The transcription factor Ifh1 is a key regulator of yeast ribosomal protein genes

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Ribosomal protein (RP) genes in eukaryotes are coordinately regulated in response to growth stimuli and environmental stress, thereby permitting cells to adjust ribosome number and overall protein synthetic capacity to physiological conditions[1–5]. Approximately 50% of RNA polymerase II transcription is devoted to RP genes[5]. The transcriptional regulator Rap1 binds most yeast RP promoters[6], and Rap1 sites are important for coordinate regulation of RP genes[7–10]. However, Rap1 is not the specific regulator that controls RP transcription because it also functions as a repressor, and many Rap1-activated promoters are not coordinately regulated with RP promoters[11,12]. Here we show that the transcription factors Fhl1 and Ifh1 associate almost exclusively with RP promoters; association depends on Rap1 and (to a lesser extent) a DNA element at many RP promoters. Ifh1 is recruited to promoters via the forkhead-associated (FHA) domain of Fhl1; the level of Ifh1 associated with RP promoters determines the level of transcription; and environmental stress causes a marked reduction in the association of Ifh1, but not Fhl1 or Rap1. Thus, Ifh1 association with promoters is the key regulatory step for coordinate expression of RP genes.

Yeast cells contain ~150 copies of the ribosomal DNA locus and 137 RP genes that encode one or two copies of each of 78 proteins. Transcription of the RP genes is coordinately regulated in accord with the cellular growth rate in a manner that requires protein kinase A and the TOR pathway[8,13–15]. In addition, RP transcription is rapidly and coordinately downregulated in response to a variety of environmental insults such as heat shock, amino-acid starvation and osmotic shock. Coordinate regulation of RP genes has a major biological impact on the overall protein synthetic capacity and growth of the cell, and 50% of RNA polymerase II transcription in yeast is devoted to RP genes[5].

Despite the biological importance of coordinate regulation of RP genes, information about the mechanism of this regulation is rather incomplete. Approximately 90% of RP promoters contain predicted Rap1 binding sites[5,16], and Rap1 is bound to essentially all such RP promoters *in vivo*[6]. Rap1 sites are important for growth-regulated expression of natural RP promoters[7–9], and a 41-base-pair (bp) fragment containing two Rap1 sites from an RP promoter is sufficient to mediate growth regulation[10]. However, Rap1 also binds and activates many non-RP promoters that are not regulated in the same manner as RP promoters. Furthermore, the Rap1-dependent activator that regulates transcription of RP genes recruits TFIID, whereas the Rap1-dependent activator that regulates transcription of glycolytic genes does not[10]. Taken together, these results strongly suggest the existence of an unknown protein(s) that is specifically involved in the coordinate regulation of RP genes.

An extensive genome-wide analysis of target sites for over 100 yeast DNA-binding transcription factors revealed Fhl1, a protein containing a fork head DNA-binding domain[17], as binding specifically to RP promoters[18]. We confirmed this result by performing chromatin immunoprecipitation (ChIP) on Fhl1 coupled with analysis on microarrays containing essentially all yeast promoter regions (Fig. 1a; Supplementary Table 1). Using a stringent cutoff of fivefold enrichment, we identified 79 targets, 76 of which are RP promoters. At this level of stringency, Fhl1 is almost exclusively specific to RP promoters. It is highly likely that Fhl1 interacts with additional RP promoters, as 80% (103 out of 129) of detectable RP promoter regions are found in the top 5% of the Fhl1-bound targets (Fig. 1a).

We also determined the genome-wide association of Ifh1, a protein that interacts genetically with Fhl1 (ref. 19). The binding profile of Ifh1 is strikingly similar to that of Fhl1, located almost exclusively at RP promoters (Fig. 1a; Supplementary Table 1). Of the 56 targets showing at least fivefold enrichment, 54 were RP promoters, and it is likely that Ifh1 associates with additional RP promoters (Fig. 1a). Remarkably, 94% (51 out of 54) of Ifh1 targets defined in this manner are also Fhl1 targets. Fhl1 and Ifh1 do not bind to non-RP, Rap1-containing promoters or to the ribosomal DNA loci (Fig. 1a, b; data not shown). Ifh1 and Fhl1 do not associate with eight of the nine RP promoters that do not bind Rap1 *in vivo*[6,16]. Thus, Fhl1 and Ifh1 bind almost exclusively to a common set, but not all RP promoters, and binding of these proteins seems to be influenced by Rap1.

