Gcn4p-mediated transcriptional repression of ribosomal protein genes under amino-acid starvation

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Gcn4p is a well-characterized bZIP transcription factor that activates more than 500 genes encoding amino acids and purine biosynthesis enzymes, and many stress-response genes under various stress conditions. Under these stresses, it had been shown that transcriptions of ribosomal protein (RP) genes were decreased. However, the detailed mechanism of this downregulation has not been elucidated. In this study, we present a novel mechanistic model for a repressive role of Gcn4p on RP transcription, especially under amino-acid starvation. It was found that Gcn4p bound directly to Rap1p, which in turn inhibited Esa1p–Rap1p binding. The inhibition of Esa1p recruitment to RP promoters ultimately reduced the level of histone H4 acetylation and RP transcription. These data revealed that Gcn4p has simultaneous dual roles as a repressor for RP genes as well as an activator for amino-acid biosynthesis genes. Moreover, our results showed evidence of a novel link between general control of amino-acid biosynthesis and ribosome biogenesis mediated by Gcn4p at an early stage of adaptation to amino-acid starvation.

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Introduction

Cells exist in various environmental conditions, some of which are beneficial for growth and proliferation and others that are harmful to the point of being life-threatening. Such environmental stresses include abnormal oxygen concentrations, temperatures, osmotic pressures and pH in addition to nutrient limitations. Cell survival during exposure to stress limits energy availability, thereby increasing the degree of metabolic depression. Therefore, cells finely tune their use of

energy and resources in order to adapt to extreme environmental changes. The energetic cost savings is accomplished through the depression of ion pumping, macromolecular synthesis and macromolecular turnover (Hand and Hardewig, 1996). These energy-saving mechanisms are widespread, from animals during hibernation to single-cell organisms such as yeasts.

The synthesis of ribosomes is the single most energy-consuming process in living cells (Warner, 1999; Warner *et al*, 2001). In yeast, the ribosome is a massive RNA–protein complex that contains 79 ribosomal proteins (RPs), four different rRNAs and over 150 additional proteins involved in various steps of ribosome maturation. This implies that ribosome biogenesis requires the coordinated activities of all three RNA polymerases (Fatica and Tollervey, 2002). In fact, it has been revealed that 60% of total transcription activity is devoted to producing rRNA, and 50% of RNA polymerase II transcription is dedicated to RP genes (Warner, 1999). Thus, cells must adjust the synthesis of ribosomes to match the amount of energy and resources available, as growth conditions change. Many previous studies reported a strong and highly significant correlation between expression of RP genes and changes in growth conditions, such as growth dynamics, carbon sources, temperature and amino-acid pools (Neuman-Silberberg *et al*, 1995; Hand and Hardewig, 1996; Natarajan *et al*, 2001; Levy *et al*, 2007).

There are many signalling pathways known to influence in ribosome biogenesis. The target of rapamycin (TOR) kinase, ras-cAMP–protein kinase A (PKA), protein kinase C and membrane secretory signalling pathways are all known to be involved in RP transcription and ribosome biogenesis (Klein and Struhl, 1994; Neuman-Silberberg *et al*, 1995; Nierras and Warner, 1999; Powers and Walter, 1999). Recent research shows that TOR pathway mediates RP expression through the RAS/cAMP pathway, independent of TAP42/SIT4 in yeast (Martin *et al*, 2004; Schmelzle *et al*, 2004; Chen and Powers, 2006). Nitrogen is a nutrient important for this signalling pathway (Crespo *et al*, 2002). In yeasts, TORC1 uses the AGC kinase Sch9p as a substrate to activate RP transcription (Urban *et al*, 2007). Otherwise, carbon is an important nutrient for activating the PKA signalling pathway (Schmelzle *et al*, 2004), which helps determine the availability of carbon and nitrogen (Neuman-Silberberg *et al*, 1995) and also affects RP expression. Other stresses such as heat shock and amino-acid starvation are also known to influence RP transcription (Li *et al*, 1999; Gasch *et al*, 2000; Causton *et al*, 2001; Natarajan *et al*, 2001). It is also important to note that the significant reduction in RP expressions is consistent with decreases in translation that occur under stressful conditions, and is explained by the redistribution of cellular resources towards the synthesis of stress-response proteins (Miller *et al*, 1979; Causton *et al*, 2001).

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In yeasts, the level of RP expression is regulated almost entirely at the transcriptional level. Although RP genes are scattered over the entire genome and have different promoter sequences, common architectures have been found in the promoter regions (Lascaris *et al*, 1999). It has been revealed that most RP genes contain one or more Rap1p and/or Abf1p binding sites (UASrpg: upstream activating sequence of RP gene) in their promoters. This element is commonly found about 250–400 bp upstream of the transcription start site. More extensive chromatin immunoprecipitation (ChIP)-chip analysis showed that RP promoters are the major functional targets of Rap1p, also known as ‘repressor and activator protein’ (Lieb *et al*, 2001). Rap1p is an abundant, sequence-specific DNA binding protein located in the nucleus, and functions as a context-dependent repressor or activator of transcription (Shore, 1994; Pina *et al*, 2003). It promotes the transcription of many heavily transcribed genes, including those encoding glycolytic enzymes, RPs and several components of the translational machinery (Goncalves *et al*, 1995; Lascaris *et al*, 1999; Joo *et al*, 2009b). As a transcriptional activator, Rap1p binds to a specific DNA sequence (ACACCCRYACAYM; Lieb *et al*, 2001). During transcription activation, it has a general role as a ‘chromatin opener’ to facilitate the binding of other transcription activators to binding sites adjacent to the T-rich region (Yu and Morse, 1999).

