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TOR Regulates Ribosomal Protein Gene Expression via PKA and the Forkhead Transcription Factor FHL1

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Summary

The regulation of ribosome biogenesis in response to environmental conditions is a key aspect of cell growth control. Ribosomal protein (RP) genes are regulated by the nutrient-sensitive, conserved target of rapamycin (TOR) signaling pathway. TOR controls the subcellular localization of protein kinase A (PKA) and the PKA-regulated kinase YAK1. However, the target transcription factor(s) of the TOR-PKA pathway are unknown. We show that regulation of RP gene transcription via TOR and PKA in yeast involves the Forkhead-like transcription factor FHL1 and the two cofactors IFH1 (a coactivator) and CRF1 (a corepressor). TOR, via PKA, negatively regulates YAK1 and maintains CRF1 in the cytoplasm. Upon TOR inactivation, activated YAK1 phosphorylates and activates CRF1. Phosphorylated CRF1 accumulates in the nucleus and competes with IFH1 for binding to FHL1 at RP gene promoters, and thereby inhibits transcription of RP genes. Thus, we describe a signaling mechanism linking an environmental sensor to ribosome biogenesis.

Introduction

Cell growth (increase in cell mass) is controlled in response to nutrients, growth factors, and other environmental conditions. A key component of cell growth control is the regulation of ribosome biogenesis, not only because ribosomes are directly required for growth, but also because ribosome biogenesis is a major consumer of cellular energy. To maintain robust growth in response to favorable conditions, cells synthesize approximately 2000 ribosomes per minute. This requires the coordinated activity of all three RNA polymerases transcribing several hundred genes, including 35S rRNA genes by Pol I, ribosomal protein (RP) genes by Pol II, and 5S rRNA and tRNA genes by Pol III (Jorgensen et al., 2004b; Nomura, 2001; Warner, 1999). Thus, in a growing cell, approximately 95% of total transcription and a large portion of total cellular energy are dedicated to ribosome biogenesis, underscoring the need for tight regulation of ribosomal genes in response to nutrient and energy conditions. Despite the fundamental importance of this regulation, it is poorly understood.

The highly conserved protein kinase TOR and its namesake signaling network control cell growth in response to nutrients (Gingras et al., 2001; Harris and Lawrence, 2003; Jacinto and Hall, 2003; Schmelzle and Hall, 2000). As a central controller of cell growth, TOR regulates several growth-related processes including ribosome biogenesis. In *S. cerevisiae*, RNA Pol I- and RNA Pol III-dependent transcription and 35S rRNA processing are strongly reduced upon TOR inhibition by rapamycin treatment. Moreover, rapamycin treatment leads to a rapid and pronounced downregulation of RNA Pol II-dependent RP genes (Hardwick et al., 1999; Powers and Walter, 1999; Schmelzle et al., 2004; Shamji et al., 2000; Zaragoza et al., 1998). Thus, TOR broadly controls ribosome biogenesis. Rapamycin-induced downregulation of ribosomal genes (RP, rRNA, and tRNA genes) is suppressed by activation of the RAS-cAMP-PKA pathway. Furthermore, TOR controls the subcellular localization of PKA and the PKA-regulated kinase YAK1. These results suggest that TOR controls ribosome biogenesis via the RAS-cAMP-PKA pathway (Schmelzle et al., 2004). However, the target transcription factor(s) and regulatory mechanism by which TOR-PKA controls ribosomal gene expression are unknown.

Several transcription factors have been implicated in the regulated expression of ribosomal genes. The rapamycin-sensitive transcription factor RRN3 mediates Pol I-dependent transcription in yeast and mammalian cells (Claypool et al., 2004; Fath et al., 2001; Mayer et al., 2004; Nomura, 2001; Peyroche et al., 2000). Upon rapamycin treatment, RRN3 dissociates from rRNA promoters and exits the nucleolus. The multifunctional ABF1 and RAP1 proteins directly control Pol II-dependent RP gene expression, but the binding of at least RAP1 to RP promoters is unaffected by nutrient limitation or rapamycin treatment (Reid et al., 2000; Rohde and Cardenas, 2003). The two histone H4 modifying factors ESA1, a histone acetylase subunit of NuA4, and RPD3, a histone deacetylase subunit of the RPD3-SIN3 complex, have been implicated in the activation and repression of RP genes, respectively. ESA1 binds and activates RP genes under good nutrient conditions, but is rapidly released from RP gene promoters upon starvation or TOR inactivation by rapamycin (Reid et al., 2000; Rohde and Cardenas, 2003). Conversely, RPD3 binds and inhibits RP and 35S rRNA genes upon TOR inactivation (Humphrey et al., 2004; Rohde and Cardenas, 2003; Tsang et al., 2003). Recently, the transcription factor SFP1 has also been shown to bind and regulate RP gene promoters in a TOR-dependent manner (Marion et al., 2004; Jorgensen et al., 2004a). The mechanism by which these factors are controlled either directly or indirectly by TOR is unknown.

A genome-wide transcription factor binding study recently revealed that the Forkhead-like, presumed transcription factor FHL1 is highly enriched at yeast RP gene promoters (Bar-Joseph et al., 2003; Lee et al., 2002). FHL1 was originally discovered as a multicopy suppressor of a mutation in RNA Pol III. An *FHL1* deletion confers a very slow growth phenotype and a defect in 35S rRNA processing (Hermann-Le Denmat et al., 1994). Furthermore, FHL1 interacts with the Pol I transcription factor

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HMO1 (Ho et al., 2002; Ito et al., 2001). These findings suggest that FHL1 is a potential regulator of ribosome biogenesis and possibly a target of TOR signaling. The essential protein IFH1 (*i*nteracting with *F*ork*h*ead *1*) was identified as a weak multicopy suppressor of an *FHL1* deletion (Cherel and Thuriaux, 1995), but the function of IFH1 and the significance of its genetic interaction with FHL1 are unknown.

In this study, we show that regulation of RP gene transcription via TOR and PKA involves the Forkhead factor FHL1 and the two cofactors IFH1 (a coactivator) and CRF1 (a corepressor). TOR controls RP gene transcription by maintaining CRF1 in the cytoplasm. Upon TOR inhibition, phosphorylated CRF1 rapidly translocates into the nucleus, competes with IFH1 for binding to FHL1 at RP gene promoters, and thereby inhibits transcription of RP genes. Importantly, CRF1 is phosphorylated in vivo and in vitro by the TOR- and PKA-regulated kinase YAK1. Thus, we describe a novel pathway from a nutrient sensor to the regulated expression of ribosomal genes.

Results

FHL1 Interacts with IFH1 and CRF1

To understand how the TOR-PKA pathway regulates expression of ribosomal genes, we focused on the presumed transcription factor FHL1 as a candidate target. To investigate the function and regulation of FHL1, we first performed a yeast two-hybrid screen to identify potential FHL1 cofactors. The N-terminal, middle, and C-terminal regions of FHL1 were used separately as bait (Figure 1A). The middle (M) region of FHL1 repeatedly and exclusively identified two interactors (each $>$10 times), IFH1 and the previously uncharacterized open reading frame YDR223W (Figure 1A). These interactions were confirmed by coimmunoprecipitation, as described below. YDR223W was named CRF1 (*cor*epressor with *F*HL*1*), for reasons also described below. A more detailed mapping of the interaction site in FHL1 revealed that residues 270–450 of FHL1 mediate the binding to IFH1 or CRF1 (Figure 1B). This region contains a conserved Forkhead-associated (FHA) domain (amino acids 300–450). FHA domains mediate protein-protein interactions in functionally diverse proteins in pro- and eukaryotes (Durocher and Jackson, 2002). Substitution of FHL1 glycine 303, a residue conserved in all FHA domains, to alanine abolished the interaction with both IFH1 and CRF1. The above results indicate that the FHA domain of FHL1 is necessary and sufficient for binding to IFH1 and CRF1 (Figure 1B). The DNA binding, Forkhead homology (FH) domain (also called FOX domain) of FHL1 was not required for the interaction with IFH1 or CRF1 (Figure 1B).