Conserved DNA sequence motifs among Fhl1- and Ifh1-bound promoters include the Rap1 binding site, an A-rich stretch implicated in RP transcription[20], and a close match to a sequence

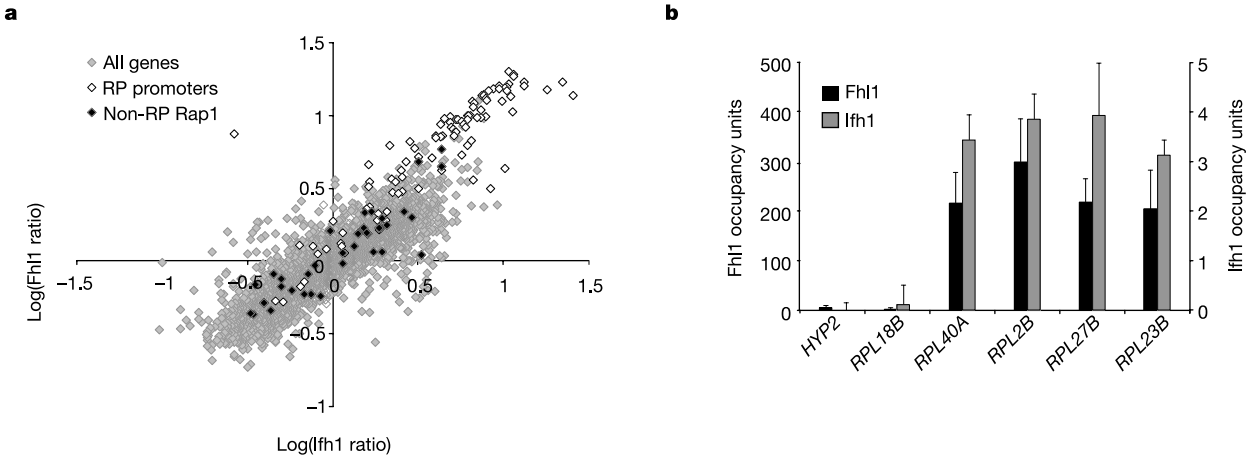

Figure 1 Promoter-specificity of Fhl1 and Ifh1. **a**, Comparison of Fhl1 and Ifh1 ChIP-chip analyses. Log ratios for Fhl1 plotted against log ratios for Ifh1 for every gene represented on the microarrays. Data for RP promoters are shown as white diamonds. Data for Rap1-bound non-RP promoters are shown as black diamonds. **b**, Fhl1 and Ifh1 occupancies at the indicated promoters. Note that *RPL18B* does not show binding of Fhl1 or Ifh1 by microarray analysis and is not bound by Rap1. Error bars reflect the standard deviation of the mean.