Several factors also have been implicated in the transcription of RP genes in yeasts. For example, Fhl1p, Ifh1p and Crf1p are among the most well-characterized transcription factors involved in the initiation of RP transcription (Martin *et al*, 2004; Schawalter *et al*, 2004; Wade *et al*, 2004; Rudra *et al*, 2005). TOR controls Fhl1p via the RAS–PKA–YAK1 effector pathway along with two Fhl1p cofactors, Ifh1p and Crf1p, the coactivator and corepressor, respectively. Sfp1p, known as a negative regulator of TORC1, specifically inhibits TOR kinase activity that acts towards Sch9p kinase (Lempiainen *et al*, 2009). Recently, the mechanisms by which the general amino-acid control (GAAC) and TOR pathways are integrated for optimal growth adaptation were reported (Staschke *et al*, 2010). Recruitment of all these factors appears to depend upon the state of histone acetylation of RP promoters, which is regulated by Esa1p and Rad3p (Reid *et al*, 2000; Rohde and Cardenas, 2003). The catalytic subunit of the NuA4 complex, Esa1p, is recruited to RP promoters for transcriptional activation under normal physiological conditions (Reid *et al*, 2000), and Rpd3p is recruited for transcriptional repression under stressful conditions such as cold shock (Robert *et al*, 2004). This correlation between factor recruitment and transcriptional regulation does not appear to be global, but rather specific for the RP promoter regions. Moreover, all the mechanisms appear to occur in a Rap1p- and UASrpg-dependent manner (Moehle and Hinnebusch, 1991; Reid *et al*, 2000; Zhao *et al*, 2006; Layer *et al*, 2010). It is also worth noting that recent studies have revealed the functional relevance of Rap1p in transcription activation through protein–protein interactions (Garbett *et al*, 2007; Bendjennat and Weil, 2008; Papai *et al*, 2010).

Gcn4p is a well-characterized bZIP-containing transcription factor that binds to a consensus sequence ATGA(C/G)TCAT (Hope and Struhl, 1985; Hill *et al*, 1986). A bZIP dimer of Gcn4p containing two parallel coiled-coils allows binding to the consensus sequence (Ellenberger *et al*, 1992). This is

essential for the induction of amino-acid and purine biosynthetic genes upon environmental signals, such as adenine limitation and amino-acid starvation induced by 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3p (Joo *et al*, 2009a; Natarajan *et al*, 2001). In such cases, the level of Gcn4p is upregulated by an intricate translational control mechanism involving Gcn2p kinase and the phosphorylation of its substrate, eIF2 α (Hinnebusch, 2005). This regulatory response is known as GAAC, in which uncharged tRNA is an upstream activator (Dong *et al*, 2000). It was also previously reported that RP genes were repressed in a Gcn4p-dependent manner along with the expression of other translational proteins (Natarajan *et al*, 2001). However, the *bona fide* mechanism of the repression has not been known.

In this study, we investigated the detailed mechanism of RP transcription mediated by Gcn4p. First, we confirmed the previously reported Gcn4p-dependent regulation of RP transcription. Next, we revealed that Gcn4p physically interacts with Rap1p, resulting in a Gcn4p recruitment on the promoter region of RP genes. We found that Gcn4p inhibited Rap1p–Esa1p interaction, which is responsible for full transcription of RP genes through histone H4 acetylation on the promoter. From these results, we hypothesized that derepressed Gcn4p under amino-acid starvation inhibits RP transcription by interacting with Rap1p, establishing an optimal and effective means for redirecting resources and apparatuses for preferential transcription of stress-response genes, thus reducing unnecessary energy consumption.

Results

Transcription of RP genes are downregulated in a Gcn4p-dependent manner under amino-acid starvation

To examine the regulation of RP transcripts under various amino-acid starvation conditions, the levels of RP transcripts were examined. Northern blotting results clearly showed that *TRP3* mRNA significantly increased under all amino-acid starvation conditions (Figure 1A). Simultaneously, transcription levels of some sets of RP genes (*RPS3*, *RPS11B* and *RPL19B* in Figure 1A; *RPS31* in Supplementary Figure S1) and *PGK1*, which contain UASrpg(s) in their promoters, were inversely proportional to that of *TRP3*. However, the transcription of two RP genes including *RPL3* and *RPL18B*, which do not possess UASrpg, did not show significant decrease under amino-acid starvation. These results showed mutually exclusive transcription of Gcn4p target genes and RP genes only containing Rap1p site on the promoter region, implying that Rap1p on the RP promoter appears to be responsible for the downregulation of RP genes under amino-acid starvation conditions.

To further examine Gcn4p-dependent regulation of RP transcription, previously studied using microarray (Natarajan *et al*, 2001), northern blot analysis was performed with *GCN4* wild-type and null strains after 3-AT treatment (Figure 1B). The level of RP mRNAs was decreased by 3-AT treatment only in the wild type. Although RP mRNA was also slightly decreased after 2 h in the *GCN4* deletion strain, the degree of downregulation was much lower than that in wild type. To exclude any off-target effects produced by 3-AT, we performed northern blot analysis with the same strains under amino-acid depletion (Figure 1C). We found that this regulation does not occur in a strain-specific manner using other

Figure 1 Transcription of RP genes decreased under amino-acid starvation in a Gcn4p-dependent manner. **(A)** Transcription of RP genes under amino-acid starvation. JS143-7D strain growing at early exponential phase was re-cultivated with 3-AT (20 mM) or with limited amino acids (10% of the concentrations originally required) or with sulfometuron methyl (SM, 10 μ g/ml). Each transcript level was examined by northern blot with specific probes. The mRNA levels were measured and normalized to the level of *ACT1*. Experiments were performed in triplicate with three independent colonies. **(B)** Gcn4p-dependent regulation of RP transcription under amino-acid starvation. Exponentially growing cells of both *GCN4* wild-type (JS143-7D, *GCN4*) and deletion strain (YJK101, Δ *gcn4*) in SC—his were treated with 3-AT (80 mM) or an equal volume of distilled water, and then harvested at the indicated time. Total RNA was precipitated and subjected to northern blotting (upper panel). Ratios of each transcript relative to *ACT1* mRNA were calculated and summarized by a graph (lower panel). **(C)** Examination of RP mRNA levels in amino acid-depleted media. Exponentially growing cells as used in **(B)** were transferred into SC—his, trp media and harvested at the indicated time. Northern blot was performed with *RPS3* and *ACT1* probes.

GCN4 wild-type and deletion strains possessing different genetic backgrounds (Supplementary Figure S2).

We next explored how Gcn4p regulates RP transcription. There are several plausible explanations for this regulation. Gcn4p could affect the steady-state level of RP mRNA. Through northern blot analysis, this hypothesis could be excluded based on the results showing that basal levels of RP transcripts were almost the same in both wild-type and null strains (Supplementary Figure S3). Gcn4p might also regulate the stabilities of RP mRNAs. We checked the half-lives of RP mRNAs using thiolutin, which inhibits all three RNA polymerases in yeast. Under both amino-acid depletion and normal physiological conditions, the stabilities of RP mRNAs were not different from each other in both isogenic strains (Supplementary Figures S4 and S5), implying that Gcn4p reduces transcription initiation of RP genes. These observations were consistent with our previous finding that transcription initiation was diminished by 3-AT treatment (Joo *et al*, 2009b). From these results, it was clearly confirmed that RP genes are negatively regulated under amino-acid starvation in a Gcn4p-dependent manner. Moreover, it was suggested that Gcn4p may exert an effect on the initiation process of RP gene transcription.