Inspection of the many isolated *IFH1* and *CRF1* prey plasmids delineated the regions in IFH1 and CRF1 required for interaction with FHL1. IFH1 and CRF1 interacted with FHL1 via an internal region common to both proteins. We refer to this homologous region as the *F*ork*h*ead-*b*inding (FHB) domain. The FHB domains fall within amino acids 644–800 of IFH1 (1086 total residues) and amino acids 279–467 of CRF1 (467 total residues). The two FHB domains display 59% identity over a core region of 69 amino acids (IFH1 amino acids 673–741, CRF1 amino acids 340–408) (see Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/119/7/969/DC1/). The FHB domains of IFH1 and CRF1 interacted with the FHA domain of FHL1 (Figures 1B and 1C). The FHB domains of IFH1 and CRF1 failed to interact with the FHA domain of the yeast Forkhead factor FKH1, as assessed by a two-hybrid assay, indicating that the FHB domains of IFH1 and CRF1 bind specifically to the FHA domain in FHL1 (data not shown). In summary, FHB domains in IFH1 and CRF1 bind the FHA domain in FHL1.

TOR Inversely Regulates the Binding of IFH1 and CRF1 to FHL1

To investigate if FHL1 is a target of TOR signaling, we examined if TOR regulates the interaction of IFH1 or CRF1 with FHL1 (Figure 2A). The interactions were assessed using a coimmunoprecipitation assay and extracts from rapamycin-treated or -untreated cells expressing epitope-tagged versions of FHL1 and IFH1 or CRF1 (see Experimental Procedures). FHL1 and IFH1 coimmunoprecpitated, but only in extracts from rapamycin-untreated cells. Conversely, FHL1 and CRF1 also coimmunoprecipitated, but only in extracts from rapamycin-treated cells. Thus, TOR promotes the FHL1-IFH1 interaction and inhibits the FHL1-CRF1 interaction in vivo, suggesting that FHL1 is indeed a target of TOR signaling. Furthermore, the finding that the binding of IFH1 and CRF1 to FHL1 appears to be mutually exclusive, combined with the earlier finding that a domain (FHB) common to both IFH1 and CRF1 binds the same domain (FHA) in FHL1, suggests that IFH1 and CRF1 compete with each other for binding to FHL1. The above results also confirm the interactions of FHL1 with IFH1 and CRF1 originally detected with the two-hybrid assay.

TOR Inversely Regulates FHL1-Dependent Recruitment of IFH1 and CRF1 to RP Gene Promoters

To investigate the functional consequence of IFH1 and CRF1 binding to FHL1, we examined the ability of FHL1, IFH1, and CRF1 to bind an RP gene promoter. Binding of FHL1, IFH1, and CRF1 to the *RPL30* promoter was examined in rapamycin-treated and -untreated cells using a chromatin-immunoprecipitation (ChIP) assay (see Experimental Procedures) (Figure 2B). FHL1 bound to the *RPL30* promoter in both rapamycin-treated and -untreated cells, confirming a previous report that FHL1 is constitutively bound to RP gene promoters (Bar-Joseph et al., 2003). IFH1 bound to the *RPL30* promoter but only in rapamycin-untreated cells. Conversely, CRF1 bound to the *RPL30* promoter only in rapamycin-treated cells. The inversely regulated binding of IFH1 and CRF1 to the *RPL30* promoter reflects the binding of these two proteins to FHL1, suggesting that the binding of IFH1 and CRF1 to the promoter is FHL1-dependent. To investigate if the binding of IFH1 and CRF1 to the RP gene promoter is FHL1-dependent, we examined promoter binding of IFH1 and CRF1 in an *FHL1* deletion (*fhl1*Δ) strain. Binding of neither IFH1 nor CRF1 to the *RPL30* promoter was detected in the *fhl1*Δ strain either treated or untreated with rapamycin, indicating that IFH1 and CRF1 require FHL1 for promoter binding (Figure 2B).

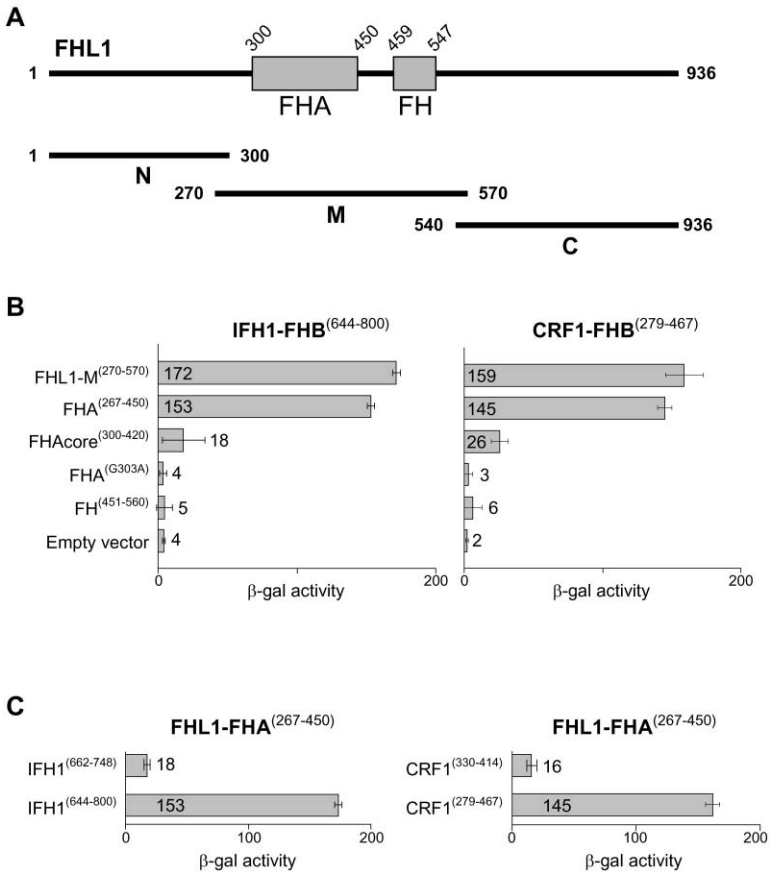

Figure 1. FHA Domain of FHL1 Binds the FHB Domains of IFH1 and CRF1

(A) FHL1 bait constructs used in the yeast two-hybrid screen. IFH1 and CRF1 (alias YDR223W) were isolated as interactors with the middle (M) fragment of FHL1.

(B) The Forkhead-associated (FHA) domain of FHL1 interacts with the FHB domains of IFH1 and CRF1. Different regions of FHL1 were assayed for interaction with the FHB domain of IFH1 (IFH1$^{(644–800)}$) and the FHB domain of CRF1 (CRF1$^{(279–467)}$). The regions of FHL1 that were assayed for interaction were FHL1-M$^{(270–570)}$, FHA$^{(267–450)}$, FHAcore$^{(300–420)}$, FHA$^{(267–450)}$ containing the G303A mutation (FHA^{G303A}), and FH$^{(451–560)}$.

(C) Highly conserved core region of IFH1 and CRF1 FHB domains is not sufficient for interaction with the FHL1 FHA domain. All interactions were assessed with the two-hybrid assay, and quantified using a β-galactosidase (*lacZ*) reporter.

