusion. **(B)** Northern blot showing levels of pre-tRNA^{Leu3} and U4 in strains with Maf1-Rpc160 or Rpc160 constructs, under conditions of repression by glucose (lanes 1–4) or induction by galactose (lanes 5–8). Expression of the Maf1-Rpc160 fusion but not Rpc160 represses pre-tRNA^{Leu3} transcription. **(C)** Quantification of lanes 5–8 of **(B)** as a ratio of tRNA/U4 band intensity with Rpc160 set to 1. Error bars represent s.d. **(D)** Expression of the Maf1-Rpc160 fusion but not Rpc160 confers a growth defect in both *RPC160*⁺ and *rpc160* Δ cells (right and left half of each plate, respectively). Cells were grown on plates containing glucose, galactose or galactose + FOA. Note that FOA only permits growth of cells lacking P_{MET25}Rpc160 as this vector carries a *URA3* marker. Two biological replicates are shown for each strain. See Materials and methods for more details. **(E)** The fusion growth defect is dependent on a functional Maf1. Strong, dephosphorylation-resistant missense mutations—*maf1-104* or *maf1-124*—in the Maf1 portion of the fusion partially suppress the fusion growth defect. **(F)** Yeast transformed with plasmids for galactose-inducible Rpc160 (p2266), Maf1-Rpc160 (p2268), *maf1-124*-Rpc160 (p2424) or *maf1-104*-Rpc160 (p2425) were grown in galactose for 4 h and RNA was isolated immediately, followed by northern blot. Relative transcription is expressed as a ratio of the pre-tRNA^{Leu3} band to U4 band, with Rpc160 set to 1. Data are from a single replicate.

although this is modest when compared with the major reduction seen following overexpression of the Maf1-Rpc160 fusion.

We also observed growth inhibition when examining Maf1-Rpc160 transformants on galactose-containing plates, as determined by colony size (Figure 1D, left and middle panels). The effect is dominant, as it was observed in WT (*RPC160*⁺) cells. The Maf1-Rpc160 fusion protein is incorporated into Pol III complexes competent for transcription in the nucleus, as it complements *rpc160* Δ when loss of the WT Rpc160 (*URA3*) vector is enforced with 5-fluoroorotic acid

(FOA) (Figure 1D, right panel), with its weak complementation due to the partial repression of Rpc160 by fused Maf1. Thus, the functional consequence of Maf1-Rpc160 expression is reduced expression of Pol III targets, conferring a slow-growth phenotype termed the ‘fusion growth defect’.

Two controls are needed to ensure that the fusion growth defect is meaningful and useful, rather than trivial. First, a trivial explanation for growth inhibition is the overexpression of Maf1 moiety, with no need for the fusion to Rpc160. However, no growth defect was observed when we used the same plasmid backbone to drive Maf1 alone on

galactose-containing plates; a modest reduction in Pol III transcription is observed, but not sufficient to affect growth (Supplementary Figure S2). More importantly, Maf1 (when fused to Rpc160) may simply impair Rpc160 by a nonspecific mechanism unrelated to Maf1 phosphorylation state, such as physical blocking. To test for this, we created missense mutations in the Maf1 portion of the fusion construct, utilizing previously characterized *maf1* alleles (*maf1-104* and *maf1-124*) conferring point mutations that resist dephosphorylation (Roberts *et al*, 2006). Notably, these point mutations within Maf1–Rpc160 fusion proteins greatly attenuated the fusion growth defect (Figure 1E) and the Pol III transcriptional repressive ability of the fusion protein (Figure 1F). These experiments and controls strongly suggest that a functional, dephosphorylated Maf1 is needed for repression in the fusion context, and support the utility of the Maf1–Rpc160 fusion tool for identifying relevant factors involved in phosphoregulation.

PP4 mediates the Maf1–Rpc160 fusion growth defect

To understand Maf1 dephosphorylation, the correct phosphatase catalytic subunit and correct phosphatase complex must be firmly established. To clarify, we defined the composition and roles of PP2A and PP4 in Maf1 dephosphorylation (Table I). Consistent with previous studies (Gingras *et al*, 2005), we found that Pph3 interacts with the PP4-specific subunit Psy2, but not the PP2A-specific subunit Tpd3 (Supplementary Figure S3), confirming that Pph3 is part of PP4 but not PP2A.

Table I Core components of yeast protein phosphatase 2A (PP2A) and protein phosphatase 4 (PP4)

| PP2A ^a | | PP4 ^b | |
|-------------------|------------|------------------|---------------------|
| Subunit | Type | Subunit | Type |
| Pph21 | Catalytic | Pph3 | Catalytic |
| Pph22 | Catalytic | | |
| Tpd3 | Scaffold | Psy2 | Scaffold/regulatory |
| Cdc55 | Regulatory | Psy4 | Scaffold/regulatory |
| Rts1 | Regulatory | | |

^aFrom Jiang (2006).

^bFrom Cohen *et al* (2005).

We then used the Maf1–Rpc160 fusion tool to assess which complex (PP4 or PP2A) and which catalytic subunits help confer the fusion growth defect. We first tested the ‘core’ members of PP4: Pph3 (catalytic), Psy2 (regulatory) and Psy4 (regulatory). Importantly, we found growth largely restored in cells lacking either Pph3 or Psy2 (Figure 2A), establishing their involvement. In contrast, the lack of Psy4 had little effect (only slight growth restoration), whereas growth was restored in the *psy2Δ psy4Δ* double mutation, suggesting that Psy4 is less important for Pol III repression than Psy2, an issue developed further below.

Next, we tested PP2A core members and regulatory subunits (Cdc55 or Rts1) (Jiang, 2006). Since Pph21 and Pph22 are redundant, we created a double null in the S288C background to test in our assays. Notably, neither the *pph21Δ pph22Δ* double mutant nor *tpd3Δ* rescued, and actually enhanced the growth defect (Figure 2B, left three panels). Furthermore, mutation in either regulatory subunit of PP2A retained pronounced growth defects (Figure 2B, right two panels). As shown previously (Roberts *et al*, 2006), *pph21Δ pph22Δ* and *tpd3Δ* mutants display clear Maf1 dephosphorylation in response to ND or rapamycin treatment (Supplementary Figure S4). Taken together, these results point to PP4/Pph3 involvement in Maf1–Rpc160 repression of Pol III, and not PP2A/Pph21/Pph22.

Pph3 is required for the acute establishment of Pol III repression

To further investigate Pph3, we assayed WT and *pph3Δ* null strains for (1) Maf1 dephosphorylation, (2) impact on Pol III transcription and (3) nuclear translocation. For Maf1 dephosphorylation, a plasmid encoding HA-tagged Maf1 (controlled by its endogenous promoter) was transformed into WT or *pph3Δ* strains. As shown previously, ND or rapamycin (125 nM) for 25 min causes Maf1 dephosphorylation, where higher molecular weight phospho-species collapse to an apparent single band of ~50 kDa in western blot analysis (Oficjalska-Pham *et al*, 2006; Roberts *et al*, 2006). This creates a prominent band of unphosphorylated Maf1 that is derived both from the dephosphorylation of existing phospho-Maf1, and the translation of new Maf1. To examine only resident Maf1 as opposed to newly translated Maf1, cells

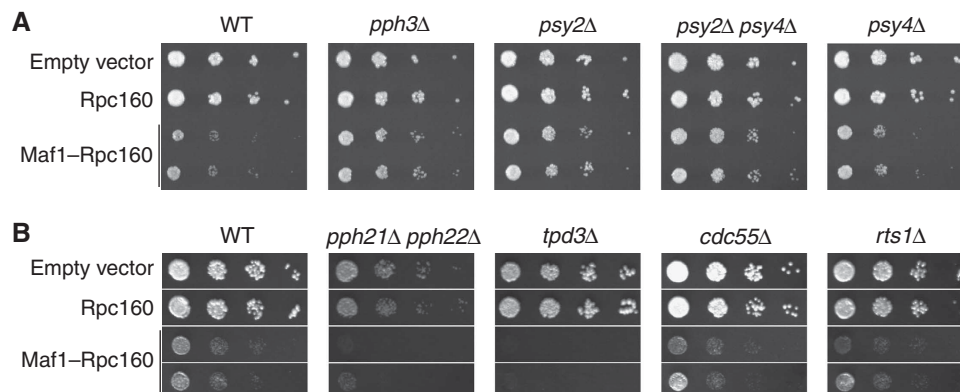


Figure 2 Maf1–Rpc160 fusion identifies Pph3 and Psy2 as Maf1-dependent repressors of Pol III. (A) WT and various PP4 mutants harbouring plasmids for empty vector, Rpc160 or Maf1–Rpc160 fusion were grown on plates containing galactose. The fusion growth defect is partially rescued in PP4 mutant strains *pph3Δ* and *psy2Δ* (two biological replicates in the bottom two rows of spot dilutions) compared with controls (top two rows). Little effect on the fusion growth defect is seen in *psy4Δ* strain. See Materials and methods for details. (B) The fusion growth defect remains (*cdc55Δ*, *rts1Δ*) or is intensified (*tpd3Δ*, *pph21Δ pph22Δ*) in PP2A mutant strains. Note that rows in (B) for each strain are from the same plate image.

