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Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Ifh1

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an Applied Biosystems 7700 sequence detector. Relative occupancy values were calculated by determining the apparent immunoprecipitation efficiency (amount of PCR product in the immunoprecipitated sample divided by the amount of PCR product in the input sample) and normalized to the level observed at the coding sequence of the *POL1* gene, which was defined as 1. This background binding was then subtracted to give a value in 'occupancy units'. Error bars shown reflect the standard deviation of the mean of independent experiments.

Western blotting

TAP (tandem affinity purification)-tagged Iff1 was detected with peroxidase-anti-peroxidase antibody (Sigma) and Fhl1 was detected with anti-myc antibody (9e10, Santa Cruz Biotechnology).

Microarray analysis

Microarrays containing duplicate spots of 6,528 PCR products corresponding to nearly all yeast intergenic regions were hybridized with a mixture of amplified immunoprecipitate (labelled with Cy5 fluorescent dye) and input (labelled with Cy3 dye) samples, as described previously²⁷. Values shown are an average of two independent experiments. Conserved DNA motifs were identified using AlignACE²⁸. WebLogo was used to generate motif logos²⁹.

Transcriptional analysis

Total RNA was purified using Qiagen RNeasy columns with DNase I treatment. First-strand cDNA was synthesized using dT₁₆, and quantitative PCR in real time was performed on the resulting first-strand complementary DNA using primers specific to the gene of interest³⁰. RNA levels were determined relative to a control gene, *ACT1*.

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Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Iff1

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Regulation of ribosome biogenesis is central to the control of cell growth¹. In rapidly growing yeast cells, ribosomal protein (RP) genes account for approximately one-half of all polymerase II transcription-initiation events¹, yet these genes are markedly and coordinately downregulated in response to a number of environmental stress conditions^{2–4}, or during the transition from fermentation to respiration⁵. Although several conserved signalling pathways (TOR, RAS/protein kinase A and protein kinase C) impinge upon RP gene transcription¹, little is known about how initiation at these genes is controlled. Rap1 (refs 6, 7) and more recently Fhl1 (ref. 8) were shown to bind upstream of many RP genes. Here we show that the essential protein Iff1 binds to and activates many RP gene promoters under optimal growth conditions in *Saccharomyces cerevisiae*. Iff1 is recruited to RP gene promoters through the forkhead-associated domain of Fhl1. Iff1 binding decreases when RP genes are downregulated either by TOR inhibition or nutrient depletion, and is restored after release from starvation or upon regulated induction of *IFH1* expression. These findings indicate a central role for Iff1 and Fhl1 in RP gene regulation.

A recent global chromatin location analysis in yeast uncovered the forkhead-like protein Fhl1 as a factor highly specific for RP gene promoters⁸. Fhl1 was originally identified as a gene dosage suppressor of RNA polymerase (Pol) III mutations required for normal growth⁹. The slow growth of *fhl1Δ* cells is suppressed by elevated gene dosages of *IFH1*, suggesting that both Fhl1 and Iff1 are involved in ribosomal RNA transcription or maturation¹⁰. In an attempt to resolve these apparently conflicting findings, we first

tested the prediction⁸ that Fhl1 is required for normal expression of the Pol-II-driven RP genes. We examined two representative genes (*RPL9A* and *RPL30*) in *fhl1Δ* cells and found reduced levels of both transcripts relative to actin (*ACT1*), which does not bind Fhl1 (ref. 8; see also Supplementary Table 1), suggesting that Fhl1 binding to RP gene upstream regulatory sequences (URS) is required for full expression (Fig. 1a). Similar results were seen in *fhl1Δ ifh1Δ* cells (Fig. 1a), and may explain why both *fhl1Δ* and *fhl1Δ ifh1Δ* strains grow extremely slowly compared with wild type (ref. 10 and data not shown).

We next used chromatin immunoprecipitation (ChIP) to test the idea that the essential Ifh1 protein is also involved in RP gene transcription, and observed specific binding to three different RP gene URS (*RPL30*, *RPL37A* and *RPL9*) under optimal growth conditions (Fig. 1b). Similarly, we confirmed that both Rap1 (ref. 6) and Fhl1 (ref. 8) also bind to these URS (Fig. 1b, c). Notably, we found that Ifh1 binding to these genes is undetectable in *fhl1Δ* cells (Fig. 1b), suggesting that Fhl1 has an essential role in the recruitment of Ifh1 to RP genes. This effect is specific to Ifh1, because neither *fhl1Δ* nor the *fhl1Δ ifh1Δ* double mutant shows any loss of Rap1 binding (Fig. 1c). Conversely, a non-RP gene bound by Rap1 (*PGK1*) shows no binding of either Fhl1 or Ifh1 (Fig. 1c), indicating that Rap1 binding itself does not lead to either Fhl1 or Ifh1 promoter association.