Thus, by controlling the FHL1-IFH1 and FHL1-CRF1 interactions, TOR controls the binding of IFH1 and CRF1 to RP gene promoters.

To determine if RP promoter binding of IFH1 and CRF1 is regulated in response to a physiologically relevant cue, we examined promoter binding in response to nutrient deprivation, in particular, glucose limitation. Similar to the above results with rapamycin treatment, glucose limitation caused IFH1 dissociation from and CRF1 binding to the *RPL30* promoter (Figure 2B). Thus, TOR appears to control RP promoter binding of IFH1 and CRF1 in response to the carbon source and possibly other nutrient signals.

CRF1 Is a Corepressor with FHL1 of RP Genes

The above finding that CRF1 binds FHL1 and thereby RP gene promoters only in rapamycin-treated or starved cells (i.e., when RP genes are repressed) suggested that CRF1 is a negative regulator of RP genes. To determine if CRF1 is a repressor, we examined expression of several RP genes (*RPL30, RPL19A, RPL16A, RPL9A, RPS26A*, and *RPS11A*) in wild-type and *crf1*Δ cells treated and untreated with rapamycin. In wild-type cells, all RP genes were strongly downregulated within 30 min of rapamycin treatment. However, in *crf1*Δ cells, rapamycin had a minor effect; all RP genes remained highly expressed after rapamycin treatment, although minor repression was still observed (Figures 3A and 3B, and data not shown). The minor repression observed in the rapamycin-treated *crf1*Δ cells could be due to additional, TOR-controlled factors such as SFP1 or RPD3. Interestingly, *fhl1*Δ cells displayed a reduced level of RP gene expression that was not further decreased upon TOR inactivation, indicating that RP genes can be neither activated nor repressed in cells lacking FHL1. A deletion of or a missense mutation (G303A) in the FHA domain of FHL1 caused the same phenotype as observed with a complete *FHL1* null mutation (*fhl1*Δ) (data not shown), suggesting that the interactions mediated by the FHA domain are essential for FHL1 function. The above phenotypes conferred by a *crf1*Δ or *fhl1*Δ mutation were abrogated upon complementation of the mutation with a wild-type, plasmid-borne version of the corresponding gene. The above findings taken together suggest that CRF1 is a corepressor with FHL1 of RP genes.

To investigate further the role of CRF1 in the TOR pathway, we examined if a *CRF1* deletion confers rapamycin resistance (Figure 3C). A *crf1*Δ *gat1*Δ *gln3*Δ strain was generated for this purpose, because the GAT1 and GLN3 transcription factors independently inhibit growth upon TOR inactivation and would thus mask a potential rapamycin-resistance phenotype conferred by *crf1*Δ (Beck and Hall, 1999; Schmelzle et al., 2004). *crf1*Δ conferred pronounced rapamycin resistance in the otherwise rapamycin-sensitive *gat1*Δ *gln3*Δ background. Deletion of *RPD3*, encoding a histone deacetylase previously implicated in RP gene expression, also conferred strong rapamycin resistance in the *gat1*Δ *gln3*Δ background. Deletion of *HOS2*, encoding a functionally unre-

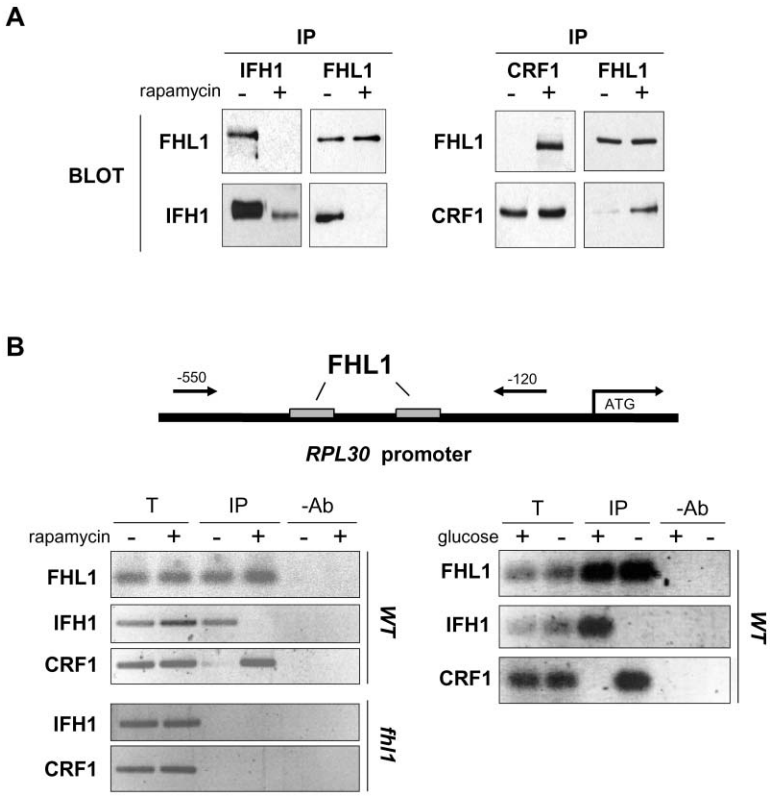

Figure 2. TOR Controls Binding of IFH1 and CRF1 to FHL1 and RP Gene Promoter

(A) TOR promotes the association of IFH1 with FHL1 and inhibits the association of CRF1 with FHL1. Strains DM64-10B (*FHL1-myc IFH1-HA*) and DM63-7A (*FHL1-myc CRF1-HA*) were grown in YPD to midlog phase, treated for 30 min with rapamycin (+) or drug vehicle alone (−), and protein interactions were analyzed by coimmunoprecipitation. Indicated are proteins immunoprecipitated (IP) and subsequently probed by Western blot (BLOT) with antibodies against corresponding epitope tags. A rapamycin-induced reduction in IFH1 protein level was consistently observed.

(B) TOR promotes IFH1 binding to RP gene promoters and inhibits CRF1 binding to RP gene promoters. The binding of IFH1 and CRF1 to RP gene promoters is FHL1-dependent. Cells were grown in YPD to midlog phase, treated for 30 min with rapamycin (+) or drug vehicle alone (−), and processed for chromatin immunoprecipitation as described in Experimental Procedures. For glucose starvation, cells were grown in SD medium containing 0.2% yeast extract and shifted to SC medium containing 0.2% yeast extract, but without glucose, for 1 hr and processed as described above. FHL1 and IFH1 were immunoprecipitated from wild-type (*WT*) strain DM64-10B (*FHL1-myc IFH1-HA*). CRF1 was immunoprecipitated from wild-type strain (*WT*) DM63-7A (*FHL1-myc CRF1-HA*). IFH1 and CRF1 were immunoprecipitated from *FHL1* deletion strains (*fhl1*) DM61-9D (*fhl1 IFH1-HA*) and DM62-5C *(fhl1 CRF1-HA*), respectively. The chromatin immunoprecipitation was probed for the presence of the *RPL30* promoter by PCR with primers that hybridize to positions −550 and −120 relative to the ATG start codon. The *RPL30* promoter contains two putative FHL1 binding sites as judged by comparison to a published putative FHL1 consensus binding site (Cliften et al., 2003; Kellis et al., 2003). T, total chromatin input; IP, ChIP with antibody against the protein listed on the left side of the figure; -Ab, control ChIP in the absence of antibody.

lated histone deacetylase, did not confer rapamycin resistance but did stimulate the appearance of spontaneous rapamycin-resistant mutants (Figure 3C). Thus, as rapamycin resistance is the expected phenotype due to the loss of a repressor in the TOR pathway, both CRF1 and the histone deacetylase RPD3 appear to have negative roles in TOR signaling.