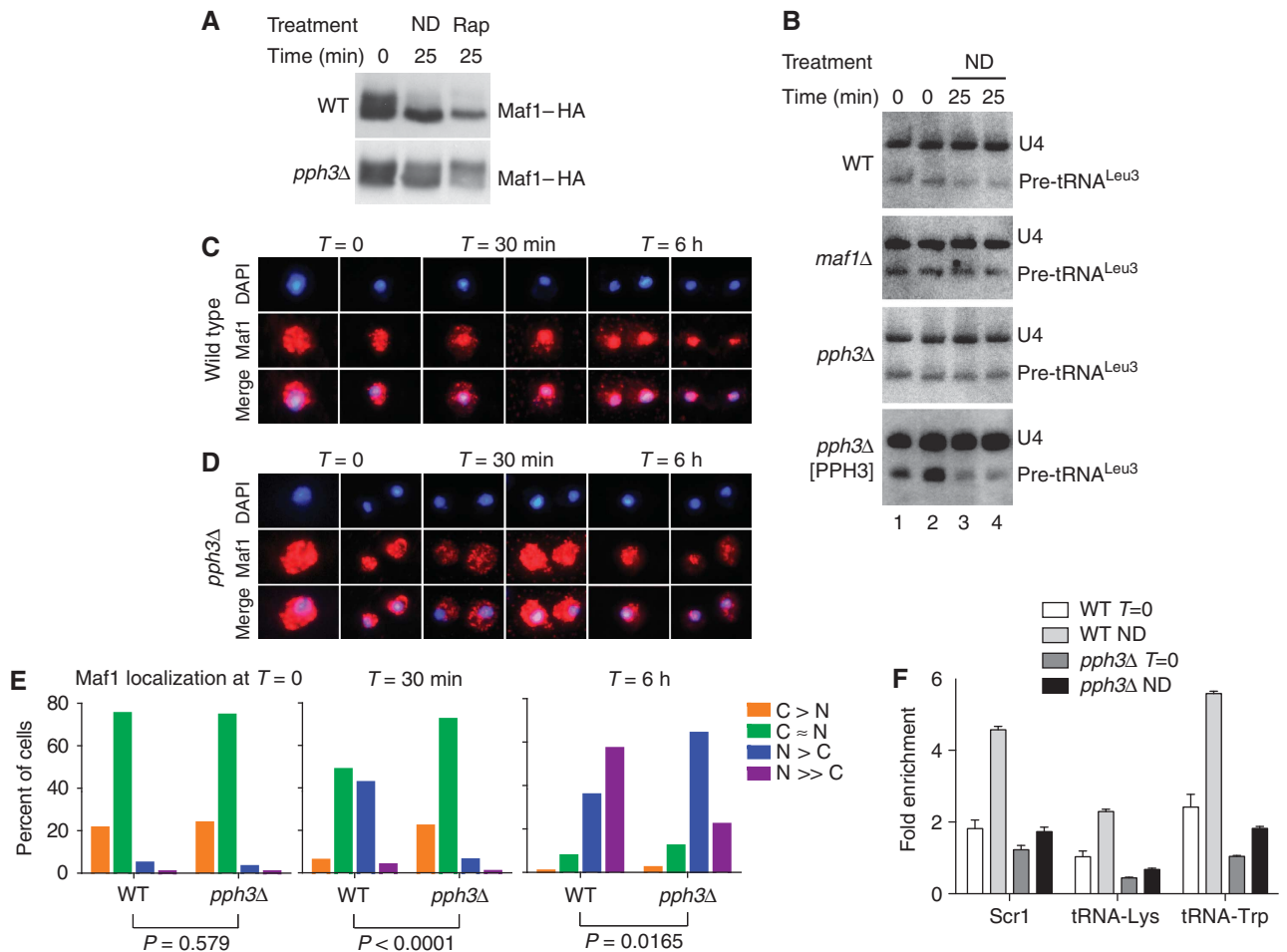


Figure 3 Pph3 mediates Pol III repression. **(A)** Western blot of WT and *pph3Δ* yeast treated with ND or rapamycin (Rap). ND and Rap treatment cause dephosphorylation of Maf1 in WT but not *pph3Δ* mutant strain. **(B)** Northern blot showing repression of pre-*tRNA*^{Leu3} transcription in WT strain (top panel) in response to ND. Repression is attenuated in *pph3Δ* strain (third panel) and repression is restored by complementing with Pph3 on a plasmid (bottom panel). Lack of Pol III repression in *maf1Δ* strain is shown for comparison (second panel). Two biological replicates are shown. **(C, D)** Nuclear localization of Maf1-HA in response to ND in **(C)** WT and **(D)** *pph3Δ*. Maf1 is stained red while nuclear DNA is stained blue (DAPI). Localization of Maf1 to the nucleus is delayed in *pph3Δ* compared with WT. **(E)** Distribution of Maf1 localization in WT and *pph3Δ* strains at *T* = 0 (left panel; *n* = 230 for WT, *n* = 150 for *pph3Δ*), *T* = 30 min (middle panel; *n* = 470 for WT, *n* = 986 for *pph3Δ*) and *T* = 6 h (right panel; *n* = 239 for WT, *n* = 341 for *pph3Δ*). Significance values calculated as difference between nuclear (*N* > *C*, *N* > *C*) and cytoplasmic (*C* > *N*, *C* ≈ *N*) Maf1 in WT and *pph3Δ* using Fisher's exact test. **(F)** ChIP of Maf1 in WT or *pph3Δ* cells at *T* = 0 or 25 min ND treatment. ChIP values are expressed as enrichment over a control locus, the *Tra1* gene.

were pretreated briefly with cycloheximide (CHX; which does not cause either Maf1 dephosphorylation or Pol III repression during acute repression, see below) prior to altering the growth medium. We found that Pph3 was required for the vast majority of the dephosphorylation of Maf1 in response to acute ND or TOR inhibition, as phosphorylated Maf1 clearly persists in the *pph3Δ* strain (Figure 3A). We also conducted ND without CHX and likewise observed a strong requirement for Pph3 in Maf1 dephosphorylation (Supplementary Figure S5). As CHX can also activate Sch9 (Urban *et al.*, 2007), a Maf1 kinase, we concurrently included a WT control to account for any differences in phosphorylation due solely to enhanced kinase activity. Importantly, Maf1 is dephosphorylated in WT cells exposed to ND or rapamycin (and CHX), suggesting that any activation of Sch9 by CHX does not affect overall dephosphorylation of Maf1. Curiously, rapamycin treatment moderately reduces resident Maf1 levels (Figure 3A), an observation that was not pursued further.

To assess the role of Pph3 in Pol III repression, we isolated RNA from WT, *pph3Δ* and *pph3Δ* cells complemented with a WT *PPH3* plasmid. Northern blot analysis revealed the *pph3Δ* strain defective for Pol III repression in ND (Figure 3B). Notably, the defect was complemented by a Pph3 derivative FLAG-tagged at the N-terminus (Figure 3B, bottom panel).

Furthermore, *pph3Δ* mutants show a defect/delay in the translocation/accumulation of Maf1 in the nucleus compared with WT (Figure 3C–E). After 30 min of ND, Maf1 is largely nuclear in WT, but largely cytoplasmic in *pph3Δ* strains (Figure 3C–E). After 6 h, the Maf1 localization profiles appear similar in WT and *pph3Δ*, consistent with a role for Pph3 in acute, but not prolonged repression of Pol III transcription. We note that independence from the phosphatase during prolonged repression is predicted, as the translation and accumulation of new Maf1 (unphosphorylated) occurs during prolonged ND (Oficjalska-Pham *et al.*, 2006; Roberts *et al.*, 2006).