Gcn4p directly interacts with Rap1p

To understand how Gcn4p represses initiation of RP transcription, we searched for Gcn4p sites in the promoter of RP genes. However, most of the *RPL* and *RPS* genes lack Gcn4p sites. Moreover, our observations of *PGK1* mRNA led us to believe that Rap1p may have a pivotal role for the down-regulation of RP genes under amino-acid starvation (Supplementary Figure S1). Thus, we preferentially focused on the transcription factor Rap1p as a target of Gcn4p, as many Rap1p target genes have been shown to be repressed by 3-AT in a Gcn4p-dependent manner (Natarajan *et al*, 2001). In addition, many recent studies had revealed that several transcription factors bind to Rap1p directly, and this interaction was important for initiation mechanism for full transcription of RP genes (Garbett *et al*, 2007; Rudra *et al*, 2007; Bendjennat and Weil, 2008; Papai *et al*, 2010). Therefore, physical interaction between Rap1p and Gcn4p was strongly expected. To investigate Rap1p–Gcn4p interaction, we generated a strain bearing N-terminal TAP (tandem affinity purification)-tagged *RAP1*. After standard TAP purification, Gcn4p was identified as a co-binding protein of Rap1p by mass spectrometry (Supplementary Figure S6). To confirm a direct interaction and identify the binding domain, *in vitro*

GST pull-down assays were performed with His₆-Rap1 and GST-Gcn4 variants or with GST-Gcn4 and His₆-Rap1 variants. Detailed mapping of the Gcn4 interaction site revealed that amino-acid residues 221–247 of Gcn4p mediate the binding with Rap1p (Figure 2A). This region

contains a DNA binding domain and a NLS2 signal. For Rap1p, the domain responsible for the interaction with Gcn4p is located between amino-acid residues of 361 and 596 (Figure 2B), which also contains the DNA binding domain of Rap1p.

A

B

C

D

Next, both proteins were tagged at the C terminus with FLAG or seven c-myc epitopes for overexpression of exogenous protein using a galactose-inducible promoter. Subsequent co-immunoprecipitation (Co-IP) with an anti-FLAG antibody clearly showed that Rap1-FLAG was associated with Gcn4-myc₇ *in vivo* (Figure 2C). Finally, we investigated this interaction using native levels of Rap1p and Gcn4p. As shown in Figure 2D, we confirmed Rap1p-Gcn4p binding repeatedly *in vivo* by Co-IP with a strain containing Gcn4-HA₃, which was regulated by an endogenous promoter. In addition, alkaline phosphatase treatment led us to believe that binding occurred in a phosphorylation-dependent manner. Although this binding was confirmed both *in vitro* and *in vivo*, we were unable to exclude the possibility that chromosomal DNA mediates the binding, as both have DNA binding activities. Therefore, Co-IP was performed in the presence of DNaseI (Figure 2C) or EtBr (Figure 2D), confirming that this interaction was chromatin DNA independent. In summary, we found that Gcn4p binds directly to Rap1p through the DNA binding domains of each protein.

The promoters of RP genes are selectively occupied by Gcn4p under amino-acid starvation

Given that Gcn4p interacts with Rap1p and this binding is responsible for transcriptional regulation of RP genes, Gcn4p may be recruited to RP promoter regions with respect to the Rap1p binding element. Other transcription factors such as Fhl1p and Ifh1p have been shown to be recruited to the RP promoter through direct binding to Rap1p (Rudra *et al*, 2007). ChIP analysis of a strain expressing tri-HA-tagged Gcn4p revealed that the promoter of RP genes was occupied by Gcn4p under amino-acid starvation (Figure 3A). As starvation time increased, the promoter occupancy of the RP genes by Gcn4-HA₃ was increased, as well as that of *HIS3* and *ADE3*, which are well known as Gcn4p target genes. Weaker binding to the RP promoters compared with *HIS3* and *ADE3* promoter binding might be due to indirect association through protein-protein interaction. Moreover, it is worth noting that promoter occupancy of *RPL18B*, which lacks a Rap1p site (UASrpg) in its promoter region, was not detected. However, that of *PGK1*, non-RP genes containing Rap1p site in the promoter, was clearly observed. These results implied promoter occupancy of RP genes by Gcn4p in a Rap1p site-dependent manner. In order to confirm Gcn4p recruitment to the RP promoter, ChIP was performed using primer pairs spanning the promoter region of one of the RP gene, *RPS3*

(Figure 3B). Promoter occupancy by Gcn4p was highest in the UASrpg region, but relatively lower in the upstream and downstream ORF regions, thus validating our hypothesis that Gcn4p was recruited to RP promoter region in a Rap1p- or Rap1p site-dependent manner.

Figure 3 Gcn4p is recruited to RP promoters. (A) Promoter occupancy of RP genes by Gcn4p. After 3-AT (80 mM) treatment, the cultivated cells (YJK102) were harvested and immunoprecipitation was performed with an anti-HA antibody. Subsequently, co-immunoprecipitated chromatin DNA with Gcn4-HA₃ was amplified by semi-qPCR using primers specific for each promoter region. Ratios of IP relative to input signal were evaluated. Average results from more than three independent experiments are summarized in a graph. ChIP was performed three times using independent colonies (** $P < 0.005$; * $P < 0.05$). (B) Mapping the RP promoter region responsible for Gcn4p recruitment. ChIP was performed as in (A). The co-purified chromosomal DNA was subjected to semi-qPCR. The number of PCR products refer to the following sequences: (I) -1576 to -1277 bp; (II) -365 to -2 bp; (III) +426 to +723 bp from translation start site (+1) of *RPS3*.