IFH1 Is a Coactivator of RP Genes

The above finding that IFH1 binds FHL1 and thereby RP gene promoters only in rapamycin-untreated cells (i.e., when RP genes are expressed) suggested that IFH1 is a positive regulator of RP genes. To determine if IFH1 is an activator, we could not simply examine RP gene expression in an *ifh1*Δ strain because IFH1 is essential. Instead, we tested if IFH1 can activate a heterologous, GAL4-driven promoter. Wild-type IFH1 or deletion variants of IFH1 lacking the FHB domain (IFH1-ΔFHB) or an N-terminal acidic domain (IFH1-ΔN) were overexpressed in cells coexpressing the FHL1-FHA domain fused to the GAL4 DNA binding domain (FHL1-FHA-GAL4-DBD). A GAL4-driven *lacZ* reporter gene was used as a readout of IFH1-dependent transcriptional activation. Wild-type IFH1 strongly activated *lacZ* expression (Figure 4A). IFH1-ΔFHB failed to activate *lacZ* expression. Interestingly, IFH1-ΔN weakly activated the reporter gene. These results suggest that IFH1 is indeed a transcriptional activator. Furthermore, they suggest that the N-terminal domain of IFH1 may function as an activation domain.

To determine if IFH1 is an activator of RP genes, we examined if IFH1 overexpression prevents CRF1-mediated repression of RP genes. Rapamycin treatment failed to downregulate RP gene expression in cells containing the *IFH1* gene on a multicopy plasmid (Figure 4B). Overexpression of IFH1-ΔFHB did not counter the effect of rapamycin on RP gene expression. Finally, we also used an *IFH1* gene driven by the strong, galactose-inducible *GAL1* promoter to analyze the effect of different IFH1 protein levels on RP gene transcription. Galactose induction (60 min) of *IFH1* led to a significant induction of RP genes (Figure 4C). The above findings taken together indicate that IFH1 is a coactivator with FHL1 of RP genes, and further suggest that IFH1 and CRF1 compete with each other for binding to FHL1.

TOR Negatively Regulates Nuclear Localization of CRF1

To investigate the mechanism by which TOR regulates the competitive binding of the IFH1 coactivator and the CRF1 corepressor to FHL1, we examined the localization of these proteins in rapamycin-treated and -untreated cells. The cellular localization of epitope-tagged FHL1, IFH1, and CRF1 was examined by indirect immu-

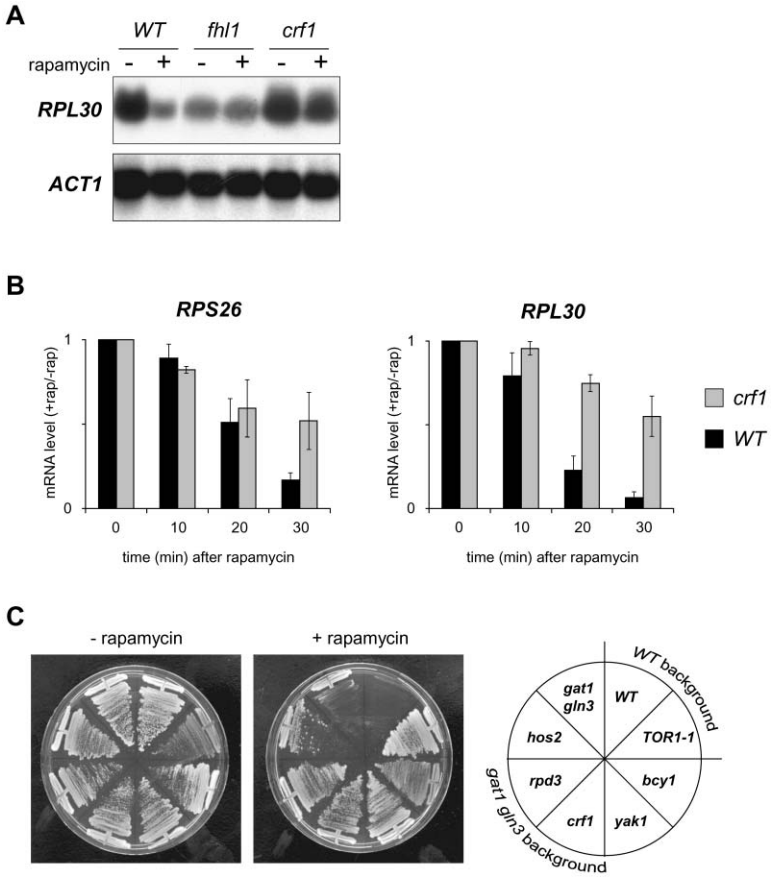

Figure 3. CRF1 Is a Negative Regulator

(A) CRF1 is a repressor of RP gene expression. Northern blot analysis was performed using cells (WT, TB50a; *fhl1*, DM46-4A; *crf1*, DM45-2C) grown in YPD to midlog phase and either treated with rapamycin (+) or drug vehicle alone (−) for 30 min. RNA was extracted and Northern analysis performed using a probe against *RPL30*.

(B) Kinetics of RP gene repression in response to rapamycin. Quantification of relative *RPS26* and *RPL30* mRNA levels from three independent Northern blot experiments with rapamycin-treated and -untreated TB50a (WT) and DM45-2C (*crf1*).

(C) Deletion of *CRF1 (crf1*) or *RPD3* (*rpd3*) confers rapamycin resistance. Deletion of *BCY1* (*bcy1*), *YAK1* (*yak1*) also confers rapamycin resistance as shown previously (Schmelzle et al., 2004). All strains except wild-type (*WT*) and *TOR1-1* contain deletions of both *GAT1* and *GLN3* (*gat1 gln3*). Strains were grown on YPD plates with (+) or without (−) rapamycin (200 ng/ml) for 5 days. Strains were as follow: *WT*, TB50a; *TOR1-1*, DM72-7A; *bcy1*, TS160-6C; *yak1*, TS135-2C; *crf1*, DM76-3B; *rpd3*, DM81-3A; *hos2*, DM80-8C.

nofluorescence on whole fixed cells. Consistent with the above observation that FHL1 is constitutively bound to RP gene promoters, FHL1 was concentrated in the nucleus in both rapamycin-treated and -untreated cells (data not shown). IFH1 was also nuclear under both conditions (data not shown). CRF1, however, was mainly cytoplasmic in rapamycin-untreated cells but rapidly accumulated in the nucleus upon rapamycin treatment (Figure 5A). CRF1 was essentially entirely nuclear within 20 to 30 min of rapamycin treatment. The localization of CRF1 in rapamycin-treated or -untreated *fhl1*Δ cells was as described above for wild-type cells, indicating that CRF1 nuclear localization was not FHL1-dependent (data not shown). As expected from the above finding that CRF1 binding to an RP gene promoter is induced upon glucose starvation, carbon source limitation also induced nuclear localization of CRF1 (see Supplemental Figure S2 available on *Cell* website). Interestingly, CRF1 required its FHB domain for nuclear localization, indicating that the FHB domain has a dual role as an FHL1 interaction domain and as a signal for nuclear import or retention (Figure 5A). Thus, TOR appears to regulate the competitive binding of IFH1 and CRF1 to FHL1 by restricting access of CRF1 to FHL1 in the nucleus.