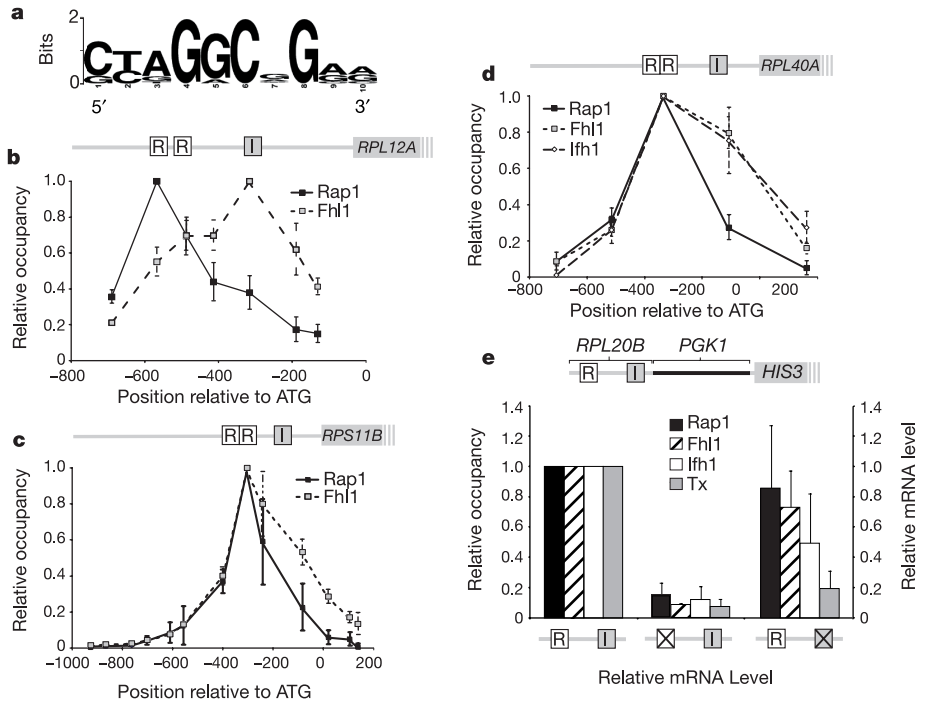

Figure 2 Role of the Rap1 site and the IFHL. **a**, The IFHL motif as derived from the Fhl1 ChIP-chip analysis. **b**–**d**, Occupancy of Rap1 and Fhl1 at various positions across the *RPL12A* (**b**), *RPS11B* (**c**) and *RPL40A* (**d**) promoters, normalized to the highest occupancy value. The positions of predicted Rap1 DNA sites and IFHL motifs are indicated above each graph by 'R' and 'I', respectively. **e**, Relative *HIS3* mRNA levels (Tx; normalized to the wild-type promoter) and Rap1, Fhl1 and Ifh1 occupancy at *RPL20B* UAS-*PGK1* core promoter-*HIS3* open reading frame (ORF) derivatives. Wild-type *RPL20B* UAS (R-I) and derivatives containing mutations in either the Rap1 site (X-I) or the IFHL motif (R-X). Error bars reflect the standard deviation of the mean.

element, 'Motif 213', recently identified in a subset of RP promoters by a computational analysis designed to predict gene expression using DNA sequence information[21] (Fig. 2a; Supplementary Fig. 1). Analysis of RP promoters that do not display fivefold enrichment of Fhl1 and Ifh1 identifies the Rap1 site and the A-rich stretch, but not the third motif. We therefore name the third motif IFHL, and a search of this motif across the yeast genome shows that it is highly over-represented at RP gene promoters ($P < 0.001$). Although the presence of the IFHL motif correlates with binding of Ifh1 and Fhl1 *in vivo*, there is not a strict one-to-one correspondence because 38 of the Fhl1 targets do not contain a clear IFHL motif.

Mapping of Fhl1 and Rap1 binding sites across three RP promoters indicate promoter-specific differences in the relative locations of these proteins (Fig. 2b–d). At *RPL12A*, Rap1 and Fhl1 associate with discrete regions, with Rap1 binding near the two predicted Rap1 sites and Fhl1 binding near the IFHL motif (Fig. 2b). In contrast, Fhl1 binding at *RPS11B* and *RPL40A* matches that of Rap1 at the upstream boundary, but it seems to bind to a broader region that spans the Rap1 sites and the IFHL motif (Fig. 2c, d). This unusual profile of Fhl1 binding at these promoters is significant because in the identical samples Rap1 is localized near the predicted Rap1 sites.

Rap1, Fhl1 and Ifh1 bind a promoter derivative comprising the *RPL20B* upstream region (contains a single Rap1 site and a single IFHL motif), the *PGK1* core promoter and the *HIS3* coding region (Fig. 2e). Mutation of the Rap1 site markedly reduces binding of Rap1, Fhl1 and Ifh1, and transcriptional activity. Mutation of the IFHL motif results in a smaller but significant decrease in the binding of Fhl1 and Ifh1, and it also causes a significant decrease in transcription. These observations indicate that Rap1 is required for Fhl1 and Ifh1 binding and that the IFHL motif contributes to binding of Fhl1 and Ifh1. In addition, they suggest that Fhl1 and Ifh1 are required for maximal transcription from an RP promoter.