Figure 2 Gcn4p directly interacts with Rap1p. (A) Mapping the binding domain of Gcn4p responsible for Rap1p binding. A diagram of the Gcn4p truncation fused to GST is presented (left panel). A GST pull-down assay was performed with His₆-Rap1 and GST or GST-Gcn4p variants overexpressed in bacteria. Bead-bound GST or GST fusion proteins were detected by CBB staining and co-precipitated His₆-Rap1 was examined by western blot with an anti-Rap1p antibody (NLS, nuclear localization signal). (B) Mapping the binding domain of Rap1p responsible for Gcn4p binding. A map of the truncation mutants of His₆-Rap1p is presented in the upper panel. Total input lysate from *E. coli*, and bead-bound GST and GST-Gcn4 were observed by CBB staining. Input and co-purified His₆-Rap1p variants were examined by western blot with anti-His₆ antibody (Tox, toxic domain; Act, activation domain; Sil, silencing domain). (C) *In vivo* association of exogenously expressed Gcn4-myc₇ and Rap1p-FLAG. Gcn4p-myc₇ and Rap1p-FLAG were expressed in the BY4741 strain by a galactose induction method as described in the Supplementary Experimental Procedures. Immunoprecipitation was performed with or without 10 μ g of DNaseI and anti-FLAG antibody. The lysate and co-precipitated proteins were examined by western blotting with anti-FLAG and anti-myc antibodies. (D) Co-immunoprecipitation with endogenous Gcn4p and Rap1p. Total protein lysates were prepared from JS143-7D (Gcn4) and YJK102 (Gcn4-HA₃) after treatment with 3-AT (80 mM). Half of the 3-AT-treated sample was incubated with 10 units of alkaline phosphatase in order to examine the phosphorylation-dependent association. To exclude chromosomal DNA-dependent association, immunoprecipitation was performed in the presence of EtBr (50 μ g/ml). Immobilized and co-precipitated proteins were detected by western blot with anti-HA and anti-Rap1p antibodies, respectively.

Binding activity to Rap1p, not *trans*-activity, is essential for the repressive role of Gcn4p in RP gene transcription

Although Gcn4p-dependent transcriptional regulation of RP genes and Rap1p-dependent Gcn4p recruitment to RP promoter were demonstrated, this does not convincingly suggest that Rap1p recruits Gcn4p through direct binding for repression of transcription. There is another possibility that Gcn4p indirectly affects RP transcription through *trans*-target genes. This is plausible as Gcn4p is the best-characterized transcription factor in yeasts and is known to have more than 500 *trans*-target genes (Hinnebusch, 2005). To investigate whether *trans*-activity has an indirect effect on RP transcription, Ser242, a highly conserved amino-acid residue in the

DNA binding domain of Gcn4p was mutated to leucine (Ellenberger *et al*, 1992; Kim and Struhl, 1995; Seong *et al*, 2007). Consequently, the transcriptional activity of this mutant was abolished (Figure 4B and E). On the other hand, both the point mutant and wild-type proteins appear to have similar binding affinity to Rap1p, as equal amount of His₆-Rap1 recombinant protein was precipitated by GST pull-down (Figure 4A). To understand whether Gcn4p directly participates in RP transcription, wild-type and mutant Gcn4p were overexpressed and the levels of RP mRNA were determined. Both exogenously expressed forms of Gcn4p repressed transcription of RP genes, although only wild-type Gcn4p induced transcription of *trans*-target genes,

Figure 4 Gcn4p inhibits the transcription of RP genes through direct interaction with Rap1p. (A) Binding affinity of wild-type Gcn4p and the S242L mutant to Rap1. GST, GST-Gcn4_{WT} and GST-Gcn4_{S242L} induced in bacteria were loaded onto GST bead followed by binding with recombinant His₆-Rap1. Purified GST proteins were examined by CBB staining and co-precipitated His₆-Rap1 was analysed by western blot with an anti-Rap1p antibody. (B) Ectopically expressed Gcn4p represses the transcription of RP genes. The BY4741Δgcn4 strain harbouring myc₇, Gcn4_{WT}-myc₇ and Gcn4_{S242L}-myc₇ were cultured in SRG media for galactose induction. The expression of Gcn4_{WT}-myc₇ and Gcn4_{S242L}-myc₇ was examined by western blotting using an anti-c-myc antibody. For the detection of RP gene transcripts, northern blot analysis was performed with specific probes as indicated. (C) Gcn4_{WT}-myc₇, Gcn4_{S242L}-myc₇ and myc₇ were overexpressed using a galactose-inducible promoter as described in (B). Subsequent ChIP analysis was performed with a c-myc antibody followed by semi-qPCR with a specific probe against each promoter region as indicated. The experiments were performed in triplicate with independent colonies (***P* < 0.005; **P* < 0.05). (D) Three exponentially growing strains harbouring different C-terminal tri-HA-tagged Gcn4p variants (YJK102, WT; YJK125, S242L; YJK126, GΔC) were treated with 3-AT (80 mM) for 2 h. Protein lysates from these cells were subjected to Co-IP with anti-HA antibody. Proteins in input lysates and Co-IP fractions were detected with specific antibodies as indicated. (E) The same strains used in (D) and the GCN4 deletion strain (YJK101, Δ) were cultivated with or without 3-AT (80 mM). After 1 h, cells were harvested and total RNA was precipitated. The RNA samples were subjected to northern blotting with specific probes for RPS3, RPS11B, RPL19B, TRP3 and ACT1. (F) The three strains containing Gcn4p variants were treated with 3-AT (80 mM) for 1 h. Lysates were extracted and subjected to ChIP analysis with an HA antibody. Co-immunoprecipitated chromatin DNA was examined by semi-qPCR with primers for the promoter region of the indicated genes or for the coding sequence of POL1.

such as *HIS4* and *ADE3* (Figure 4B). Next, we executed ChIP experiment to investigate whether wild-type and mutant Gcn4p associate with RP promoters. Statistically significant promoter occupancies of RP genes (*RPS3* and *RPL19B*) were detected in cells expressing both wild-type and mutant Gcn4p (Figure 4C). These results are consistent with the northern blot result shown in Figure 4B, confirming that Gcn4p associates with the RP promoter adjacent to UASrpg through direct binding to Rap1p. Furthermore, it was revealed that the *trans*-activity of Gcn4p and its downstream *trans*-target genes were not involved in the reduction of RP transcription under amino-acid starvation.