As an exponentially growing yeast culture begins to deplete glucose from the medium, cells initiate a global re-programming of transcription in preparation for the metabolic transition from fermentation to respiration¹¹. Well before this 'diauxic shift', a marked drop in RP gene transcript levels is observed⁵, the mechanism of which is unknown. To address this issue we used ChIP to measure association of Rap1, Fhl1 and Ifh1 at three different RP gene promoters as cells prepared for the diauxic shift. We observed a

marked decline in Ifh1 promoter binding at *RPL30* (and the two other RP genes tested; data not shown) that coincided with, or immediately preceded, the observed drop in messenger RNA level (Fig. 1d). In contrast, promoter association of Rap1 and Fhl1 remains constant during this period. The protein levels of all three factors are largely unchanged during the period of RP gene repression (Supplementary Fig. S1a). These results establish a tight temporal correlation between loss of Ifh1 promoter association and downregulation of RP gene transcription in a wild-type setting, and suggest that controlled promoter association of Ifh1 may be a key regulatory mechanism for RP genes. We also examined the RP gene association of the three proteins as stationary phase cells resume exponential growth after inoculation into fresh medium. As expected, *RPL30* transcripts accumulate rapidly (approximately sevenfold increase by 15 min; Supplementary Fig. S1b). Notably, at this early time point we observed a roughly threefold increase in Ifh1 URS association, whereas both Fhl1 and Rap1 association remained constant. These data reinforce the notion that Ifh1 binding at RP genes is associated with (and possibly required for) full activation of these genes (see below).

To investigate whether reversible Ifh1 binding is more generally associated with RP gene regulation, we examined the response to rapamycin treatment, which inhibits TOR, a highly conserved phosphatidylinositol-kinase-like growth regulator in yeast¹². As previously reported^{13–15}, we found that rapamycin causes a rapid drop in the mRNA levels of RP genes (Fig. 1e). Notably, Ifh1 association with three different RP gene promoters begins to drop by the earliest time point tested (5 min after rapamycin addition), and is tenfold lower compared with untreated cells by 30 min. This drop in Ifh1 promoter association precedes any measurable change in RP gene mRNA levels (perhaps by as much as 10 min), suggesting that it may be a cause of transcriptional downregulation. Notably,

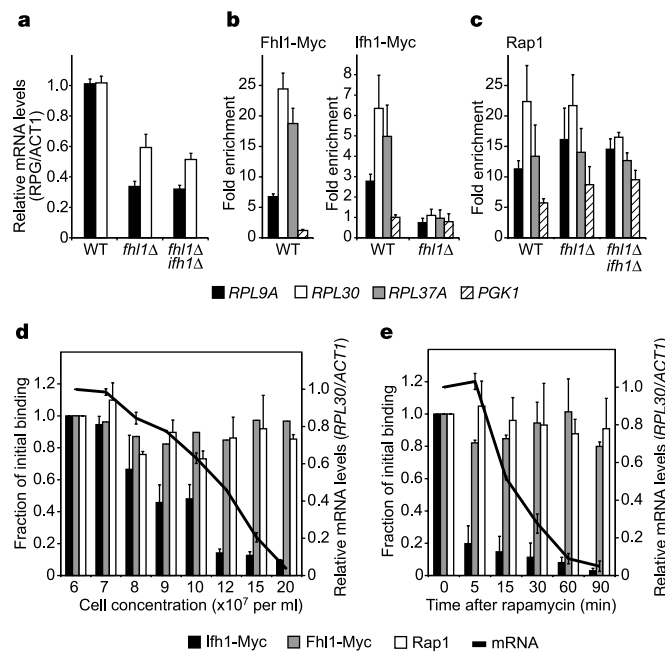


Figure 1 Ifh1, Fhl1 and Rap1 binding at RP gene (RPG) promoters, and mRNA levels during logarithmic growth, before the diauxic shift or after TOR inhibition. **a**, *RPL9A* and *RPL30* transcript levels relative to actin in wild-type (WT), *fhl1Δ* and *fhl1Δ ifh1Δ* strains (logarithmic growth: 2×10^7 cells ml⁻¹). **b**, Promoter association of Fhl1- and Ifh1-Myc at three RP gene promoters and the glycolytic gene *PGK1* in logarithmic wild-type and *fhl1Δ* strains. Mean values and standard deviations (s.d.) of three independent experiments are shown. **c**, Binding of Rap1 in wild-type, *fhl1Δ* and *fhl1Δ ifh1Δ* strains, as in **b**. **d**, Diauxic shift. Fhl1- or Ifh1-Myc strains were processed for ChIP and mRNA