The mutational analysis and the promoter-specific Fhl1 binding profiles are interesting in connection with the observation that Fhl1 and Ifh1 bind poorly at RP promoters lacking Rap1 sites, but can bind well at a number of RP promoters lacking a good match to the IFHL motif. RP promoters often contain multiple Rap1 sites or a single Rap1 site together with an element strongly resembling an IFHL motif[21]. We suggest that association of Ifh1 and Fhl1 is mediated by interactions (direct or indirect) with Rap1 and/or the IFHL motif. Multiple Rap1 interactions with Fhl1 or Ifh1 minimize the need for the IFHL motif, whereas a protein–DNA interaction between Fhl1, Ifh1 and the IFHL motif minimizes the need for multiple interactions with Rap1. The precise location of Fhl1 and Ifh1 would depend on the quality of the IFHL motif and other sequences in the vicinity of the Rap1 site(s).

We investigated the role of Ifh1 in regulation of the RP genes using a strain in which the only copy of *IFH1* is under the control of the *GAL1* promoter. Galactose-induced expression of *IFH1* specifically increases transcription of several RP genes, but has little or no effect on two non-RP, Rap1-regulated genes (*ENO2* and *HYP2*), and *RPL18B*, whose promoter is not bound by Fhl1 or Ifh1 (Fig. 3a, b). Furthermore, upon galactose induction, Ifh1 associates with all transcriptionally activated promoters tested, whereas it does not associate with *ENO2*, *HYP2* and *RPL18B* (Fig. 3c). Rap1 association with RP and non-RP promoters was not altered significantly upon induction (Supplementary Fig. 2). Thus, Ifh1 is a transcriptional activator that is specific for RP genes that are targets of Ifh1 and Fhl1.

Fhl1 contains a forkhead-associated (FHA) domain that can be functionally replaced by an FHA domain from a transcriptional activator from tobacco[22]. A hybrid protein containing the Gal4 DNA-binding domain and the FHA domain of Fhl1 activates transcription of *GAL7* (Fig. 3d). Furthermore, the Gal4–FHA domain fusion recruits Ifh1 to the *GAL7* promoter, whereas the Gal4 DNA binding domain alone or a Gal4 fusion to the Gcr2 activator does not (Fig. 3e). Thus, the FHA domain of Fhl1 is