Pph3 promotes the association of Maf1 with Pol III genes

To determine whether Pph3 plays a role in recruitment of Maf1 to Pol III, we performed ChIP of Maf1 in WT and *pph3Δ* cells both before and following 30 min ND (Figure 3F). First, we found that basal Maf1 ChIP levels are lower in *pph3Δ* cells compared with WT. Second, recruitment of Maf1 during ND is greatly attenuated in *pph3Δ* cells compared with WT cells. Taken together, these results are consistent with a model for PP4 in maintaining a basal level of Maf1 at Pol III loci, as well as enabling enhanced recruitment of Maf1 in response to stress.

Maf1 dephosphorylation and Pol III repression requires particular subunits of PP4, but not PP2A

Above, the Maf1-Rpc160 fusion growth defect was greatly attenuated in cells lacking Pph3 or Psy2, but not Psy4. In keeping with this, we find Psy2 required for Maf1 dephosphorylation, but not Psy4 (Figure 4A). Notably, Pph3 and Psy2 still physically interact the *psy4Δ* strain (Figure 4B). Pph3 likely functions through PP4, as Psy2 is similarly required for Pol III transcriptional repression in response to ND, rapamycin or methyl methanesulphonate (MMS) (Figure 4C).

To further investigate PP4, we used the fusion construct to screen other proteins that regulate PP4. The yeast protein tyrosyl phosphatase activators Rrd1 and Rrd2 activate PP2A-like phosphatases (Van Hoof *et al.*, 2005). Rrd1 activates Pph3, Sit4 and Ppg1, while Rrd2 activates Pph21/22. Consistent with a role for Rrd1 in activating PP4, *rrd1Δ* mutants largely rescue the fusion growth defect (Figure 5A) and are defective for full Maf1 dephosphorylation in ND and rapamycin treatment (Figure 5B). In contrast, *rrd2Δ* mutants are not rescued (similar to *pph21Δ pph22Δ* or *tpd3Δ* mutants) and display full Maf1 dephosphorylation in ND or rapamycin (Figure 5). Tip41 is a TOR signalling pathway member that physically interacts with PP2A family phosphatases, including Pph3, Pph21/22 and Sit4 (Jacinto *et al.*, 2001; Santhanam *et al.*, 2004; Gingras *et al.*, 2005). Upon inhibition of TOR, Tip41 binds and activates these phosphatases (Jacinto *et al.*, 2001; Gingras *et al.*, 2005). We find that *tip41Δ* mutants rescue the fusion growth defect (Figure 5A) and are defective in full dephosphorylation of Maf1 (Figure 5B), consistent with the results of the fusion growth assay. Taken together, strains with mutations in PP2A components enhance the fusion growth defect and retain rapid Maf1 dephosphorylation, whereas strains with PP4 mutations suppress the fusion growth defect and are defective for full Maf1 dephosphorylation—displaying the utility of the Maf1-Rpc160 fusion for revealing involved factors.

Pph3 interacts with Maf1

We next tested whether Pph3 interacts with Maf1 via co-IP in extracts. As effective polyclonal antibodies are not available, we cloned a FLAG-tagged Pph3 derivative under the control of the *MET25* promoter (enabling moderate overexpression) and co-expressed with HA-tagged Maf1. We note that this moderate Pph3 overexpression does not cause Maf1 dephosphorylation or Pol III repression, consistent with its incorporation (see Supplementary Figure S3) and regulation by other PP4 components. We examined interaction by co-IP in unstressed cells as well as cells stressed with MMS for 30 min

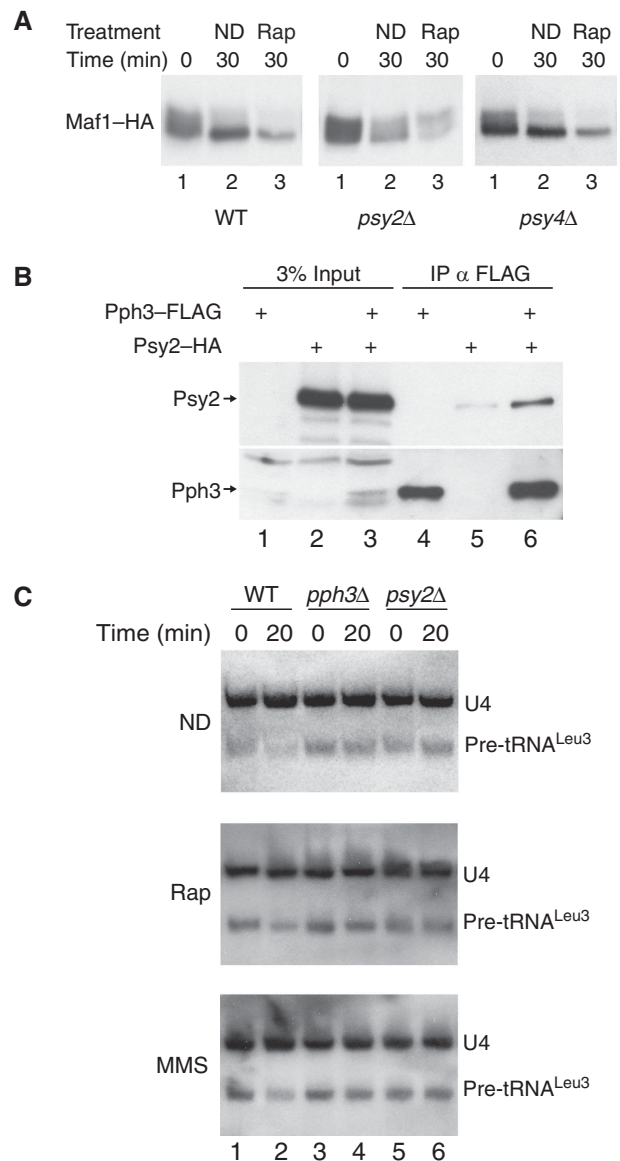


Figure 4 PP4 complex members play a role in steps of Pol III repression. (A) Western blot of WT, *psy2Δ* and *psy4Δ* yeast treated with ND or rapamycin (Rap). Maf1 is dephosphorylated in WT and the *psy4Δ* mutant, but not in the *psy2Δ* mutant in response to ND and Rap treatment. (B) Co-IP of tagged Pph3-FLAG and Psy2-HA in *psy4Δ* mutant strain, revealed by western blot. Psy2 and Pph3 can interact in the absence of Psy4. (C) Northern blot of WT, *pph3Δ* and *psy2Δ* strains treated with ND, Rap or MMS. Pph3 and Psy2 are required for reduction of nascent tRNA levels in ND, Rap and MMS treatments.

to activate the phosphatase. In both stressed and unstressed cells, we detect a specific interaction of Maf1 with Pph3, enriched well above background (Figure 6A and B), but representing only a small fraction of total, consistent with its other roles and substrates. A similar level of association was detected in crosslinked (1% formaldehyde, to capture the enzyme-substrate complex; Figure 6A and B) and uncrosslinked extracts (Supplementary Figure S6).

PP4 is localized in the nucleus

During DNA damage recovery, PP4 dephosphorylates nuclear Rad53 and γ H2AX (Keogh *et al.*, 2006; O'Neill *et al.*, 2007).

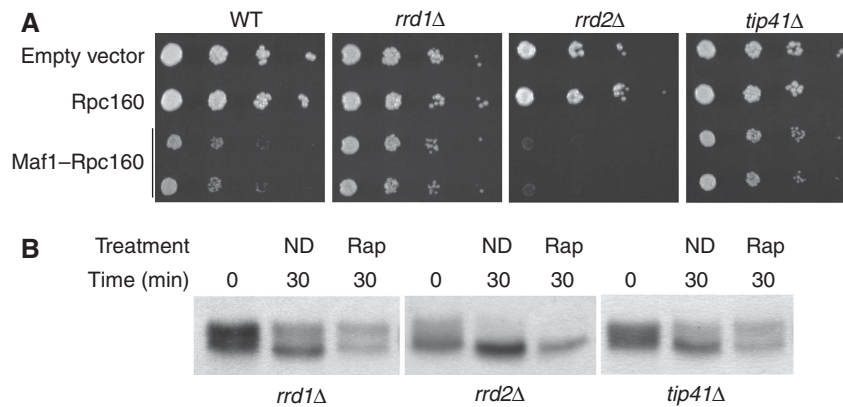


Figure 5 Pol III repression requires PP4 accessory factors Rrd1 and Tip41. (A) The fusion growth defect is rescued in *rrd1Δ* and *tip41Δ* mutants, but not *rrd2Δ*. See Materials and methods for details. (B) Western blot showing PP4-associated factors Tip41 and Rrd1 are required for dephosphorylation of Maf1 during ND or exposure to rapamycin, whereas PP2A-associated Rrd2 is not required, correlating to the fusion growth data in (A).