To investigate the *in vivo* relevance of Rap1p–Gcn4p binding, strains were generated in which either Gcn4_{S242L}–HA₃ or Gcn4ΔC–HA₃ allele was expressed at normal physiological levels as the only form of Gcn4p. The Gcn4ΔC–HA₃ mutant is the same truncation mutant used in Figure 2A (GST–Gcn4Δ5). As shown in Figure 4D, both the wild-type and point mutant of Gcn4p could bind to Rap1p *in vivo*, but as expected, the Gcn4ΔC–HA₃ mutant could not (Figures 2A and 4A). Next, downregulation of RP transcription was investigated in four *GCN4* isogenic strains using northern blotting analysis (Figure 4E). Induction of *TRP3* by 3-AT was detected only in the wild-type strain. On the other hand, RP transcription was repressed in both wild-type and Gcn4_{S242L}–HA₃ mutant strains, but not in the Gcn4ΔC–HA₃ or *gcn4*-null strains. Moreover, it was revealed by ChIP analysis that both wild-type and S242L mutant Gcn4p were recruited to RP promoters, but not Gcn4ΔC (Figure 4F). Given the fact that expression levels of these Gcn4p variants were almost all identical (Supplementary Figure S7), these data led us to hypothesize that Gcn4p was recruited to the RP promoter for transcription repression through direct binding to Rap1p under amino-acid starvation.

Promoter occupancy of RP genes by Esa1p was decreased in a Gcn4p-dependent manner under amino-acid starvation condition

We next wanted to understand how Gcn4p exerts a negative effect on RP transcription through direct binding to Rap1p. Previous reports found that transcriptional regulators, such as Fhl1p, Ifh1p, Sfp1p and Esa1p, were recruited to RP promoter in a Rap1p-dependent manner (Shore and Nasmyth, 1987; Reid *et al*, 2000; Martin *et al*, 2004; Kasahara *et al*, 2007; Lempiainen *et al*, 2009). It is possible that Gcn4p influences these recruitments. Therefore, we generated strains expressing Ifh1–HA₃, Fhl1–HA₃, Sfp1–HA₃, Esa1–HA₃ and TAP–Rap1 in each JS143-7D (*GCN4*) and YJK101 (*Δgcn4*) genetic background, respectively. First, the expression levels of these factors were investigated by immunoblotting (Figure 5A). As expected, repressed basal expression and significant induction of Gcn4p were clearly observed following 3-AT treatment. There were no changes in Rap1p, Esa1p or Prg1p expression. However, the expression of other factors was significantly reduced by 3-AT treatment. Although the reduction of Sfp1p was less than that of other factors, the patterns of reduction for three proteins (Ifh1p, Fhl1p and Sfp1p) were observed. To investigate Gcn4p-dependent factor recruitment to RP promoters, ChIP was performed in the same strains (Figure 5B–F). The results shown in Figure 5F revealed that promoter occupancy by Rap1p was unchanged following 3-AT treatment.

The recruitment of Fhl1p was decreased under amino-acid starvation, but does not seem to be related to Gcn4p (Figure 5B). It is important to note that the recruitment of Esa1p was diminished in a Gcn4p-dependent manner (Figure 5D). However, Ifh1p and Sfp1p bindings were not detected in our ChIP experimental scale (Figure 5C and E). These results strongly suggested that Esa1p binding to RP promoters is suppressed in a Gcn4p-dependent manner under amino-acid starvation, indicating that Gcn4p may directly influence Esa1p recruitment.

Gcn4p inhibits the physical interaction of Esa1p–Rap1p

Direct involvement of Gcn4p in RP transcription was observed in our studies. In addition, previous studies reported that recruitment of Esa1p to RP promoters is mapped to Rap1p binding sites, which implies that Esa1p is recruited by Rap1p (Reid *et al*, 2000; Rohde and Cardenas, 2003). Therefore, physical interaction between Esa1p and Rap1p is strongly suggested. To validate this prediction, an *in vitro* GST pull-down assay was performed, resulting in confirmation of Esa1p–Rap1p binding (Figure 6A). This binding was also revealed *in vivo* by Co-IP analysis in cells with overexpressed (Figure 6B) and endogenous levels of Rap1p and Esa1p (Supplementary Figure S8).

To verify Gcn4p-dependent regulation of Rap1p–Esa1p binding, another Co-IP analysis was performed with isogenic strains, BY4741 and BY4741Δ*gcn4*. As shown in Figure 6C and D, the association between ectopically expressed HA–Esa1 and Rap1–FLAG was repressed only in when Gcn4p was overexpressed. This was verified in cells expressing native levels of Rap1p and Esa1p (Figure 6E, Supplementary Figure S9). Interestingly, the decreased association between the two factors in the absence of sulfometuron methyl or 3-AT was observed in the *gcn4*-null mutant. This suggested that a tight interaction between Esa1p and Rap1p is not required under nonstarvation conditions in cells lacking *GCN4* for efficient recruitment of Esa1p to RP promoters. The observations of Gcn4p–Rap1p and Esa1p–Rap1p binding led us to predict an inhibitory role of Gcn4p for Esa1p–Rap1p binding. Derepressed Gcn4p expression under amino-acid starvation may be able to hinder the Esa1p–Rap1p interaction as a consequence of diminished Esa1p recruitment to RP promoter by Rap1p. To validate this model, an *in vitro* binding competition assay was performed. Co-precipitation of His₆–Rap1 was decreased as the amount of Gcn4–His₆ in the binding mixture was increased (Figure 6F). Likewise, when Gcn4–His₆ was mixed with the His₆–Rap1–GST–Esa1 binding complex, His₆–Rap1 was eluted from the pre-existing binding complex by Gcn4–His₆, but not by BSA (Figure 6G). The amount of His₆–Rap1 eluted was increased depending on the concentration of Gcn4–His₆. We also determined the molar ratio of the three proteins under amino-acid starvation (Supplementary Figure S10). Although the level of Rap1p was about four- to five-fold higher than others, the amount of Gcn4p was sufficient for our mechanistic model. Taken all together, our data clearly showed that Gcn4p physically bound to Rap1p, which in turn inhibited Rap1p–Esa1p interaction.