analysis at the indicated cell concentrations (bottom). Binding for each factor at the initial time point (fold enrichment; see **b**, **c**) was set to 1. Three independent ChIP reactions and mRNA measurements for each point were performed (mean values and s.d. for one such experiment are shown). **e**, TOR inhibition. Fhl1- or Ifh1-Myc strains were grown to 2×10^7 per ml, when rapamycin was added to 200 ng ml⁻¹ ($t = 0$). Preparation and analysis of samples was as in **d**, and data shown are the average and s.d. of three independent experiments.

and like the diauxic shift, neither Rap1 nor Fhl1 promoter binding appear to change after TOR inhibition, and total Ifh1 protein levels are unchanged at the time that the protein disappears from the RP gene promoters (Supplementary Fig. S1a).

To determine whether Fhl1 is directly involved in recruiting Ifh1 to RP genes, we fused parts of Fhl1 to the Gal4 DNA-binding domain (GBD). We then asked whether tethering of these GBD–Fhl1 hybrids (Fig. 2a) to a promoter containing Gal4-binding sites, where Fhl1 is normally not bound, would be sufficient to recruit Ifh1 there. ChIP analysis showed that this is indeed the case (Fig. 2b). Ifh1 binding at the *GAL7* promoter in cells expressing GBD–Fhl1 hybrids is quantitatively similar to that observed at native RP gene promoters (data not shown), suggesting that Fhl1 is sufficient for robust Ifh1 recruitment at an ectopic site. Significantly, we found that the forkhead-associated (FHA) domain of Fhl1, and perhaps a small amount of flanking sequence, is sufficient for Ifh1 recruitment, whereas all hybrids tested that lack this domain fail to recruit Ifh1. Furthermore, GBD–Fhl1-mediated Ifh1 recruitment is sensitive to rapamycin, suggesting that a primary downstream effect of TOR inhibition may be to interfere with the ability of Ifh1 to associate with Fhl1 at RP gene promoters. Finally, although recruitment of Ifh1 is consistently associated with activation of the *GAL7-lacZ* reporter (and lack of recruitment is associated with no activation) there is no strict quantitative relationship between the amount of Ifh1 binding and the extent of activation (Fig. 2c). The carboxy terminus of Fhl1, and to a lesser extent the FH domain, appear to have a repressive function in this context.

To ask whether the Fhl1–Ifh1 interactions described above at three RP gene promoters are common to most, or all, RP genes, we conducted a chromosomal binding analysis of both Ifh1 and Fhl1 through hybridization of immunoprecipitated DNA to yeast intergenic microarrays^{6,16,17}. As reported previously⁸, we found that Fhl1 chromatin binding *in vivo* is highly specific for RP genes. We found the same to be true for Ifh1, the chromosomal binding sites of which remarkably overlap those found for Fhl1 (Supplementary Fig. S2a, b). Fhl1 and Ifh1 bound to 53% (72) and to 45% (61) ($P < 0.001$) of the 137 RP gene promoters, respectively. Moreover, 34% (46) of RP gene promoters were bound by both factors. Owing to the stringency of our analysis and technical limitations, we believe that these data actually underestimate the co-localization of these factors. We repeated our analysis with cells that had been

treated with rapamycin before crosslinking, and found a markedly different result for Ifh1 binding, as predicted by our study of three specific RP genes. Specifically, rapamycin treatment reduced binding to background levels at all but one RP gene, and no new sites of Ifh1 binding were identified (Supplementary Fig. S2 and Supplementary Table 1). As expected from our initial studies, we observed very little effect of rapamycin on global Fhl1 binding.