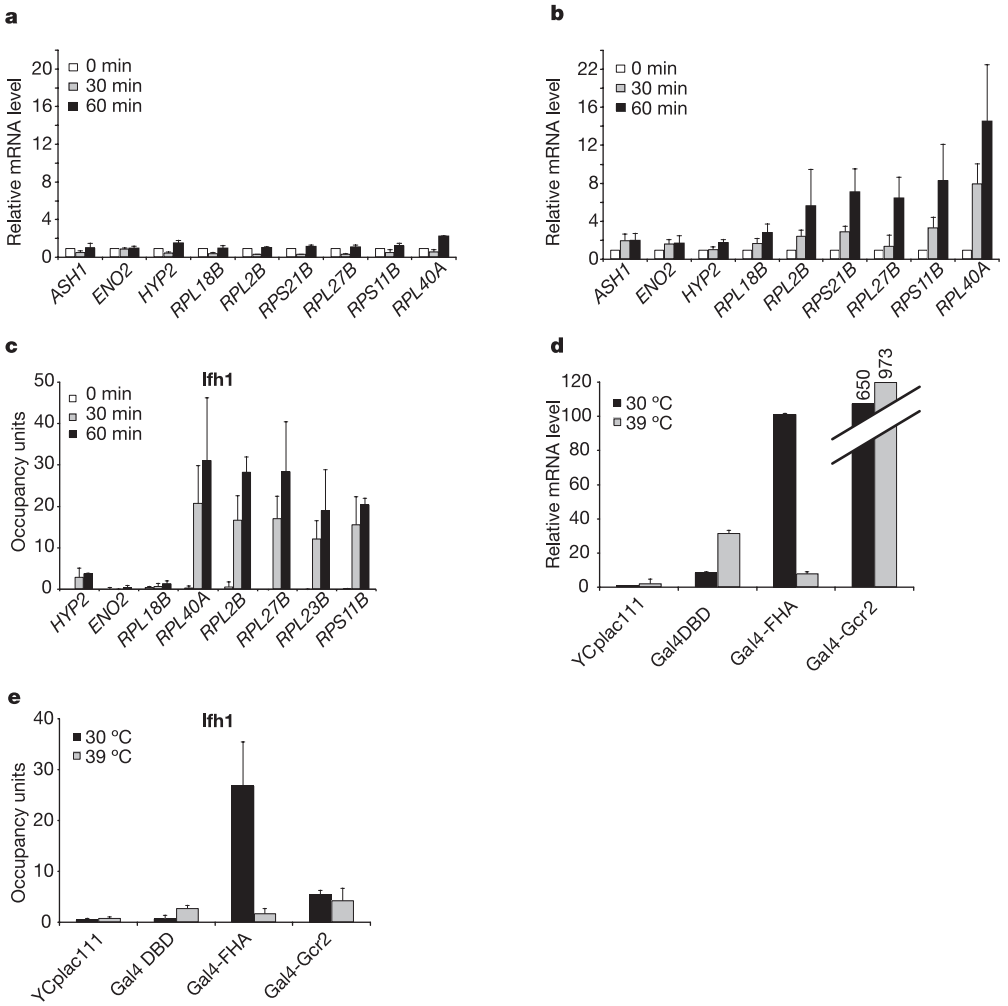

Figure 3 Ifh1-dependent induction of transcription at Ifh1-bound RP genes. **a**, Relative RNA levels of indicated genes 0, 30 and 60 min after galactose addition in a strain expressing a chromosomal copy of *IFH1* under the control of the *IFH1* promoter. **b**, Relative RNA levels of indicated genes 0, 30 and 60 min after galactose addition in a strain expressing a plasmid-borne copy of *IFH1* under the control of the *GAL1* promoter. **c**, Occupancy of Ifh1 at the indicated promoters in the same cells. **d**, *GAL7* mRNA levels in cells containing either empty vector (YCplac111), Gal4 DBD, Gal4-FHA or Gal4-Gcr2, before and after a heat shock. **e**, Ifh1 occupancy at the *GAL7* promoter in the same cells. Error bars reflect the standard deviation of the mean.

sufficient to recruit Ifh1 and activate transcription.

We examined the association of Rap1, Fhl1 and Ifh1 under three conditions of environmental stress: heat shock, osmotic shock and inhibition of the TOR pathway by rapamycin. As expected, these environmental stresses result in decreased RP gene expression (Fig. 4a). Interestingly, the three stress conditions cause a rapid dissociation of Ifh1, but not of Rap1 or Fhl1 association, at RP promoters (Fig. 4b–d) whereas the cellular level of Ifh1 does not decrease (Fig. 4e). The observation that Ifh1 is not required for association of Fhl1 strongly suggests that Rap1 interacts (directly or indirectly) with Fhl1. In addition, heat shock causes a large decrease in *GAL7* transcription (Fig. 3d) and Ifh1 recruitment (Fig. 3e; Supplementary Fig. 3) mediated by the hybrid protein containing the Gal4 DNA-binding domain and the FHA domain of Fhl1. Thus, in the presence of the Gal4–FHA fusion, the *GAL7* promoter responds to environmental stress indistinguishably from RP promoters.

Coordinate regulation of RP genes is of fundamental biological importance as it represents approximately 50% of all RNA polymerase II transcription, and it represents the primary mechanism by which protein synthesis and hence cell growth is regulated appropriately in response to optimal or stressful environmental conditions. Fhl1 and Ifh1 are the first proteins specifically linked to coordinate regulation of RP genes, and regulated association and dissociation of Ifh1 is a key step at which this control is exercised. Ifh1 dissociation, coupled with the short messenger RNA half-life of many RP mRNAs permits the rapid and efficient regulation of RP gene expression. However, Fhl1 and Ifh1 do not seem to associate with some RP promoters that are coordinately expressed, suggesting the existence of alternative regulatory mechanisms. Nevertheless, our results indicate that Fhl1 and particularly Ifh1 are key proteins that regulate most RP promoters in response to growth signals and environmental stress. □