TOR Controls CRF1 via the RAS-PKA Pathway

Previous findings indicated that TOR controls RP gene expression via the RAS-PKA pathway (Schmelzle et al., 2004). Does TOR control CRF1 via RAS-PKA? To answer this question, we investigated if an activated RAS-PKA pathway prevents rapamycin-induced nuclear localization of CRF1. To activate the RAS-PKA pathway, we knocked out the gene (*BCY1*) encoding the negative regulatory subunit of PKA or introduced an activated allele of *RAS2* (*RAS2*^{Val19}). Rapamycin failed to induce nuclear localization of CRF1 in both *bcy1*Δ and *RAS2*^{Val19} cells (Figure 5B). Rapamycin also failed to induce nuclear translocation of CRF1 in cells containing a rapamycin-resistance *TOR1* (*TOR1-1*) allele. To further investigate a role for the RAS-PKA pathway, we examined CRF1 localization in the temperature sensitive *cdc25-5* and *cdc35-10* mutants. CDC25 is the guanine nucleotide exchange factor required for RAS2 activation, and CDC35/CYR1 is adenylate cyclase required for cAMP production and PKA activation. Upon inactivation of CDC25 and CDC35 by shifting the *cdc25-5* and *cdc35-10* mutants to nonpermissive temperature, CRF1 accumulated in the nucleus (see Supplemental Figure S3 available on *Cell* website). These results suggest that TOR signals to CRF1, and ultimately FHL1, via the RAS-PKA effector pathway. However, we cannot exclude the possibility that CRF1 localization is controlled by PKA in a TOR-independent fashion. Further evidence that TOR signals to CRF1 via PKA is provided by the below finding that CRF1 is controlled by YAK1.

YAK1 Phosphorylates and Activates CRF1

PKA negatively regulates, probably directly, the dual-specificity protein kinase YAK1 (Garrett and Broach,

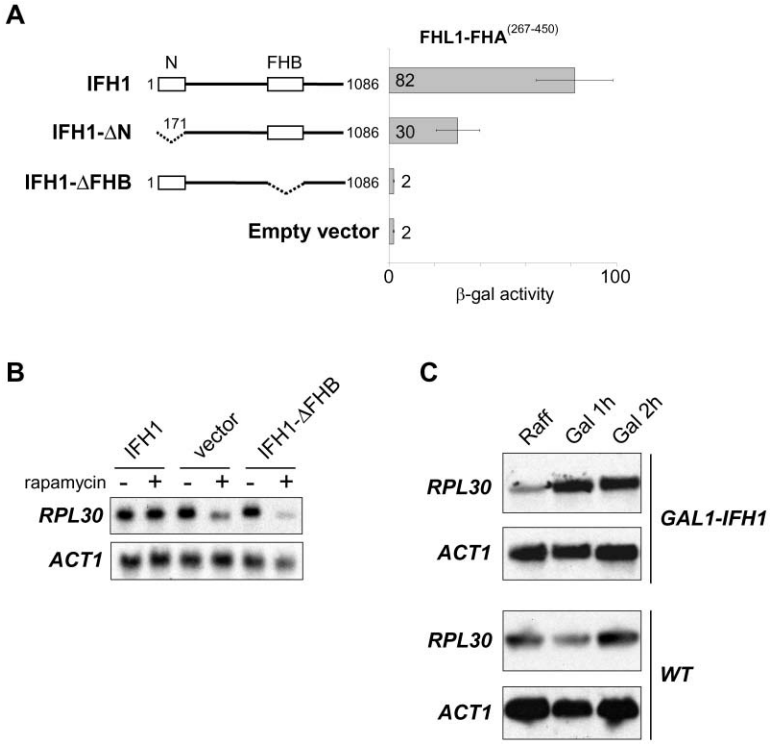

Figure 4. IFH1 Is a Transcriptional Activator

(A) IFH1 activates transcription of a heterologous promoter. IFH1 was recruited to a GAL4-driven, heterologous promoter via interaction with the FHL1-FHA domain fused to the GAL4 DNA binding domain. Strain PJ69-4A, expressing a hybrid protein consisting of the FHL1-FHA domain fused to the GAL4 DNA binding domain (pDM103), was transformed with a plasmid containing full-length *IFH1* (pSA001) or the *IFH1* deletion variant *IFH1*-ΔN (pSA002) or *IFH1*-ΔFHB (pSA003) or the empty vector (pHAC181). Activation was assayed as expression of the *lacZ* reporter gene.

(B) Multicopy *IFH1* counters rapamycin-induced repression of RP genes. Northern blot analysis was performed on wild-type cells (TB50a) harboring a multicopy plasmid containing either full-length *IFH1* (IFH1) or the IFH1-ΔFHB deletion variant. Cells were grown in SD medium to midlog phase and treated with rapamycin or drug vehicle alone for 30 min. RNA was extracted and Northern analysis performed using a probe against *RPL30*.

(C) Transcriptional induction of *IFH1* under control of a heterologous promoter induces RP gene transcription. *IFH1* under control of the strong, galactose-inducible *GAL1* promoter was induced by shifting cells from raffinose (Raff) to galactose (Gal). Strains DM70-1B (*IFH1* driven by the *GAL1* promoter) and TB50a (WT) were grown in YPRaffinose medium to an OD of 0.5, filtered, resuspended in YPGalactose, and incubated for an additional 1 or 2 hr. RNA was extracted and Northern analysis was performed using a probe against *RPL30*.

1989; Griffioen et al., 2001; Moriya et al., 2001; Zappacosta et al., 2002). Furthermore, TOR controls YAK1 localization, and a *YAK1* deletion confers rapamycin resistance (Schmelzle et al., 2004) (Figure 3B). These findings suggest that TOR and PKA may control CRF1 via YAK1. More specifically, they suggest that YAK1 may activate CRF1 upon reduced signaling through the TOR-PKA pathway. To investigate this possibility, we first examined if YAK1 has a role in downregulating RP gene expression upon TOR inactivation. A *YAK1* deletion prevented rapamycin-induced nuclear localization of CRF1 and downregulation of *RPL30*, *RPL16*, and *RPS26* (Figures 5B and 6A). Thus, upon TOR inactivation, YAK1 appears to activate CRF1 and, thereby, to negatively regulate RP gene expression.

To investigate further the control of CRF1 by YAK1, we examined if YAK1 phosphorylates CRF1 in a TOR-sensitive manner. CRF1 was immunoprecipitated from rapamycin-treated and -untreated wild-type and *yak1*Δ cells, and subsequently probed with antiphosphothreonine antibodies. Threonine-phosphorylated CRF1 was detected only in rapamycin-treated, wild-type cells (Figure 6B), suggesting that YAK1 phosphorylates CRF1 in vivo.

To investigate if YAK1 phosphorylates and thus controls CRF1 directly, we examined if purified YAK1 phosphorylates purified CRF1 FHB domain in vitro (see Experimental Procedures). We speculated that YAK1 phosphorylates the Forkhead binding (FHB) domain of CRF1 because this domain mediates the regulated nuclear import and FHL1 binding of CRF1. myc-tagged YAK1 was immunopurified from rapamycin-treated and -untreated yeast cells. GST-tagged CRF1-FHB domain was purified from *E. coli* cells. myc-YAK1 isolated from rapamycin-treated cells, but not from rapamycin-untreated cells, strongly phosphorylated GST-tagged CRF1-FHB but not GST or BSA (Figure 6C and data not shown). A mock purification of untagged YAK1 from either rapamycin-treated or -untreated cells failed to yield any kinase activity toward any of the examined substrates (Figure 6C and data not shown). Furthermore, kinase dead (K398R) YAK1 ($YAK1^{KD}$) purified from rapamycin-treated cells failed to phosphorylate CRF1-FHB, indicating that YAK1 and not a copurifying kinase phosphorylated CRF1-FHB (Figure 6D). $YAK1^{KD}$ also failed to promote nuclear localization of CRF1 in rapamycin-treated cells (data not shown), suggesting that YAK1 kinase activity is required for CRF1 regulation. YAK1 autophosphorylates on one or more tyrosine residues (Moriya et al., 2001), and we also detected rapamycin-induced YAK1 autophosphorylation on tyrosine residues (Figure 6C). These results suggest that YAK1 specifically phosphorylates and thereby activates CRF1 directly. Furthermore, the observation that YAK1 autophosphorylation is rapamycin-induced suggests that the TOR-PKA pathway negatively controls intrinsic YAK1 kinase activity.