It has been known that the recruitment of Esa1p to RP promoters along with the acetylation of histone H4 by NuA4 coordinately regulate transcription (Reid *et al*, 2000). Therefore, disturbance of Rap1p–Esa1p binding by Gcn4p

Figure 5 Gcn4p influences recruitment of Esa1p to RP promoters. (A) Expression level of factors involved in RP transcription. The *GCN4* wild-type and deletion strain containing Ifh1-HA₃ (YJK115 and YJK116), Fhl1-HA₃ (YJK113 and YJK114), Sfp1-HA₃ (YJK119 and YJK120), Esa1-HA₃ (YJK105 and YJK106), TAP-Rap1 (YJK109 and YJK110) and Gcn4-HA₃ (YJK102) were treated with 3-AT (80 mM) and harvested at the indicated times (details of strains are shown in Supplementary Table S1). Whole-cell lysates from these strains were subjected to western blotting with specific antibodies against HA, TAP and Pgc1p. (B–F) Promoter occupancies of RP genes by factors involved in RP transcription. The same strains expressing the proteins as in (A) were cultivated with or without 3-AT (80 mM). After a 1 h incubation, the cells were harvested for preparation of input lysates. ChIP assay was performed with anti-HA or anti-TAP antibodies, and amount of co-immunoprecipitated chromatin DNA was investigated by semi-qPCR with primers against the promoter regions of *RPS3*, *RPS11B*, *RPL19B*, and against the ORF region of *POL1*. JS143-7D and YJK101 strains were used as a negative control for the IP experiment. Analysis was performed in triplicate and averages of IP/input signal are summarized in a graph (** $P < 0.005$; * $P < 0.05$).

may lead to a decrease in histone H4 acetylation specifically in the RP promoter. ChIP analysis with an anti-acetyl-histone H4 antibody revealed that histone H4 acetylation of RP promoters was decreased only in the *GCN4* wild-type strain

under amino-acid starvation (Figure 7A), consistent with the Esa1p ChIP data in Figure 5D. In summary, we concluded that derepressed Gcn4p binds to Rap1p under amino-acid starvation, thereby inhibiting Rap1p-Esa1p interaction and

resulting in decreased histone H4 acetylation of the promoter. Taken together, our results clearly concluded that transcription of RP genes was repressed by Gcn4p through direct binding to Rap1p. This regulation appears to give yeast cells a means to re-direct resources from major energy-consuming RP transcription process for energy conservation under amino-acid starvation.

Discussion

In this study, we uncovered a novel role for Gcn4p as a negative regulator of transcription, and proposed a novel link between GAAC and ribosome biogenesis. Gcn4p–Rap1p binding was confirmed both *in vitro* and *in vivo*, and its biological relevance was investigated by ChIP and northern blot analyses. This interaction promotes promoter occupancy of RP genes by Gcn4p during amino-acid-limiting conditions. We also found target proteins influenced by Gcn4p–Rap1p binding based on the hypothesis that this interaction is responsible for the reduction of RP transcription under amino-acid starvation. ChIP analysis using five putative target proteins confirmed that the recruitment of Esa1p was inhibited in a Gcn4p-dependent manner. Taken together, we generated a simple mechanistic model defining the roles of Gcn4p under amino-acid starvation (Figure 7B). When yeast cells are grown in an amino-acid-rich condition, GAAC signalling is turned off and RP expression becomes activated by Rap1p and Esa1p, as well as by many other transcription factors such as Ifh1p, Fhl1p and Hmo1p. On the contrary, Gcn4p is induced through GAAC signalling when amino acids are limited, initiated by uncharged tRNA and propagated by activation of eIF2 α kinase, Gcn2p (Hinnebusch, 2005). Subsequently, derepressed Gcn4p activates the transcription of many target genes encoding enzymes responsible for amino acid and purine biosyntheses, or that of amino-acid permeases in order to compensate for limitations in amino-acid availability (Joo *et al*, 2009a; Hinnebusch and Natarajan, 2002). Upon activation, transcription of RP genes is suppressed by Gcn4p through the direct binding of Gcn4p to Rap1p.

Cross-talk between GAAC and ribosome biogenesis

Several benefits can be provided by a present Gcn4p-dependent mechanism. In yeasts, regulation of gene expression occurs predominantly at the transcriptional level, and reprogramming of transcriptions is pivotal for encountering drastic changes in environment. Among all transcription processes in yeasts, the mechanism of RP transcription is the best characterized with respect to growth rate and various environmental cues (Jorgensen *et al*, 2004), and also known to be the most energy-consuming process. Therefore, the transcription of RP genes must be finely regulated to avoid wasting resources. Under amino-acid starvation, cells must stop the synthesis of general proteins. It is noteworthy that inhibition of general protein expression and induction of amino-acid biosynthetic enzymes are thought to be executed by *trans*-acting proteins that control *GCN4* translation. Moreover, the molecular events that stimulate *GCN4* translation also inhibit the rate of general protein synthesis (Hinnebusch, 2005).

Reduction of ribosome biogenesis can be executed by degradation of protein and protein complexes. Adaptation to severe environmental stresses is related to metabolic

depression, such as the ability to reduce ion pumping activities, macromolecular synthesis and macromolecular turnover (Hand and Hardewig, 1996). Furthermore, it was reported that turnover of mature ribosomes proceeds non-selective and selective autophagy, called ‘ribophagy’, under severe and long-term amino-acid starvation (Beau *et al*, 2008; Kraft *et al*, 2008). Therefore, these previous data along with our observations suggest that the reduction of RP transcription and activation of amino-acid biosynthetic enzymes by Gcn4p is responsible for rapid and short-term stress adaptation. Otherwise, when the starvation period is extended, cells might induce ribophagy resulting in the breakdown of excess mature ribosomes into materials for the synthesis of other essential molecules. The repression of RP transcription by Gcn4p discovered here has therefore been assigned a new role in adaptations, as it may lead yeast cells to save and redirect available energy, resources and transcriptional machineries in a quick response to amino-acid depletion. Accordingly, it is important to note that Gcn4p has more than 500 target genes that require lots of transcriptional machinery for activation under stress conditions. Therefore, plenty of transcriptional apparatuses as well as resources and energy are needed for their transcriptional induction. However, as previously mentioned, 50% of RNA polymerase II transcription as well as 90% of mRNA splicing are devoted to producing RPs (Warner, 1999; Warner *et al*, 2001). Reduction of this massive transcriptional process leads to the efficient induction of its target genes, as well as other genes related with adaptation. This cross-talk between GAAC and ribosome biogenesis facilitated by Gcn4p can occur under conditions other than amino-acid starvation. Gcn4p can be stimulated by ultraviolet light and methyl methane-sulfonate (Hill *et al*, 1986; Natarajan *et al*, 2001), and expression of ribosome biosynthetic genes is highly responsive to environmental changes (Levy *et al*, 2007). For a full understanding of regulation between massive biological processes, Gcn4p-dependent regulation of RP genes provides a sophisticated explanation for how regulation between major biological processes may be accomplished.