Methods

Yeast strains and DNAs

Two strains were used for ChIP-chip analysis, both derived from BY4727 (ref. 23) with either Fhl1 (strain JTW1) or Ifh1 (strain JTW2) carboxy-terminally epitope-tagged with nine myc epitopes linked to a *TRP1* marker[24]. JTW2 was also used for experiments involving Gal4 derivates binding to the *GAL7* promoter. Gal4 derivates were constructed in plasmid DH021, a derivative of YCplac111 (ref. 25) that contains the Gal4 DNA-binding domain (residues 1–147) amino-terminally tagged with three haemagglutinin (HA) epitopes, expressed from the *EFT2* promoter and upstream of the *CYC1* terminator. The Gal4 DNA-binding domain was fused either to residues 266–400 of Fhl1 (FHA domain) or Gcr2. Derivates of *RPL20B* were constructed in plasmid HPIpV4 containing the *PGK1* core promoter (−246 to −7) upstream of the *HIS3* coding region[10]. The *RPL20B* upstream activating sequence (UAS) (−377 to −245) contains a Rap1 site and an IFHL motif (modified from CTAGGCCGCGG to CTAGGCGGAAG to facilitate cloning with a

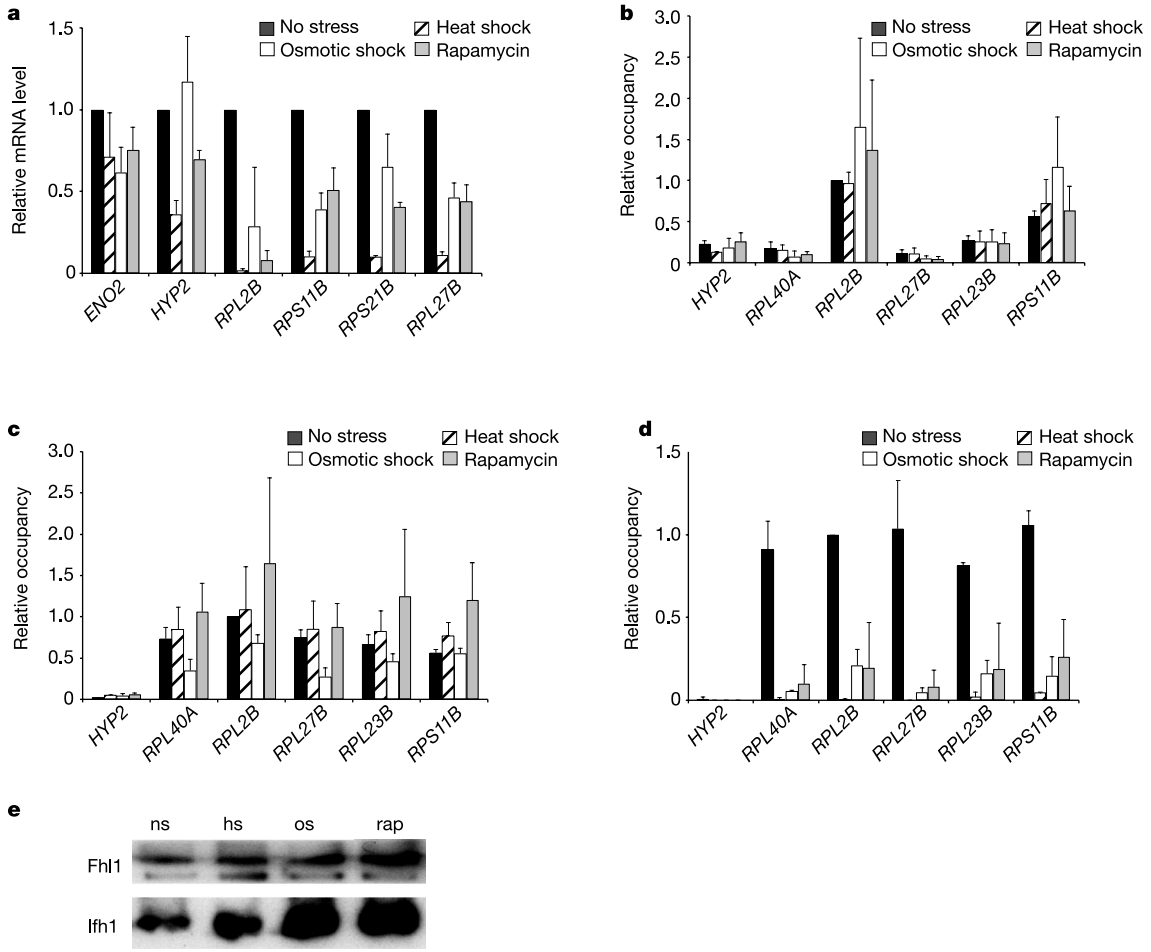

Figure 4 Effect of environmental stress on transcription and binding of Rap1, Fhl1 and Ifh1. **a**, RNA levels of indicated genes under various conditions relative to unstressed cells. **b**, Rap1 occupancy relative to the level at *RPL2B* in unstressed cells. **c**, Fhl1 occupancy relative to the level at *RPL2B* in unstressed cells. **d**, Ifh1 occupancy relative to the level at *RPL2B* in unstressed cells. **e**, Western blot showing the cellular levels of Fhl1 and Ifh1 under conditions of no stress (ns), heat shock (hs), osmotic shock (os) and rapamycin treatment (rap). Equal amounts of cell extract were loaded in each lane. Error bars reflect the standard deviation of the mean.

*Sac*II restriction site). Wild-type Rap1 site: ACCCGTACA; mutated Rap1 site: AAAAGTACA; wild-type IFHL motif: CTAGGCGGAAG; and mutated IFHL motif: CTATTACTCGG. JTW3, which was used for galactose induction of *IFH1*, was created by sporulating a derivative of the diploid BY4743 (ref. 23) that contains one wild-type *IFH1* allele and one deleted *IFH1* allele, and also contains a derivative of plasmid YCPlac33 (ref. 25) that has the *GAL1* promoter driving expression of *IFH1* N-terminally epitope-tagged with three HA epitopes (plasmid JZW1). JTW3 was selected