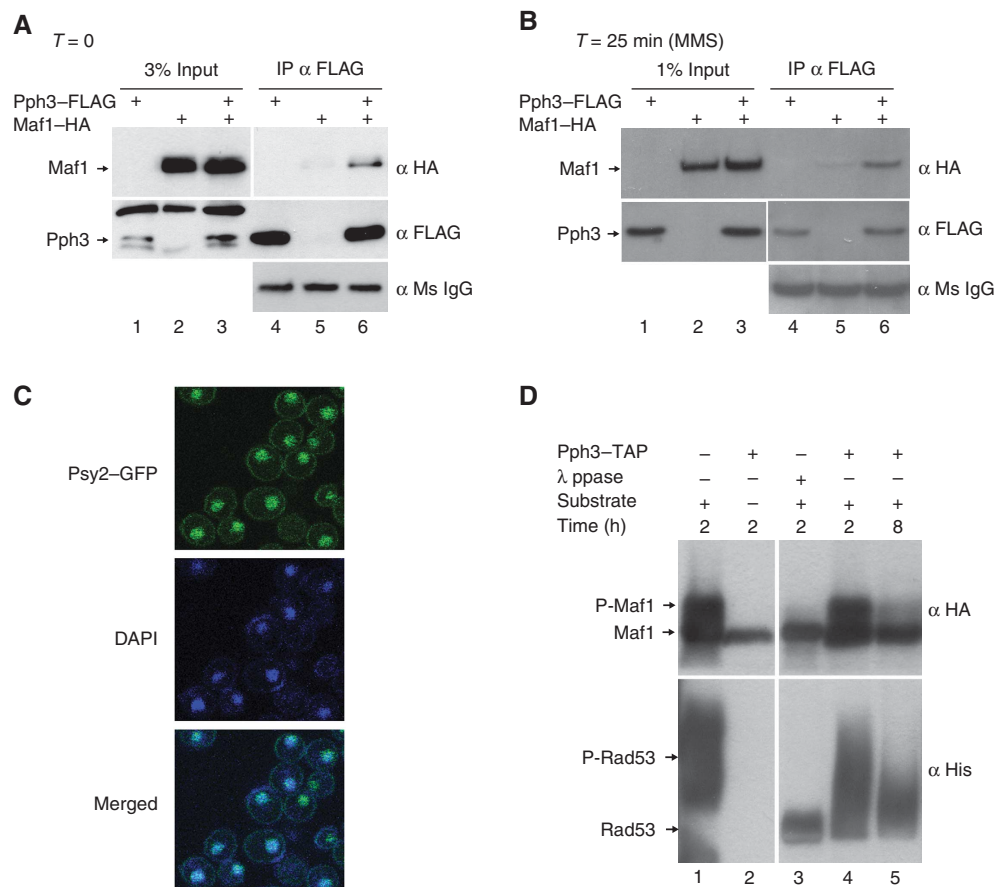


Figure 6 Pph3 directly dephosphorylates Maf1 in the nucleus. (A, B) Co-precipitation of Maf1 and Pph3 in (A) unstressed cells and (B) MMS-treated cells. Cells transformed with tagged Maf1 and/or Pph3 were isolated after brief (15 min) formaldehyde crosslinking. Extracts were incubated with FLAG beads to immunoprecipitate Pph3 and bound Maf1. Maf1 is enriched in Pph3-FLAG-containing extracts (lane 6) over background (lane 5). (C) Psy2-GFP localizes largely to the nucleus (and partially to the cell membrane) in unstressed cells. (D) Pph3-TAP complexes are active on purified phosphorylated Maf1 (top panels) and Rad53 (bottom panels). Left and right panels are from the same gel. The band seen in the top panel, lane 2 is likely crossreacting Pph3-TAP, due to the protein A epitope present on Pph3-TAP, which runs near dephosphorylated Maf1. See text and Supplementary data for details.

Interestingly, in the nuclear exportin *msn5Δ* mutant strain, Maf1 is constitutively nuclear but still requires dephosphorylation to repress Pol III (Towpik *et al*, 2008). Thus, the Maf1 phosphatase should be present in the nucleus. To determine

the location of PP4, we obtained GFP-tagged Pph3 or Psy2 strains. Psy2-GFP strains show growth similar to WT in camptothecin, which activates PP4-requiring steps in the DNA damage response, whereas Pph3-GFP strains show

growth similar to *pph3Δ* (Supplementary Figure S7), suggesting impairment due to GFP fusion. Accordingly, we focussed on Psy2–GFP, which shows strong nuclear localization (Figure 6C), consistent with previous results (Huh *et al*, 2003). This supports PP4 action on Maf1 in the nucleus, with the accumulation of Maf1 in the nucleus during stress due to the inability to shuttle dephosphorylated Maf1 out of the nucleus by Msn5 (Towpik *et al*, 2008).

To further address Pph3 action in the nucleus, we utilized a Maf1 mutant derivative, Maf1^{7A} (or *maf1-7A*), in which seven serines known to be phosphorylated in Maf1 are replaced with alanine (Huber *et al*, 2009). In nutrient replete conditions, this derivative localizes largely to the nucleus, shows increased (but not complete) association with Pol III and confers slight tRNA repression while remaining highly responsive to Pol III repression during rapamycin treatment (Huber *et al*, 2009). Therefore, *maf1-7A* possesses a weak gain-of-function for repression, yet retains full responsiveness. We find *maf1-7A*–Rpc160 fusion more potent at growth inhibition than Maf1–Rpc160, consistent with dephosphorylation promoting repression (Supplementary Figure S8A and B). We also observe partial growth suppression of the *maf1-7A*–Rpc160 by *pph3Δ* (Supplementary Figure S8A), and attenuated tRNA^{Leu3} repression (Supplementary Figure S8C), consistent with the possible presence of additional phosphoserine residues on *maf1-7A* that require Pph3 action for full repression.

We tested for association of Pph3 with a set of Pol III target genes by ChIP in both nutrient replete and deprivation conditions, and did not observe significant association (Supplementary Figure S9), suggesting that PP4 action on Maf1 occurs in the nucleoplasm, though the lack of positive controls (established PP4-associated loci) prevents definitive assessment.

PP4 dephosphorylates Maf1 *in vitro*

To test whether PP4 can directly dephosphorylate Maf1, we reconstituted Maf1 dephosphorylation by PP4 *in vitro* from partially purified components. PP4 was isolated as described previously, by forming IgG-bound complexes from a Pph3–TAP strain, which provides highly purified material (O'Neill *et al*, 2007). Pph3–TAP tagged proteins bind stably to the other components of PP4 (e.g., Pph3, Psy2 and Psy4) and confer phosphatase activity (O'Neill *et al*, 2007), and yielded the same spectrum of proteins provided previously. The substrate—phosphorylated Maf1—was isolated by purification from a *pph3Δ* strain, using a 10 × His tag and a high-stringency procedure (see Materials and methods). To calibrate PP4 activity on Maf1, we isolated phosphorylated Rad53, a known native substrate of PP4 (O'Neill *et al*, 2007). Indeed, tests for a new enzyme substrate are often accompanied by assays on a known substrate to determine relative turnover numbers. We also used λ phosphatase to provide a positive control for a highly active (though non-specific) phosphatase enzyme. We note that reconstitution biochemistry typically utilizes *in vitro* incubation times that are much longer than the *in vivo* kinetics of a reaction, for obvious reasons.

In our *in vitro* reconstitution, we observed clear dephosphorylation of the known substrate, Rad53, by Pph3–TAP at 2 and 8 h (Figure 6D, bottom panels). By quantifying the change in Rad53 mobility, Pph3–TAP has 22.2% (± 0.7) of

the activity of λ phosphatase at 2 h, and 34.8% (± 3.1) of the activity at 8 h (when comparing the change in the bottom band as a percentage of total Rad53 signal per lane; see Materials and methods). For Maf1, dephosphorylation is not easily seen or quantified at 2 h, due to the modest separation of the bands (Figure 6D, top panels). However, at 8 h, dephosphorylation is clearly observed, representing 43.0% (± 4.1) of the activity of λ phosphatase. Furthermore, the activity observed on Maf1 is similar to the known substrate Rad53. We note that determination of dephosphorylation kinetics in certain preparations of Maf1 was prevented by variable levels of a contaminating protease. Taken together, the PP4 complex (containing Pph3) dephosphorylates Maf1 *in vitro* at rates similar to Rad53, consistent with its identity as an authentic substrate.