Discussion

We have presented several observations indicating that TOR controls the expression of ribosomal protein genes via the Forkhead transcription factor FHL1. Furthermore, our findings provide a molecular mechanism by which

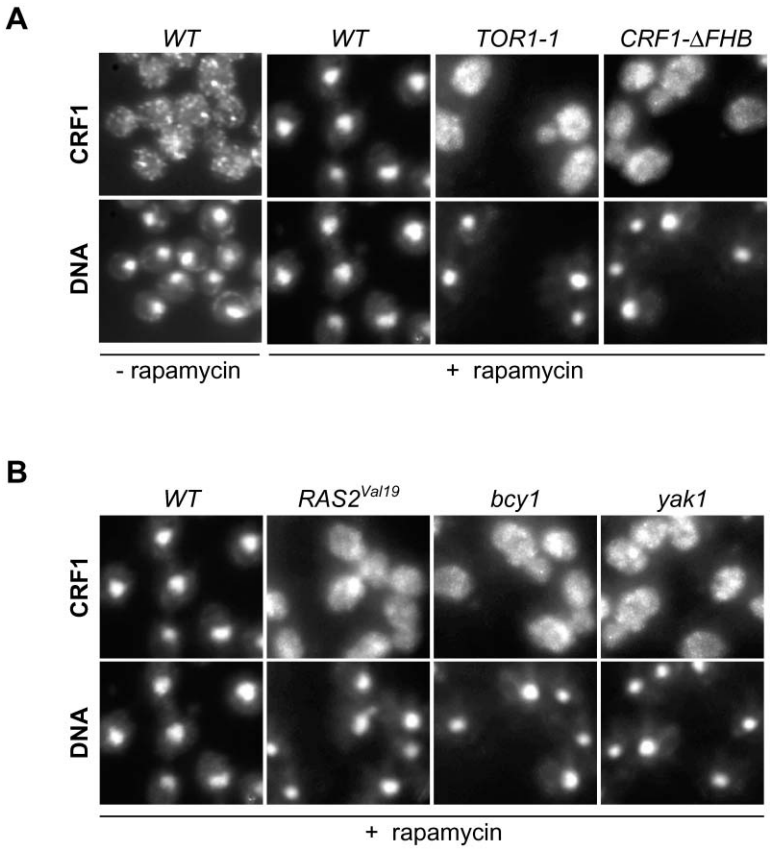

Figure 5. TOR Controls CRF1 Nuclear Localization via the RAS-PKA Pathway

(A) CRF1 accumulates in the nucleus upon TOR inhibition. Cells were grown in SD medium to midlog phase and treated with rapamycin or drug vehicle alone for 30 min. CRF1 localization was examined by indirect immunofluorescence on whole fixed cells. Strains were as follow: *WT*, DM63; *TOR1-1*, DM72-7A; *CRF1-ΔFHB*, DM45-2C harboring plasmid pDM41. In all cases, CRF1 was tagged with the HA epitope and a monoclonal anti-HA antibody was used as a primary antibody for indirect immunofluorescence.

(B) TOR controls CRF1 nuclear localization via PKA and YAK1. Rapamycin-induced CRF1 nuclear accumulation is inhibited by constitutively active RAS-PKA signaling (*bcy1* and $RAS2^{Val19}$) or by deletion of *YAK1* (*yak1*). Cells were grown in SD medium to midlog phase, treated with rapamycin or drug vehicle alone for 30 min, and examined by indirect immunofluorescence. Strains were as follow: *WT*, DM63; *bcy1*, DM79-7B; $RAS2^{Val19}$, DM90-4B harboring plasmid pTS118; *yak1*, DM71-9C.

TOR regulates FHL1. A model summarizing our findings and illustrating this mechanism is shown in Figure 7. According to this model, TOR controls FHL1 via the RAS-PKA-YAK1 effector pathway and the two FHL1 cofactors IFH1, a coactivator, and CRF1, a corepressor. Under favorable growth conditions, TOR signals via PKA to inhibit the kinase YAK1. In the absence of active YAK1, the corepressor CRF1 is cytoplasmic and inactive. Consequently, IFH1 binds FHL1 at RP gene promoters and activates transcription, possibly by recruiting other transcription factors such as the histone acetylase subunit ESA1. The binding of IFH1 to the promoter appears to be a key event in the expression of RP genes since targeting IFH1 to a heterologous promoter is sufficient to activate that promoter. Upon unfavorable growth conditions, TOR and PKA are inactive and, consequently, YAK1 is active. Active YAK1 phosphorylates CRF1. Phosphorylated CRF1 accumulates in the nucleus and binds FHL1, thereby dislodging the coactivator IFH1 from RP gene promoters. The binding of CRF1 to promoter bound FHL1 may lead to repression of RP genes, firstly, by releasing the factors formerly recruited by IFH1 and, secondly, by recruiting new factors such as the histone deacetylase subunit RPD3. Consistent with this notion, an *RPD3* deletion confers rapamycin resistance and, as shown by others, the binding of ESA1 and RPD3 to RP gene promoters is inhibited and induced, respectively, by rapamycin (Humphrey et al., 2004; Rohde and Cardenas, 2003). Thus, our findings provide a pathway from TOR to RP gene expression and, thereby, a molecular link between the growth environment and the regulation of ribosome biogenesis.

The two FHL1 cofactors IFH1 and CRF1 contain a so-called FHB (*F*ork*h*ead *b*inding) domain that binds the FHA domain in FHL1. The mutually exclusive binding of IFH1 and CRF1 to FHL1 determines whether the Forkhead factor, which is constitutively bound to target promoters, acts as a transcriptional activator or repressor. The interplay of two opposing cofactors provides a highly sensitive and fine-tunable regulatory mechanism. The yeast Forkhead factor FKH2, via its FHA domain, recruits the coactivator NDD1 to activate transcription of cyclin genes, but in this case a corepressor remains to be identified (Darieva et al., 2003; Koranda et al., 2000; Reynolds et al., 2003). To our knowledge, a Forkhead cofactor other than IFH1, CRF1 and NDD1 has yet to be described in any organism. Thus, we propose a novel mode of action for a Forkhead factor. Given the advantages of opposing regulatory cofactors and the widespread conservation of the FHA domain, we speculate that the model proposed above for FHL1 will prove to be a common paradigm.