as having *IFH1* expressed solely from plasmid JZW1 (note that this strain grows in medium containing glucose, presumably due to leaky expression of *IFH1*). JTW4, a derivative of JTW1 with Ifh1 C-terminally tagged with a tandem affinity purification (TAP) tag linked to a *URA3* marker, was used for all other experiments. For galactose induction, cells were grown at 30 °C in YP (yeast-peptone)media containing 2% raffinose to an absorbance of 0.7 at 600 nm (A_{600}=0.7), followed by addition of 2% galactose. For all other experiments cells were grown in YP media containing 2% glucose. For experiments involving environmental stress, cells were subjected to the following treatments: shift to 39 °C for 8 min; addition to 0.4 M NaCl for 12 min; addition to 100 nM rapamycin for 20 min.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out with a modified version of a procedure described previously[26]. Cells (A_{600}=0.7) were fixed in 1% formaldehyde for 20 min at room temperature, quenched for 5 min with glycine and lysed with zirconia-silica beads (BioSpec Products) in a mini-bead beater (BioSpec Products). Chromatin was first pelleted by centrifugation and then solubilized by sonication (Branson Sonifier 350, three times, 100% duty, power 5, 30 s for each cycle). Crosslinked chromatin was immunoprecipitated with protein A-sepharose beads (Amersham) and polyclonal antibody against Rap1, monoclonal antibodies against the HA (F7) and myc (9e10, all from Santa Cruz Biotechnology) epitopes, or with IgG-sepharose beads (Amersham). Quantitative polymerase chain reaction (PCR) analyses were performed in real time using 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 Ifh1 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 previously[27]. Values shown are an average of two independent experiments. Conserved DNA motifs were identified using AlignACE[28]. WebLogo was used to generate motif logos[29].

Transcriptional analysis

Total RNA was purified using Qiagen RNeasy columns with DNase I treatment. First-strand cDNA was synthesized using dT_{16}, and quantitative PCR in real time was performed on the resulting first-strand complementary DNA using primers specific to the gene of interest[30]. RNA levels were determined relative to a control gene, *ACT1*.

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