We next asked whether Ifh1 functions as a direct transcriptional activator of native RP genes, as the above results strongly imply. To address this question we constructed a strain in which the endogenous *IFH1* gene is controlled by the galactose-inducible *GAL1* promoter. This strain grows slowly and contains very low levels of Ifh1 protein on medium containing glucose (which represses

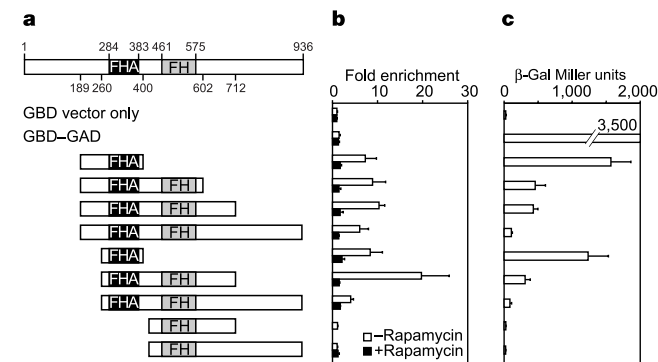


Figure 2 Rapamycin-sensitive recruitment of Ifh1 to an ectopic site (*GAL7*) via the FHA domain of GBD–Fhl1 hybrids. **a**, Schematic representation of native Fhl1 (ref. 28) and GBD–Fhl1 hybrid proteins. Numbers above the bar indicate amino acid positions of domain boundaries; those below indicate the fusion endpoints of GBD–Fhl1 hybrids. **b**, Ifh1–Myc binding at *GAL7* as measured by ChIP in cells growing exponentially or 90 min after rapamycin treatment. Cells express control (GDB alone or GBD–GAD) or GBD–Fhl1 hybrid proteins, as indicated in **a**. The mean values and s.d. of three independent experiments are shown. **c**, β -Galactosidase activity in cells expressing the proteins indicated in **a**.

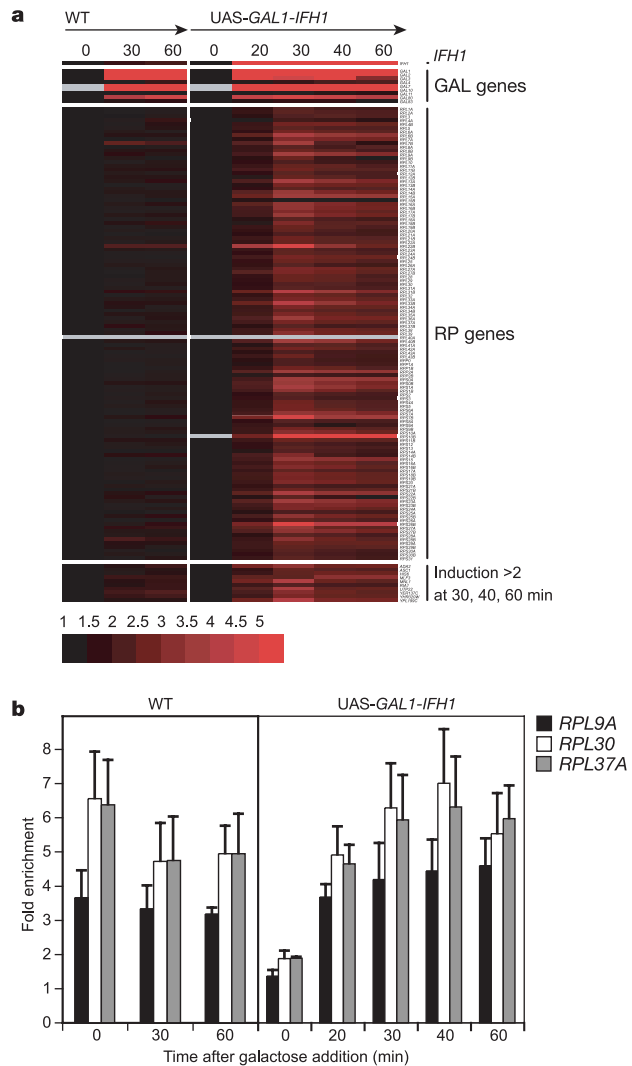


Figure 3 Ifh1 directly activates RP gene transcription. Isogenic wild-type and UAS-*GAL1-IFH1* cells were grown to 2×10^7 cells ml^{-1} in YPLG medium (containing lactic acid and glycerol) and $t = 0$ samples collected. Galactose was then added to 2% and samples collected at the indicated time points (in minutes). **a**, mRNA expression ratios of induced genes relative to the initial YPLG culture ($t = 0$), as measured by microarray analysis, are shown for each time point. Genes specifically induced in UAS-*GAL1-IFH1* cells (>twofold) at $t = 30, 40$ and 60 were grouped. Red indicates >onefold increase (induction) and grey signifies that no reliable measurement was obtained. Functional classes of genes are indicated, except from the ten genes grouped at the bottom (see text and Supplementary Fig. S3 for details). **b**, Ifh1–Myc binding to three RP gene promoters (*RPL9A*, *RPL30* and *RPL37A*) for each time point as measured by ChIP. The mean values and s.d. of three independent experiments are shown. See Supplementary Information for further details.