YAK1 is required for the nuclear localization of CRF1. How does YAK1 control the nuclear localization of CRF1? YAK1 phosphorylates CRF1 and this phosphorylation coincides with nuclear accumulation of CRF1. Furthermore, YAK1 phosphorylates the CRF1 FHB domain which is required by CRF1 for both FHL1 binding and nuclear accumulation. Finally, YAK1 like CRF1 also accumulates in the nucleus upon rapamycin treatment

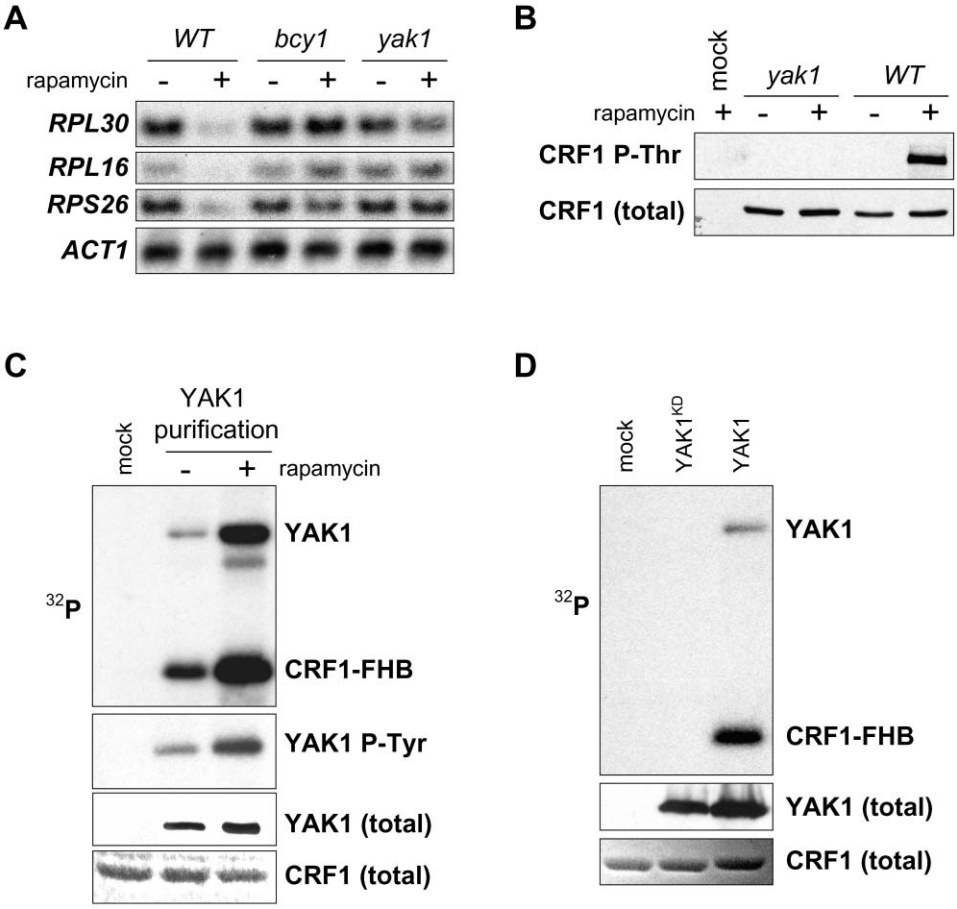

Figure 6. YAK1 Phosphorylates and Activates CRF1

(A) YAK1 is required for RP gene repression upon TOR inhibition. Cells were grown in YPD to midlog phase and treated with either rapamycin or drug vehicle alone for 30 min. RNA was extracted and Northern Blot analysis was performed with probes against the ribosomal protein genes *RPL30*, *RPL16*, and *RPS26A*.

(B) YAK1 phosphorylates CRF1 in vivo. CRF1 was immunoprecipitated from extracts of rapamycin-treated or -untreated *WT* or *yak1* cells and subjected to immunoblotting analysis using anti-HA or anti-phosphothreonine antibodies.

(C) YAK1 phosphorylates CRF1 in vitro. YAK1 autophosphorylates and transphosphorylates CRF1 upon TOR inhibition. YAK1-myc was immunopurified with antibodies directed against the myc epitope (see Experimental Procedures). As a control, a mock purification was performed with cells expressing untagged YAK1. Cells expressing untagged (TB50a) or myc-tagged YAK1 (TS129-8C) were grown in YPD medium and treated with rapamycin or drug vehicle alone for 30 min. The CRF1-FHB domain was expressed in *E. coli* as a GST fusion protein. Equal amounts of the YAK1 kinase preparations and the CRF1-FHB fusion protein were used in each kinase reaction, as described in Experimental Procedures. The reaction products (^{32}P-labeled YAK1 and CRF1-FHB) were detected by autoradiography. YAK1 autophosphorylation (YAK1 P-Tyr) was also detected with antiphosphotyrosine antibody. Total YAK1-myc (YAK1) added to the reactions was detected by immunoblotting. Total GST-CRF1-FHB (CRF1) was detected by Coomassie blue stain.

(D) YAK1 kinase activity is required for CRF1 phosphorylation in vitro. *yak1* cells (TS127-1A) were transformed with plasmid pDM76 (YAK1) or pDM72 (YAK1^{KD}), grown in SD medium, and treated with rapamycin for 30 min. YAK1-HA and YAK1^{KD}-HA (K398R) were immunopurified with antibody directed against the HA epitope (see Experimental Procedures). Equal amounts of the YAK1 kinase and the CRF1-FHB fusion protein were used in each kinase reaction, as described in Experimental Procedures. Otherwise experiment was performed as in 6C.

(Schmelzle et al., 2004). These observations suggest that YAK1-mediated phosphorylation of CRF1 enhances FHL1 binding and, thereby, nuclear retention of CRF1. However, CRF1 still accumulates in the nucleus in the absence of FHL1 (in an *fhl1*Δ strain). Furthermore, CRF1 still interacts with FHL1 in the two-hybrid system without rapamycin treatment (i.e., without YAK1 activation); providing CRF1 with a heterologous nuclear localization signal, as in the two-hybrid system, is sufficient to obtain an interaction with FHL1. These findings suggest that YAK1 stimulates CRF1 nuclear import rather than retention. It remains to be determined whether the phosphorylation of CRF1 stimulates the binding of CRF1 to FHL1, to a factor mediating nuclear import, or to both. The significance of YAK1 nuclear accumulation also remains to be determined.

Ribosome biogenesis requires the coordinated activity of all three RNA polymerases. How is Pol II-dependent transcription of RP genes coordinated with Pol I- and Pol III-dependent transcription? Since the TOR-PKA signaling pathway broadly controls ribosome biogenesis by regulating all three RNA polymerases (Powers and Walter, 1999; Schmelzle et al., 2004; Zaragoza et al., 1998), FHL1, IFH1, and CRF1 may also regulate Pol