Complicated regulation of RP transcription through multiple pathways

All ribosomal components exist in equimolar levels in yeasts as a consequence of a common regulatory mechanism. The rate of ribosome synthesis responds to not only nutritional cues but also signals associated with other macromolecular synthesis pathways. Recent results have revealed that many factors, such as Rap1p, Abf1p, Fhl1p, Ifh1p, Crf1p, Hmo1p, Sfp1p and Rpd3p, participate directly in the transcription of RP genes, but their detailed mechanisms are not fully described. Such complications could be due to the complexity and heterogeneity that are common in RP promoter architectures. Computational research revealed that all RP promoters are not identical, although duplicated Rap1p sites are common (Lascaris *et al*, 1999). RP genes can be divided into several subclasses based on the abundance of Hmo1p and the dependence of Fhl1p and Rap1p promoter binding to Hmo1p (Kasahara *et al*, 2007). However, there are some questions about the involvement of Hmo1p. For instance, a previous study found that loss of Hmo1p abolishes Fhl1p binding but does not significantly affect RP transcription (Hall *et al*, 2006), an unexpected result that challenges previous

observations (Martin *et al*, 2004; Schawalter *et al*, 2004; Wade *et al*, 2004; Rudra *et al*, 2007). Moreover, the response of RP promoters to TOR inhibition is independent of the Iff1p-related protein Crf1p, indicating that the role of this protein is strain-specific (Zhao *et al*, 2006). Other groups revealed that histone acetylation is selectively increased in the promoter

regions of RP genes (Reid *et al*, 2000; Rohde and Cardenas, 2003). The histone acetyl transferases Gcn5p and Esa1p are recruited to the promoters of actively transcribing genes, including those encoding RP (Robert *et al*, 2004). However, other studies have found that histones are essentially absent from the Rap1p sites of RP promoters (Zhao *et al*, 2006).

Here, we showed several lines of evidence demonstrating parallel mechanisms of RP gene downregulation under amino-acid starvation. Western blot analysis in Figure 5A clearly revealed that the levels of Fhl1p and Ifh1p were decreased by 3-AT in both wild-type and null strains of *Gcn4*. Moreover, expression levels of Fhl1p seemed to be regulated in a Gcn4p-dependent manner. It was possible that Gcn4p might have an effect on RP gene regulation indirectly through the modulation of Fhl1p expression level. Fhl1p and Ifh1p reduction by 3-AT could explain why RP mRNA (Figure 1B) and promoter occupancy by Fhl1p (Figure 5B) were both decreased in the *gcn4*-null strain, although to a lesser degree compared with wild type. These results showed that yeast cells have many parallel pathways that regulate RP transcription, and that the novel regulatory mechanism shown here appears to be one of these. However, it is worth noting that all these phenomena occurred in a Rap1p-dependent manner, although no specific regulator controls RP transcription.

Our observations also showed several lines of evidence that other factor(s) can be involved in the repressive mechanism of Gcn4p. First of all, RP transcriptions were decreased in the absence of *Gcn4* (Figure 1B), and transcripts of RP genes that contain no Rap1p sites in their promoters also were slightly reduced (Figure 1A). Interestingly, the degree of reduction in both cases was almost the same (80% of the control). These results indicated that 20% of RP mRNA appears to be decreased by other mechanisms not yet elucidated. Moreover, the binding domain of Rap1p, which is responsible for Gcn4p binding, is exactly identical to that for TBP binding (Bendjennat and Weil, 2008). These led us to hypothesize that Gcn4p could hinder the association of Rap1p with general transcription machineries, such as TFIID. Furthermore, we also found that the phosphorylation status of Gcn4p had an effect on Rap1p binding (Figure 2D). This implies that an unknown upstream kinase also participates in this process. Moreover, in the case of the S242L mutant, the repressive effect on RP transcription and recruitment to RP promoters were slightly diminished, although binding affinity to Rap1p was equal *in vitro* (Figure 4D and E). These results imply that other factors should be involved in Gcn4p–Rap1p binding to RP promoters *in vivo*. Therefore,

further research into Rap1p binding partners to the RP promoter region will be performed in order to fully understand how yeast cells regulate RP expression.

Materials and methods

Yeast strains and culture conditions

Characteristics of the *Saccharomyces cerevisiae* strains used in this study are summarized in Supplementary Table S1. All strains containing tri-HA-tagged and N-terminal TAP-tagged proteins were generated using PCR-based gene modification methods as previously described (Longtine *et al*, 1998). For N-terminal TAP tagging, cassettes were amplified by PCR with pBS1761–RAP1pro or pBS1761–ESA1pro generated from pBS1761 (EUROSCRAF). To generate Gcn4_{S242L}–HA₃ containing strain (YJK125), a general two-step PCR-based targeted mutagenesis method was performed. For generation of a tagging cassette containing point mutation, genomic DNA from a strain harbouring Gcn4–HA₃ (YJK102) was used as a PCR template. Strain generation was confirmed by genomic DNA sequencing. Yeast cells were grown in proper SC media (synthetic complement; contained 2% glucose, 0.67% yeast nitrogen base without amino acids (BD) and 15 or less proper amino acids). For galactose induction, cells were grown in SRG media (SC with raffinose and galactose; contains 2% raffinose, 2% galactose, 0.67% yeast nitrogen base without amino acids (BD) and 15 or less proper amino acids), and detailed procedures are described in the Supplementary Experimental Procedures.

Primers and plasmids

Primers used in this research are summarized in Supplementary Table S2. A pESC yeast epitope tagging system (Stratagene) was used for the exogenous expression of c-myc-, HA- and FLAG-tagged proteins using a galactose-inducible promoter. For the *in vitro* pull-down assay, a pGEX-5X-1 plasmid (GE Healthcare) was used for generating GST fusion proteins. In addition, pET21a and pET15b plasmids (Novagen) were used for hexa-histidine tagging. Detailed vector constructions are described in Supplementary Experimental Procedures.

Total RNA preparation and northern blotting

Total yeast RNA was obtained using a phenol/freeze RNA preparation methods as previously described (Schmitt *et al*, 1990). A total amount of 5–10 µg RNA was subjected to general northern blot analysis using digoxigenin (DIG; Boehringer Mannheim; DIG system User's Guide for Filter Hybridization) as previously described (Joo *et al*, 2009a).