UAS-*GAL1-IFH1*), but grows normally on galactose (inducing) medium (data not shown). We examined, by genome-wide microarray analysis, the effect of inducing *IFH1* expression in this strain by shifting cells from non-inducing (lactic acid and glycerol) to inducing medium. Nearly all mRNAs of RP genes were detectably upregulated only 20 min after galactose addition, and by 30 min were typically two- to fivefold induced, in all cases exceeding the modest induction observed for some RP genes in the control (*IFH1* wild type) strain (Fig. 3a). In the three cases examined directly by ChIP, this increase in RP gene transcript was associated with increased *Ifh1* promoter binding, again unlike the control strain where *Ifh1* binding remained constant (Fig. 3b). Because the RP genes were among the primary targets after induction of *Ifh1*, we conclude that *Ifh1* is a direct transcriptional activator of these genes. Only ten other Pol II protein-encoding genes were induced as rapidly and as strongly as the RP genes (Fig. 3a). Among these genes *UTP22*, *ASC1* and *RIA1* are bound by both Rap1 and Fhl1 (ref. 8; see also Supplementary Fig. S2) and are involved in ribosome biogenesis. Our data thus point to a possible role for *Ifh1* in coordinating RP gene activation with other key steps in ribosome biogenesis.

The results reported here support a simple model in which normal RP gene expression under favourable growth conditions requires recruitment of the essential (co)activator *Ifh1* to RP gene promoters through an interaction with the FHA domain of promoter-bound Fhl1. This model shares several features with that proposed to explain the action of two other FH proteins, Fkh1 and Fkh2, together with the essential protein Ndd1 in activation of the 'CLB2 cluster' of G2/M-specific genes^{18,19}. One curious property of both systems is that the requirement of the co-activator for cell growth (*Ifh1* here; Ndd1 for the Fkh1/2 system) can be bypassed by deletion of the DNA-binding recruitment factor¹⁰. In cells lacking *Ifh1*, Fhl1 may repress RP gene transcription, either alone or with a co-repressor, and thus block cell growth. In the absence of both proteins, activation of RP genes is low but sufficient to permit cell growth.

Our data also imply that the Fhl1-*Ifh1* interaction constitutes a major regulatory node in the system: both glucose (possibly through the RAS/protein kinase A pathway) and TOR activity are required for full recruitment of *Ifh1* to RP gene promoters. Significantly, inactivation of either one of these two major growth stimulatory pathways results in a rapid loss of *Ifh1* promoter binding, which is followed by a large drop in RP gene mRNA levels. The mechanism(s) by which this occurs are unknown. The fact that *Ifh1* is recruited by the FHA domain of Fhl1 (Fig. 2b), and that FHA domains in various other proteins are phospho-peptide-binding motifs²⁰, suggests that *Ifh1* recruitment at RP gene promoters may be sustained by active, reversible phosphorylation of a putative Fhl1-binding domain in *Ifh1*. Whether this occurs through the direct action of protein kinase A, TOR, or both kinases, is at present unknown.

The findings reported here do not exclude the possibility that other important control mechanisms act directly at RP gene promoters. Indeed, the coordinated downregulation of RP genes under a wide range of stress conditions, and the importance to the cell of this regulation from an energetic standpoint¹, suggests that multiple mechanisms are probably involved. An obvious additional candidate for regulatory input at RP gene promoters is Rap1 (ref. 7, 21), which could function in promoting proper Fhl1 binding, or in the recruitment of the *Esal* histone acetyltransferase and/or the TAF-containing form of TFIID²²⁻²⁴. Finally, the recently identified growth regulator Sfp1 is an additional candidate regulator of RP genes²⁵⁻²⁷. An understanding of RP gene regulation will provide a detailed picture of how the yeast cell integrates various growth signals at the level of the promoter. This knowledge should help to

dissect further the signal pathways that regulate ribosome biogenesis and growth in yeast, and is likely to have important implications in more complex multicellular organisms. □

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