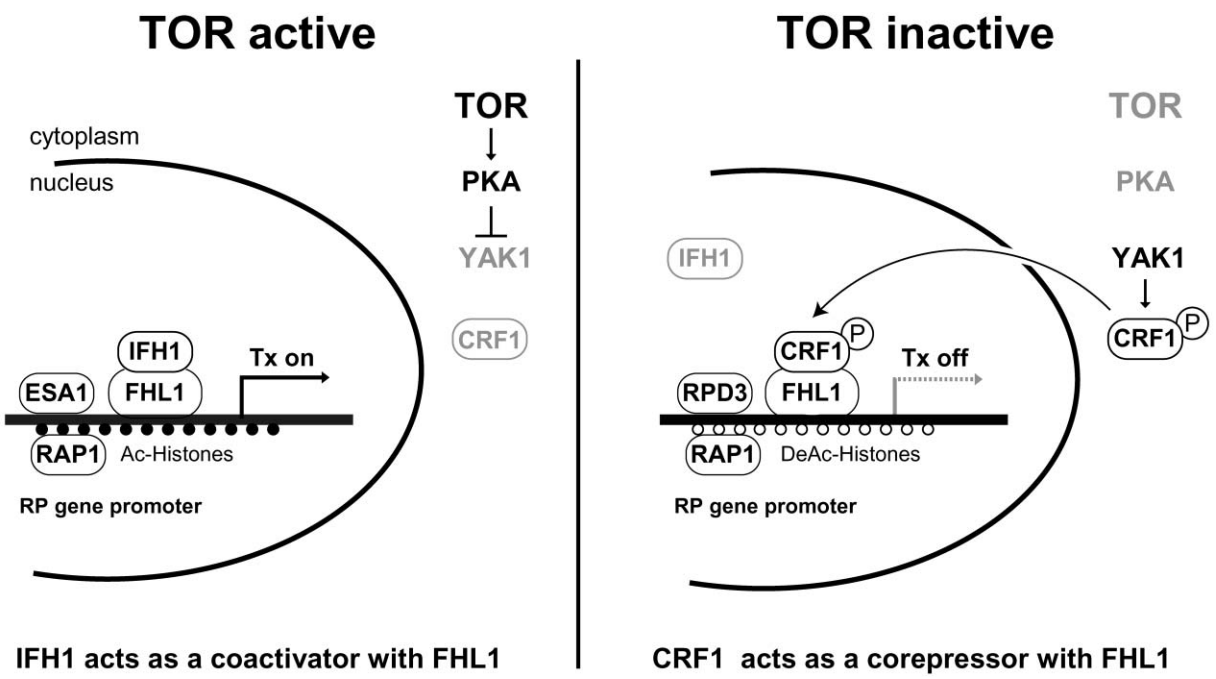

Figure 7. Model of RP Gene Regulation by TOR and the Forkhead Transcription Factor FHL1
See Discussion for details.

I and Pol III. Consistent with this notion, *FHL1* was originally isolated as a multicopy suppressor of a mutation affecting Pol III, and the FHL1 protein was subsequently shown in two genome-wide interaction studies to interact with the Pol I transcription factor HMO1 (Ho et al., 2002; Ito et al., 2001). Furthermore, RPD3 binds Pol I-dependent promoters in addition to Pol II-dependent RP gene promoters (Humphrey et al., 2004; Rohde and Cardenas, 2003; Tsang et al., 2003). These observations suggest that the TOR-PKA pathway may indeed coordinate all three RNA polymerases via FHL1, IFH1, and CRF1.

TOR signals as part of two structurally and functionally distinct complexes, TORC1 and TORC2 (Loewith et al., 2002). Only TORC1 is rapamycin sensitive, indicating that TORC1 mediates the regulation of ribosome biogenesis. TORC1, YAK1, and FHA domain-containing Forkhead transcription factors (MNF and ILF1/2 in human) are conserved from yeast to human (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Durocher and Jackson, 2002) (D.E.M., A.S., M.N.H., unpublished observation for YAK1), suggesting that the mechanism by which TORC1 regulates ribosome biogenesis may, at least in part, also be conserved.

Experimental Procedures

Strains, Plasmids, and Media

The *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in Supplemental Tables S1 and S2 (available on *Cell* website), respectively. All strains from our laboratory are isogenic derivatives of TB50 or JK9-3d. The yeast two-hybrid analysis was performed with strain PJ69-4A (James et al., 1996). Rich medium (YPD) or synthetic medium (SD or SC) were prepared as described previously (Beck et al., 1999; Sherman, 1991), with the exception that the synthetic media were supplemented with 0.2% yeast extract. Rapamycin was added to a final concentration of 200 ng/ml (200 nM) from a 1 mg/ml stock in 90% ethanol-10% Tween20. Rapamycin treatment was always for 30 min unless otherwise indicated.

Molecular Biology and Genetics

Restriction enzyme digestions, ligations, isolation of plasmids, PCR reactions, and DNA sequencing were performed by standard methods (Ausubel et al., 1998). PCR cassettes were used to generate gene deletions and modifications as described previously (Longtine et al., 1998). To generate partial gene deletions of *CRF1* and *IFH1*, an overlap extension PCR technique was used as described previously (Ho et al., 1989). Site-directed mutagenesis was performed using the QuickChange method (Stratagene). All generated mutations were verified by PCR or DNA sequencing. Chromatin immunoprecipitation (ChIP) analyses were carried out as described previously (Hecht et al., 1996) with the following slight modifications. PCR products were resolved on 2.5% agarose gels, visualized by SYBRGREEN staining, and subsequently analyzed using Syngene Gene Quant and Gene Tool software. Cycle numbers and template concentrations were adjusted to ensure that PCR product formation was in the linear range (22–35 cycles). Total chromatin input DNA was 1/20 to 1/500 of DNA used for ChIP analysis. Monoclonal antibodies against the HA epitope (clone 12C5) and the myc epitope (clone 9E10) were used to precipitate epitope-tagged IFH1, CRF1, and FHL1. Primers amplifying a 330 bp fragment of the *RPL30* gene promoter were used. Primer pairs against a 200 bp fragment of the *ACT1* coding region served as an internal control. The *ACT1* control PCR product after ChIP was not detected upon ChIP analysis under the conditions used. Yeast two-hybrid analysis and quantitative β-galactosidase assays were performed as described previously (Ausubel et al., 1998; James et al., 1996). All positive interactions were confirmed by retransformation of plasmids and analysis of β-galactosidase expression upon loss of bait plasmids on 5-FOA plates.

RNA Isolation and Northern Blot Analysis

RNA from cells treated with rapamycin or drug vehicle alone was isolated as described previously (Schmitt et al., 1990). Probe synthesis by PCR using digoxygenin-modified dUTP and subsequent

Northern Blot analysis was performed according to the manufacturer's protocol (DIG labeling and detection, Roche).

Coimmunoprecipitation and Immunoblotting

Whole-cell extracts for SDS-PAGE analysis were prepared by glass bead lysis as described previously (Beck et al., 1999). Lysis buffer containing phosphatase and protease inhibitors were used as described previously (Schmelzle et al., 2004). For detection of HA- and myc-tagged proteins, a mouse anti-HA antibody (clone 12CA5) or mouse anti-myc antibody (clone 9E10) was used. For detection of threonine phosphorylation an antiphosphothreonine antibody (Q7, Qiagen) was used. For detection of tyrosine phosphorylation an antiphosphotyrosine antibody was used (P-Tyr-100, Cell Signaling). Signals were detected using horseradish peroxidase-conjugated secondary goat antimouse antibodies and ECL reagents (Amersham Pharmacia Biotech).

Fluorescence Microscopy

Fluorescence microscopy and indirect immunofluorescence on whole fixed cells were performed as described previously (Schmelzle et al., 2004). Monoclonal anti-HA (12CA5) and anti-myc (9E10) antibodies were used as the primary antibody to detect HA- and myc-tagged proteins, respectively. Antibodies were always checked for specificity in each experiment using wild-type cells lacking the corresponding epitope.

YAK1 Kinase Assay

myc-tagged YAK1 was purified from strain TS129-8C. A mock purification was performed with the isogenic derivative TB50a expressing untagged YAK1. YAK1-HA and YAK1^{KD}-HA were purified from strain TS127-1A (*yak1Δ*) transformed with plasmid pDM76 (*YAK1*) or pDM72 (*YAK1*^{KD}). Cells were grown in YPD and treated with rapamycin or drug vehicle alone for 30 min. Protein purification was performed according to a standard protocol using coupled anti-myc-protein G-Sepharose or anti-HA protein A-Sepharose. The extended CRF1-FHB domain (amino acid residues 279–467), which was shown to interact with FHL1 in a yeast two-hybrid analysis, was fused to glutathione-S-transferase (GST) using plasmid pGEX4T (Amersham Pharmacia Biotech). Fusion protein expression was induced with 1 mM IPTG for 4 hr in *E. coli* BL21 cells. Protein purification was performed according to a standard protocol (Qiagen Expressionist). Kinase reactions with varying substrate concentrations were performed as described (Moriya et al., 2001). GST and BSA were used as control substrates for the specificity of phosphorylation.

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