PP4 is an integrator of multiple signals for Maf1 dephosphorylation

PP4 might be one of many Maf1 phosphatases with relatively equal impact, or instead be responsible for the vast majority of Maf1 dephosphorylation during the acute response to known stresses—essentially functioning as a co-integrator with Maf1. To address this, and to extend beyond our examination of ND (lacking glucose) or rapamycin treatment, extracts were prepared from WT and *pph3Δ* cells stressed with one of multiple conditions/agents: the DNA-damaging agent MMS, CHX, chlorpromazine (CPZ), dithiothreitol (DTT) or phosphate deprivation—all of which have been associated with Pol III repression (except phosphorus deprivation) (Willis *et al*, 2004). Phosphorus deprivation was included as it causes nuclear accumulation of tRNAs, similar to DNA damage (Ghavidel *et al*, 2007; Hurto *et al*, 2007), although whether it causes Pol III transcription repression or Maf1 dephosphorylation has not been tested. Notably, we find that DNA damage or replication fork stress (with MMS), endoplasmic reticulum stress (with DTT), cell wall stress (with CPZ) and phosphorus deprivation are clearly Pph3-dependent stresses (Figure 7A and B). With CHX treatment, dephosphorylation of Maf1 begins between 30 and 60 min, and this dephosphorylation is Pph3 dependent (Figure 7C). These findings demonstrate that PP4/Pph3 phosphatase is involved in the integration of multiple nutrient and stress signals to affect the dephosphorylation of Maf1.

Discussion

Maf1 is a conserved and central regulator of Pol III repression in all organisms tested, making its mode of regulation an issue of high interest. Here, the rapid repression of Pol III is likely important for conserving protein synthesis resources when unfavourable conditions are encountered. The rapid establishment of Pol III repression is correlated with the extent of Maf1 dephosphorylation in all conditions tested, and mutations that impair dephosphorylation impair repression, making the dephosphorylation mechanism a key feature of Maf1 regulation. Thus, it is critical to identify the correct phosphatase complex, the precise catalytic subunit, and which attendant subunits are needed for Maf1 dephosphorylation. Here, we establish PP4 as the major Maf1 phosphatase complex for acute Pol III repression, identify Pph3 as the involved catalytic subunit, identify the PP4 core and regulatory/interacting proteins involved in Maf1 dephosphorylation, show physical PP4 interaction with Maf1, and

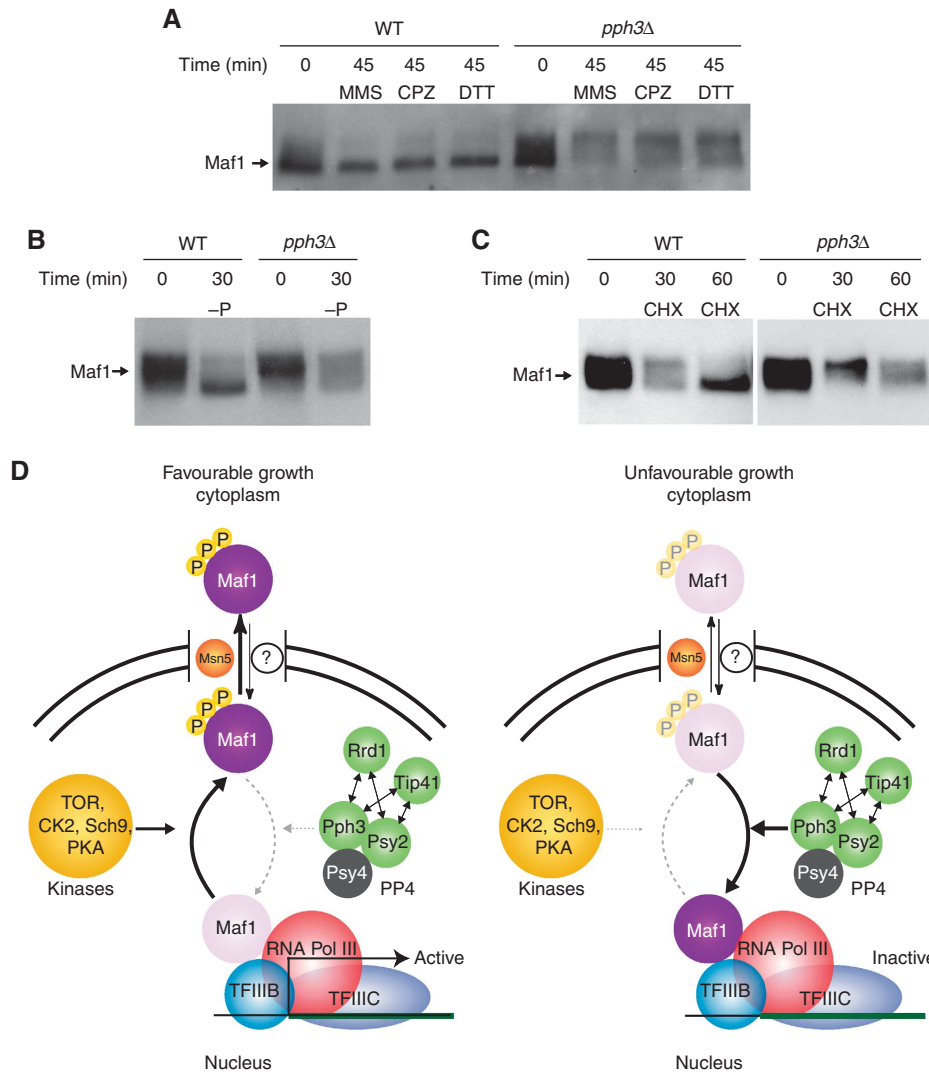


Figure 7 Pph3 is required for dephosphorylation of Maf1 in multiple stress conditions. **(A–C)** Involvement of Pph3 in Maf1 dephosphorylation in **(A)** MMS, CPZ, DTT, **(B)** phosphorus deprivation (–P) and **(C)** extended CHX treatment. See Materials and methods for details on growth conditions. **(D)** Model for PP4-dependent regulation of Maf1 action in repression of Pol III. In favourable growth conditions (left panel), activity of Maf1 kinases predominates over activity of PP4, inhibiting Maf1–Pol III interaction and allowing Pol III to remain active. Phosphorylated Maf1 is exported from the nucleus in an Msn5-dependent manner in S288C background yeast. Maf1 is shuttled into the nucleus due to nuclear localization sequences. Hyperphosphorylated Maf1 is represented with multiple P's, while hypophosphorylated Maf1 is represented by Maf1 lacking P's. Darkly coloured Maf1 illustrates the predominating species of Maf1: hyperphosphorylated form in favourable growth (left), and hypophosphorylated form in unfavourable growth (right). In conditions where Maf1 is predominately phosphorylated (left panel), the rate of export by Msn5 is high, leading to mostly cytoplasmic localization. Lightly coloured Maf1 represents a small or diminishing pool of Maf1, due to action by kinases in favourable growth (left panel) or by the PP4 phosphatase in unfavourable growth (right panel). In unfavourable growth conditions (right panel), activity of PP4 phosphatase predominates over kinase activity, producing hypophosphorylated Maf1, which binds tightly to Pol III and inhibits its activity. Double arrows between Rrd1, Tip41 and components of PP4 represent physical interaction based on experimental evidence (Gingras *et al*, 2005; Van Hoof *et al*, 2005; Krogan *et al*, 2006; Collins *et al*, 2007). Core and noncore subunits of PP4 are coloured based on their requirement for Maf1 phosphatase activity: green-coloured subunits are required, while the black-coloured subunit is not required. In the diagram, the various Maf1 kinases are depicted in the nucleus, though their location of action has not been determined, and may function in the cytoplasm.

reconstitute PP4-dependent Maf1 dephosphorylation *in vitro*, providing evidence that the effect is direct (Figure 7D).