In vitro pull-down assay with GST fusion proteins

All recombinant proteins used in this study were overexpressed in *E. coli* (BL21-CodonPlus (DE3)-RIL; Stratagene). Bacterial lysates

Figure 6 Gcn4p inhibits Rap1p–Esa1p binding. (A) Esa1p binds directly to Rap1p *in vitro*. GST pull-down analysis using recombinant His₆–Rap1, and GST or GST–Esa1 proteins was performed. Proteins input or immunoprecipitated were investigated by western blot or CBB staining. (B) Whole-cell lysates of the BY4741 strain containing indicated combinations of ectopically expressed HA–Esa1 and/or Rap1–FLAG were subjected to co-immunoprecipitation with anti-FLAG or anti-HA antibodies, respectively. Input lysates and precipitated immuno-complexes were examined by western blotting with antibodies against the corresponding epitope tags. (C–E) Association of Esa1p with Rap1p was decreased by Gcn4p under amino-acid starvation. (C) BY4741 cells containing Rap1–FLAG, HA–Esa1 and myc₇ or Gcn4-myc₇ were harvested after galactose induction. FLAG–Rap1 was immobilized with an anti-FLAG antibody and protein-A agarose, followed by western blotting with antibodies against FLAG, HA and c-myc to detect proteins in input lysates and precipitated fractions. (D) The BY4741 (*Gcn4*) and BY4741Δ*gcn4* (Δ*Gcn4*) strains harbouring the indicated combinations of HA–Esa1 and Rap1–FLAG were cultivated in SRG media for galactose induction, and treated with 10 µg/ml SM or drug vehicle alone. Cells were harvested at the indicated times. Associations of proteins were analysed by co-immunoprecipitation with HA antibody, and input lysates and co-immunoprecipitated proteins were detected with anti-FLAG, anti-HA, anti-Gcn4p and anti-Pgk1p antibodies. (E) *Gcn4* wild-type and deletion strains harbouring N-terminal TAP-tagged Esa1 (YJK127 and YJK128) were cultivated and treated with 3-AT (80 mM) or drug vehicle alone. After 2 h of incubation, cells were harvested and protein lysates were subjected to pull-down analysis with IgG sepharose beads. Proteins in input lysates and precipitated fractions were examined by western blot with antibodies against Rap1p, TAP and Pgk1p. JS143-7D and YJK101 strains were used as the negative control. (F, G) Competition between Esa1p and Gcn4p for Rap1p binding. (F) A GST pull-down analysis was performed with lysates from cells overexpressed His₆–Rap1, GST or GST–Esa1 with increasing amounts of Gcn4–His₆. GST or GST–Esa1 loaded onto beads was examined by CBB staining and the level of co-purified His₆–Rap1 was detected by western blotting with an anti-His₆ antibody. (G) An *in vitro* binding competition assay was performed as in (A) with some modifications. After the second binding reaction of GST pull-down analysis with GST–Esa1 and His₆–Rap1 (500 µg, 5.36 nmol), the beads were washed with 1 ml PBS and an increasing amount of purified Gcn4–His₆ (100 µg, 3.11 nmol; 300 µg, 9.34 nmol; 500 µg, 15.56 nmol) or BSA (1 mg, 15.06 nmol) was added into the binding mixture. Following a 1 h incubation at RT, the eluted fraction was separated by centrifugation. His₆–Rap1 in elution and bead-bound fractions was examined by western blot with an anti-His₆ antibody.

Figure 7 Dual roles of Gcn4p in response to amino-acid starvation. (A) Histone H4 acetylation on RP promoter regions under amino-acid starvation. JS143-7D (*Gcn4*) and YJK101 (*Δgcn4*) were grown to an early log phase, treated with 3-AT (80 mM) and harvested after 1 h. ChIP was then performed with antibodies specific for acetyl-histone H4 (AcH4) or rabbit pre-immune serum (IgG). The semi-qPCR analysis was performed to examine co-immunoprecipitated chromatin DNA with primers specific for the promoter region of *RPS3*, *RPS11B* and *RPL19B*, or for the coding sequence of *POL1 CDS*. Experiments were performed in triplicate with independent colonies (** $P < 0.005$; * $P < 0.05$). (B) Schematic diagram for the roles of Gcn4p under amino-acid starvation.

containing GST or GST fusion proteins were applied to 30 μ l of glutathione Sepharose 4B resin (Amersham Pharmacia) and incubated for 30 min at room temperature (RT). After beads were washed twice with 1 ml of PBS, other bacterial lysates containing overexpressed His₆-Rap1p or its variants were added, followed by incubation with rotation for 2 h at RT. Finally, the binding mixtures were washed three times with PBS. Proteins bound to the GST resins were recovered by boiling for 5 min and subjected to 10% SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining or western blotting.

Preparation of protein lysates, western blotting and Co-IP

Harvested cells along with PBS washing were resuspended in FA lysis buffer as previously described (Kuras and Struhl, 1999),

complemented with protease inhibitors (1 mM PMSF, 1 μ g/ml pepstatin A, 5 μ g/ml leupeptin and 1 μ g/ml aprotinin). All procedures were performed at 4°C. An equal volume of glass beads were added. The cells were lysed by vortexing with a bead beater (MP) for 5 min on ice. Whole-cell lysates were obtained by centrifugation at 10 000 g for 10 min. An equal amount of total lysate protein was analysed by western blot with specific antibodies against HA (Santa Cruz), c-myc (Santa Cruz), His₆ (Santa Cruz), Pgk1p (Molecular Probes), FLAG (Sigma), Rap1p (Santa Cruz), Esa1p (Santa Cruz) and TAP (OPEN Biosystems). For Co-IP, 6 mg of protein lysate was incubated with antibodies against the indicated epitopes for 2 h. The immuno-complexes were immobilized by the addition of 35 μ l of protein-A agarose (Roche). Following incubation for 2 h on a rotator, precipitants were

recovered and subjected to 10% SDS-PAGE for further western blotting.

Chromatin immunoprecipitation

Chromatin DNA preparation, immunoprecipitation and semi-quantitative PCR (semi-qPCR) were performed as previously described with some modifications (Joo *et al*, 2009a). Cells were harvested and lysed by glass beads, as previously described (Hecht and Grunstein, 1999). Chromatin DNA was sheared by sonication, and input lysates containing fragmented chromatin DNA were fractionated by centrifugation at 10 000 g for 10 min. A total protein amount of 3 mg for each input sample was subjected to immunoprecipitation with the indicated antibodies, and 500 µg of total input samples were saved for input control PCR. Semi-qPCR was performed with 1/40 of the immunoprecipitated DNA and 1/1200 of the total input chromatin DNA as a template. Also included were primers specific for the promoter regions of *RPS3*, *RPS11B*,

RPL19B, *RPL18B*, *HIS3*, *ADE3*, *HIS4*, *ACT1* and the coding sequence of *POL1* (sequences are summarized in Supplementary Table S2).

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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