Our identification of PP4 as the major Maf1 phosphatase was enabled by a fusion protein, Maf1–Rpc160, which allowed us to test candidate PP2A-related complexes and components, and provided clear evidence for participation by certain PP4 members, but not PP2A. This was confirmed by extensive additional experiments on PP4 and PP2A, and their impact on Maf1 and Pol III repression in the natural (nonfused) context. Prior studies have shown that certain phosphorylation sites on Maf1 are involved in nuclear–

cytoplasmic shuttling, raising the possibility that yeast simply use Maf1 phospho-dynamics to control the shuttling aspect of Maf1 regulation, but not the execution of Pol III repression in the nucleus. However, studies in yeast have shown that nuclear accumulation of Maf1 is insufficient to repress Pol III (Moir *et al*, 2006; Towpik *et al*, 2008). In addition, vertebrate Maf1, although under phosphoregulation, is constitutively nuclear (Kantidakis *et al*, 2010). These results suggest that Maf1 dephosphorylation is critical for repression, but is not used to control nuclear localization in all species. We note that although overexpression of Maf1

confers modest Pol III repression, no growth defect is observed, suggesting that cells must cross a threshold of very low tRNA abundance before conferring a clear growth defect.

Our results add to our understanding of Maf1 localization during repression of Pol III. Although initial models suggested that Maf1 is dephosphorylated in the cytoplasm prior to nuclear localization, the mechanism is likely more complex. Maf1 contains two nuclear localization signals, one closer to the amino-terminus (NtNLS) and one closer to the carboxy-terminus (CtNLS). The NtNLS has been shown to be adjacent to serines phosphorylated by PKA and Sch9, and this NtNLS is rendered nonfunctional in the phosphorylated state (Moir *et al.*, 2006; Huber *et al.*, 2009; Lee *et al.*, 2009). However, based on studies of the *msn5Δ* null, lacking the Maf1 exportin, Maf1 can be phosphorylated and dephosphorylated within the nucleus (Towpik *et al.*, 2008). Consistent with this, we find an essential subunit of PP4 complex, Psy2, located in the nucleus, supporting Maf1 dephosphorylation in the nucleus. Importantly, even in *maf1* mutants with inactivated NtNLS (by either deletion of the NLS signal or phosphomimetic 6SE mutation), nuclear accumulation can occur readily upon stress (Moir *et al.*, 2006). In addition, since the CtNLS is not adjacent to known or predicted phosphorylation sites, it is possible that the CtNLS is constitutively active in Maf1 nuclear translocation, regardless of Maf1 phosphorylation state. One possible model involves constitutive, low-level translocation of phosphorylated Maf1 to the nucleus during favourable growth conditions, countered by constitutive export by Msn5. In unfavourable conditions, Maf1 is dephosphorylated in the nucleus, preventing its export by Msn5, thus leading to nuclear accumulation (Figure 7D). Importantly, in mutants where Maf1 is constitutively nuclear (e.g., *msn5Δ*, *maf1-6SA*, *maf1-7A*), Pol III is not constitutively repressed, and *maf1-7A* mutants respond robustly to rapamycin (Huber *et al.*, 2009), suggesting an additional required step beyond nuclear localization/retention, such as dephosphorylation at additional positions to confer Pol III repression. Although PP4 is clearly involved in nuclear localization of Maf1, it is also likely involved in this additional required step, since the fusion growth defect of *maf1-7A*–Rpc160 is partially rescued in *pph3Δ* mutants, and Pph3 is required for full Pol III repression by *maf1-7A* mutant yeast in ND treatment (Supplementary Figure S8).

Whereas multiple kinases act on Maf1, PP4 appears to mediate the majority of Maf1 dephosphorylation during the acute response to rapamycin, DNA damage, CPZ, CHX and ND—and is needed for the acute establishment of Pol III repression. (We note, however, that long-term Pol III repression appears to be independent of PP4, which likely involves the *de novo* translation and accumulation of unphosphorylated Maf1 over time.) Taken together, one straightforward interpretation of our data is that PP4 functions with Maf1 as a co-integrator of environmental conditions. To further test this, the interplay between the various Maf1 kinases and PP4 in favourable versus unfavourable growth conditions should be investigated. Specifically, it is not clear whether PP4 constitutively dephosphorylates Maf1—with the Maf1 kinases attenuated during stress—or whether PP4 activity towards Maf1 is increased in poor growth conditions. These models are not exclusive, and both could be involved. If PP4 is relatively constitutive, Maf1 activity could be kept in balance by kinase activation/deactivation in response to

growth conditions, where poor growth conditions would lead to kinase inhibition, and PP4 removing the phosphorylated pool. Indeed, TORC1 activity is inhibited by ND and rapamycin, consistent with this model. However, in our view, some regulation of PP4 activity during stress seems likely, as a single type of stress (like DNA damage) causes Pol III repression even though the Maf1 kinases that assess nutrient availability remain active. Furthermore, there are known roles for TOR and Tap42 in regulating phosphatase activities. Our data does not support a major change in PP4–Maf1 interaction during stress, so if PP4 activation occurs, it likely involves improving its catalytic turnover. Therefore, understanding PP4 regulation and substrate selection is an important next step.

PP4 participates in the DNA damage response in multiple species, including yeast, human, *Caenorhabditis elegans* and *Drosophila* (Cohen *et al.*, 2005; Gingras *et al.*, 2005; Kim *et al.*, 2007). This involves dephosphorylation of Rad53, which helps overcome G2/M arrest (O'Neill *et al.*, 2007). In *Dictyostelium discoideum*, the PP4 complex is activated in response to starvation conditions to enable differentiation into fruiting bodies (Mendoza *et al.*, 2005, 2007). In addition to its role in the DNA damage response, *C. elegans smk-1* (homologue of Psy2) is required for the response to oxidative and innate immune stress required for long-lived worms (Wolff *et al.*, 2006). Given the role for PP4 in the environmental stress response of higher organisms, a role for PP4 in Maf1 dephosphorylation in higher organisms should be explored.

Our findings also inform the compositional requirements of PP4 for dephosphorylation of Maf1. Here, PP4 ‘core’ components Pph3 and Psy2 are required for dephosphorylation of Maf1, while Psy4 is not. Notably, Psy4 is required for dephosphorylation of γ H2AX but not Rad53, while Psy2 is required for all known substrates (Keogh *et al.*, 2006; O'Neill *et al.*, 2007). Less is known regarding the use of ‘noncore’ PP4 subunits in substrate selection, and here we identify Tip41 and Rrd1 as important for full Maf1 dephosphorylation. Tip41 acts in concert with PP4 in DNA damage (Gingras *et al.*, 2005) and activates Sit4 and PP2A catalytic subunits towards Gln3 (Jacinto *et al.*, 2001) and Msn2 (Santhanam *et al.*, 2004), respectively. Rrd1 and Rrd2 are yeast homologues of human phosphotyrosyl phosphatase activator (PTPA); Rrd1 activates several PP2A family phosphatases *in vitro*, but selectively binds Pph3 (and not Pph21/22) *in vivo* (Van Hoof *et al.*, 2005). PTPA has prolyl isomerase activity, which activates PP2A catalytic subunits (Jordens *et al.*, 2006). The proline residue involved in human PP2A_C is Pro190, which is conserved in Pph3, suggesting a conserved mechanism of activation.

Our data does not support a role for PP2A as the main phosphatase of Maf1 (Oficjalska-Pham *et al.*, 2006), or as a phosphatase needed for acute repression. In both *tpd3Δ* (scaffold) and *pph21Δ pph22Δ* (alternative catalytic) double mutants, Maf1 is completely dephosphorylated under stress conditions and with rapid kinetics, and the fusion growth defect is not rescued in these genetic backgrounds (Figure 2; Supplementary Figure S4). This is also true in *rrd2Δ* mutants, which lack a PP2A-specific activator (Figure 5). In fact, in PP2A mutants, we observe an enhanced fusion growth defect, suggesting that disruption of PP2A causes cellular stress, which elicits, rather than compromises, Pol III

repression. The work can be reconciled by noting that all genotypes defective for Maf1 dephosphorylation involved combinations of *pph21* or *pph22* along with *pph3Δ*, which at the time was erroneously considered a redundant PP2A catalytic subunit; *pph3Δ* was never examined in isolation, which we confirm here as exclusively a subunit of PP4. Although Pph3/PP4 is identified as the main and required Maf1 phosphatase, the slight Maf1 dephosphorylation observed during ND in *pph3Δ* cells (Figure 3A) leaves open the possibility for alternative minor phosphatases for Maf1 dephosphorylation.

Recently, the co-structure of Maf1 with yeast Pol III was solved (Vannini *et al.*, 2010), and showed Maf1 binding the clamp domain of Rpc160 (aa 1–245). Therefore, Maf1 fused to the amino-terminus of Rpc160 is an ideal position to poise the complex for repression. Binding of Maf1 causes a shift in the position of the C34 subunit that prevents Brf1 binding, effectively inhibiting TFIIB–Pol III interactions. Other structural studies of Maf1 protein have shown that the phosphorylated form of Maf1 is correlated with absence of interaction between its amino-terminal A domain and its carboxyl-terminal BC domain, while dephosphorylation is correlated with their interaction (Gajda *et al.*, 2010). Thus, dephosphorylation may create a more compact Maf1 that binds in the pocket between C34 and C82 in the Pol III complex. These structural studies may help us understand the utility of our Maf1–Rpc160 fusion tool. First, the Maf1–Rpc160 fusion growth defect can be intensified to complete growth arrest by addition of 10 nM rapamycin (unpublished observations), or instead attenuated by deletion of PP4 phosphatase components. We suggest that in unfavourable growth conditions, Maf1 in the fusion context becomes fully dephosphorylated, enhancing its regulated interaction with the Pol III complex, further inhibiting Pol III interaction with TFIIB and further decreasing transcription. Accordingly, in PP4 mutants, Maf1 is phosphorylated, eliminating A and BC domain interaction, allowing Brf1 association and normal Pol III activity and growth. We envision ourselves and others utilizing the Maf1–Rpc160 fusion to further investigate factors involved in Pol III repression.

Materials and methods

Growth conditions

We used standard culture methods. See Supplementary Table SI for strains. For monitoring phosphorylation status of Maf1, we grew initial cultures to OD₆₀₀ ~0.5–0.7 (*T* = 0), after which we applied a stress treatment. We added CHX (5 μg/ml) to cultures 5 min prior to application of stress treatment, unless otherwise indicated. Stress treatments are as follows: rapamycin (125 nM; Sigma); methyl methanesulphonate (0.13%; Sigma); CPZ (250 μM; Sigma); ND (0.15 × synthetic complete (SC), no glucose); phosphorus deprivation (SC made without nitrogen base mix (Difco), re-adding ammonium sulphate, biotin, calcium pantothenate, inositol, all trace elements, magnesium sulphate, magnesium chloride, sodium chloride and calcium chloride but not potassium phosphate) (Sherman, 2002); DTT (5 mM). We harvested a portion of the culture before applying stress (*T* = 0) and after the specified time of stress treatment. At harvest, we crosslinked cells for 15–30 min in 1% formaldehyde at room temperature (RT; 23°C), and washed three times with TBS. For co-IP, we grew cultures to OD₆₀₀ ~1 and did not crosslink them, unless otherwise indicated. For northern blot, we grew cells to OD₆₀₀ ~0.5, then applied treatment. For all experiments, we froze cells in liquid nitrogen, or prepared extracts immediately. For fusion growth assay spot dilutions, we prepared overnight cultures in SC dropout plus glucose and spotted to plates

with glucose, galactose and/or FOA. We incubated plates at RT (~23°C) for 4–6 days, until the size of control colonies (i.e., those containing plasmid p2266 or p518) was equivalent between strains. The fusion growth defect was then assayed in strains harbouring the Maf1–Rpc160 fusion (plasmid p2268).

Extract preparation, co-IP and western blot

For Maf1 phosphorylation western blot and crosslinked co-IP, we broke cells with 1 mm glass beads in ChIP lysis buffer (Roberts *et al.*, 2006), sonicated six times with microprobe for 30 s on highest setting and clarified by centrifugation. For noncrosslinked co-IP, we prepared extracts in a similar manner, except using lysis buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 0.1% Triton, 1 mM EDTA, 10% glycerol, 0.1 mM DTT and protease inhibitors) and no sonication. We measured protein concentration with Bio-Rad assay and loaded equal protein amounts (usually 30 μg per lane). For co-IP, we incubated 40 μl Dynabeads (Invitrogen; preincubated with BSA plus 2 μg anti-HA [12CA5] antibody) or FLAG-agarose beads (Sigma; prewashed with lysis buffer) with 1 mg of extract for 4–6 h at 4°C, eluted with 2 × SDS sample buffer or FLAG peptide (Sigma) and used half of the eluate for western blot. For Maf1 phosphorylation western blot, we used 10% gels with a 1:125 bis-acrylamide ratio. We performed western blot with anti-HA antibody (either 12CA5 or Abcam 9110), anti-FLAG (Sigma) or anti-Myc (9E10).

Northern blot

We isolated RNA and performed northern blot as described (Roberts *et al.*, 2006), using 20 μg per lane and overnight hybridization with end-labelled probes. Blots were exposed to phosphorimager screen (GE Healthcare) for 24 h, scanned with Typhoon (GE Healthcare), and the images were quantified using ImageQuant or ImageQuant TL (IQTL; GE Healthcare).

Fluorescence microscopy

We performed immunofluorescence for Maf1 as described (Roberts *et al.*, 2006). We treated live Psy2–GFP yeast cells with Hoechst to stain nuclei and visualized with confocal microscope.

Chromatin immunoprecipitation

ChIP assay was performed as described (Roberts *et al.*, 2006; Oler *et al.*, 2010). In all, 1 mg of sonicate was incubated with mouse or rabbit Dynabeads (Invitrogen) and 5 μg of anti-myc (9E11; Abcam ab56) or anti-HA (Abcam 9110) antibody overnight. Beads were washed and eluted with TES (10 mM Tris, pH 8.0, 1 mM EDTA, 1% SDS). Eluate was subjected to qPCR with serially diluted yeast genomic DNA used for primer standard curves to calculate starting copy numbers using iCycler software (Bio-Rad). Primers used for qPCR were described previously (Roberts *et al.*, 2006; Graczyk *et al.*, 2011).

In vitro dephosphorylation assay

We incubated autophosphorylated yeast 6 × His–Rad53 (purified from *Escherichia coli*) or phosphorylated yeast 10 × His–3 × HA–Maf1 (overexpressed in *pph3Δ* strain) with Pph3–TAP complexes or λ phosphatase for 2 or 8 h. See Supplementary data for more details.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: BRC and AJO conceived of the study, designed the experiments and prepared the manuscript. AJO performed the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Boguta M, Czerska K, Zoladek T (1997) Mutation in a new gene MAF1 affects tRNA suppressor efficiency in *Saccharomyces cerevisiae*. *Gene* **185**: 291–296
- Boisnard S, Lagniel G, Garmendia-Torres C, Molin M, Boy-Marcotte E, Jacquet M, Toledano MB, Labarre J, Chedin S (2009) H₂O₂ activates the nuclear localization of Msn2 and Maf1 through thioredoxins in *Saccharomyces cerevisiae*. *Eukaryot Cell* **8**: 1429–1438
- Cohen PT, Philp A, Vazquez-Martin C (2005) Protein phosphatase 4—from obscurity to vital functions. *FEBS Lett* **579**: 3278–3286
- Collins SR, Kemmeren P, Zhao XC, Greenblatt JF, Spencer F, Holstege FC, Weissman JS, Krogan NJ (2007) Toward a comprehensive atlas of the physical interactome of *Saccharomyces cerevisiae*. *Mol Cell Proteomics* **6**: 439–450
- Desai N, Lee J, Upadhyay R, Chu Y, Moir RD, Willis IM (2005) Two steps in Maf1-dependent repression of transcription by RNA polymerase III. *J Biol Chem* **280**: 6455–6462
- Dieci G, Fiorino G, Castelnovo M, Teichmann M, Pagano A (2007) The expanding RNA polymerase III transcriptome. *Trends Genet* **23**: 614–622
- Felton-Edkins ZA, Kenneth NS, Brown TR, Daly NL, Gomez-Roman N, Grandori C, Eisenman RN, White RJ (2003) Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc. *Cell Cycle* **2**: 181–184
- Gajda A, Towpik J, Steuerwald U, Muller CW, Lefebvre O, Boguta M (2010) Full repression of RNA polymerase III transcription requires interaction between two domains of its negative regulator Maf1. *J Biol Chem* **285**: 35719–35727
- Ghavidel A, Kislinger T, Pogoutse O, Sopko R, Jurisica I, Emili A (2007) Impaired tRNA nuclear export links DNA damage and cell-cycle checkpoint. *Cell* **131**: 915–926
- Gingras AC, Caballero M, Zarske M, Sanchez A, Hazbun TR, Fields S, Sonenberg N, Hafen E, Raught B, Aebersold R (2005) A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity. *Mol Cell Proteomics* **4**: 1725–1740
- Gomez-Roman N, Grandori C, Eisenman RN, White RJ (2003) Direct activation of RNA polymerase III transcription by c-Myc. *Nature* **421**: 290–294
- Goodfellow SJ, Graham EL, Kantidakis T, Marshall L, Coppins BA, Oficjalska-Pham D, Gerard M, Lefebvre O, White RJ (2008) Regulation of RNA polymerase III transcription by Maf1 in mammalian cells. *J Mol Biol* **378**: 481–491
- Graczyk D, Debski J, Muszynska G, Bretner M, Lefebvre O, Boguta M (2011) Casein kinase II-mediated phosphorylation of general repressor Maf1 triggers RNA polymerase III activation. *Proc Natl Acad Sci USA* **108**: 4926–4931
- Huber A, Bodenmiller B, Uotila A, Stahl M, Wanka S, Gerrits B, Aebersold R, Loewith R (2009) Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. *Genes Dev* **23**: 1929–1943
- Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O’Shea EK (2003) Global analysis of protein localization in budding yeast. *Nature* **425**: 686–691
- Hurto RL, Tong AH, Boone C, Hopper AK (2007) Inorganic phosphate deprivation causes tRNA nuclear accumulation via retrograde transport in *Saccharomyces cerevisiae*. *Genetics* **176**: 841–852
- Jacinto E, Guo B, Arndt KT, Schmelzle T, Hall MN (2001) TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. *Mol Cell* **8**: 1017–1026
- Jiang Y (2006) Regulation of the cell cycle by protein phosphatase 2A in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **70**: 440–449
- Jordens J, Janssens V, Longin S, Stevens I, Martens E, Bultynck G, Engelborghs Y, Lescrinier E, Waelkens E, Goris J, Van Hoof C (2006) The protein phosphatase 2A phosphatase activator is a novel peptidyl-prolyl cis/trans-isomerase. *J Biol Chem* **281**: 6349–6357
- Kantidakis T, Ramsbottom BA, Birch JL, Dowding SN, White RJ (2010) mTOR associates with TFIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc Natl Acad Sci USA* **107**: 11823–11828
- Keogh MC, Kim JA, Downey M, Fillingham J, Chowdhury D, Harrison JC, Onishi M, Datta N, Galicia S, Emili A, Lieberman J, Shen X, Buratowski S, Haber JE, Durocher D, Greenblatt JF, Krogan NJ (2006) A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. *Nature* **439**: 497–501
- Kim SH, Holway AH, Wolff S, Dillin A, Michael WM (2007) SMK-1/PPH-4.1-mediated silencing of the CHK-1 response to DNA damage in early *C. elegans* embryos. *J Cell Biol* **179**: 41–52
- Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, Punna T, Peregrín-Alvarez JM, Shales M, Zhang X, Davey M, Robinson MD, Paccanaro A, Bray JE, Sheung A, Beattie B *et al.* (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* **440**: 637–643
- Lee J, Moir RD, Willis IM (2009) Regulation of RNA polymerase III transcription involves SCH9-dependent and SCH9-independent branches of the target of rapamycin (TOR) pathway. *J Biol Chem* **284**: 12604–12608
- Mendoza MC, Booth EO, Shaulsky G, Firtel RA (2007) MEK1 and protein phosphatase 4 coordinate Dictyostelium development and chemotaxis. *Mol Cell Biol* **27**: 3817–3827
- Mendoza MC, Du F, Iranfar N, Tang N, Ma H, Loomis WF, Firtel RA (2005) Loss of SMEK, a novel, conserved protein, suppresses MEK1 null cell polarity, chemotaxis, and gene expression defects. *Mol Cell Biol* **25**: 7839–7853
- Michels AA, Robitaille AM, Buczynski-Ruchonnet D, Hodroj W, Reina JH, Hall MN, Hernandez N (2010) mTORC1 directly phosphorylates and regulates human MAF1. *Mol Cell Biol* **30**: 3749–3757
- Moir RD, Lee J, Haeusler RA, Desai N, Engelke DR, Willis IM (2006) Protein kinase A regulates RNA polymerase III transcription through the nuclear localization of Maf1. *Proc Natl Acad Sci USA* **103**: 15044–15049
- Moss T, Stefanovsky VY (2002) At the center of eukaryotic life. *Cell* **109**: 545–548
- Nguyen VC, Clelland BW, Hockman DJ, Kujat-Choy SL, Mewhort HE, Schultz MC (2010) Replication stress checkpoint signaling controls tRNA gene transcription. *Nat Struct Mol Biol* **17**: 976–981
- O’Neill BM, Szyjka SJ, Lis ET, Bailey AO, Yates 3rd JR, Aparicio OM, Romesberg FE (2007) Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. *Proc Natl Acad Sci USA* **104**: 9290–9295
- Oficjalska-Pham D, Harismendy O, Smagowicz WJ, Gonzalez de Peredo A, Boguta M, Sentenac A, Lefebvre O (2006) General repression of RNA polymerase III transcription is triggered by protein phosphatase type 2A-mediated dephosphorylation of Maf1. *Mol Cell* **22**: 623–632
- Oler AJ, Alla RK, Roberts DN, Wong A, Hollenhorst PC, Chandler KJ, Cassidy PA, Nelson CA, Hagedorn CH, Graves BJ, Cairns BR (2010) Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. *Nat Struct Mol Biol* **17**: 620–628
- Orioli A, Pascali C, Pagano A, Teichmann M, Dieci G (2011) RNA polymerase III transcription control elements: Themes and variations. *Gene* **493**: 185–194
- Pluta K, Lefebvre O, Martin NC, Smagowicz WJ, Stanford DR, Ellis SR, Hopper AK, Sentenac A, Boguta M (2001) Maf1p, a negative effector of RNA polymerase III in *Saccharomyces cerevisiae*. *Mol Cell Biol* **21**: 5031–5040
- Reina JH, Azzouz TN, Hernandez N (2006) Maf1, a new player in the regulation of human RNA polymerase III transcription. *PLoS ONE* **1**: e134
- Roberts DN, Wilson B, Huff JT, Stewart AJ, Cairns BR (2006) Dephosphorylation and genome-wide association of Maf1 with Pol III-transcribed genes during repression. *Mol Cell* **22**: 633–644
- Santhanam A, Hartley A, Duvel K, Broach JR, Garrett S (2004) PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. *Eukaryot Cell* **3**: 1261–1271
- Schramm L, Hernandez N (2002) Recruitment of RNA polymerase III to its target promoters. *Genes Dev* **16**: 2593–2620
- Sherman F (2002) Getting started with yeast. *Methods Enzymol* **350**: 3–41
- Towpik J, Graczyk D, Gajda A, Lefebvre O, Boguta M (2008) Derepression of RNA polymerase III transcription by phosphorylation and nuclear export of its negative regulator, Maf1. *J Biol Chem* **283**: 17168–17174

- Upadhy R, Lee J, Willis IM (2002) Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol Cell* **10**: 1489–1494
- Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN, Loewith R (2007) Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. *Mol Cell* **26**: 663–674
- Van Hoof C, Martens E, Longin S, Jordens J, Stevens I, Janssens V, Goris J (2005) Specific interactions of PP2A and PP2A-like phosphatases with the yeast PTPA homologues, Ypa1 and Ypa2. *Biochem J* **386**: 93–102
- Vannini A, Ringel R, Kusser AG, Berninghausen O, Kassavetis GA, Cramer P (2010) Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell* **143**: 59–70
- Wei Y, Tsang CK, Zheng XF (2009) Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1. *EMBO J* **28**: 2220–2230
- Willis IM, Desai N, Upadhy R (2004) Signaling repression of transcription by RNA polymerase III in yeast. *Prog Nucleic Acid Res Mol Biol* **77**: 323–353
- Wolff S, Ma H, Burch D, Maciel GA, Hunter T, Dillin A (2006) SMK-1, an essential regulator of DAF-16-mediated longevity. *Cell* **124**: 1039–1053
- Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**: 471–484
- Zaragoza D, Ghavidel A, Heitman J, Schultz MC (1998) Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol Cell Biol* **18**: 4463–4470



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