Mini review

Yeast Crf1p: An activator in need is an activator indeed

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Ribosome biogenesis is an energetically costly process, and tight regulation is required for stoichiometric balance between components. This requires coordination of RNA polymerases I, II, and III. Lack of nutrients or the presence of stress leads to downregulation of ribosome biogenesis, a process for which mechanistic target of rapamycin complex I (mTORC1) is key. mTORC1 activity is communicated by means of specific transcription factors, and in yeast, which is a primary model system in which transcriptional coordination has been delineated, transcription factors involved in regulation of ribosomal protein genes include Ifh1p and its cofactors, Ifh1p and Crf1p. Ifh1p is an activator, whereas Crf1p has been implicated in maintaining the repressed state upon mTORC1 inhibition. Computational analyses of evolutionary relationships have indicated that Ifh1p and Crf1p descend from a common ancestor. Here, we discuss recent evidence, which suggests that Crf1p also functions as an activator. We propose a model that consolidates available experimental evidence, which posits that Crf1p functions as an alternate activator to prevent the stronger activator Ifh1p from re-binding gene promoters upon mTORC1 inhibition. The correlation between retention of Crf1p in related yeast strains and duplication of ribosomal protein genes suggests that this backup activation may be important to ensure gene expression when Ifh1p is limiting. With ribosome biogenesis as a hallmark of cell growth, failure to control assembly of ribosomal components leads to several human pathologies. A comprehensive understanding of mechanisms underlying this process is therefore of the essence.

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Abbreviations: CK2, casein kinase 2; Crf1, corepressor with forkhead like; FHA, forkhead-associated; FHB, forkhead-binding; Fh1, forkhead like; Fpr1, FK506-sensitive proline rotamase; FKBP, FK506 binding protein; Hmo1, high mobility group; Ifh1, interacts with forkhead like; mTORC1, mechanistic target of rapamycin complex 1; Rap1, repressor/activator protein; RASTR, ribosome assembly stress response; RIB1, ribosome biogenesis; RP, ribosomal protein; Slp1, split finger protein; WGD, whole genome duplication.

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1. Introduction

Ribosomes are subcellular machines required for protein synthesis. The absolute requirement for ribosomes during the cellular lifespan makes their biosynthesis essential, and it is an energy-consuming process. For example, during normal growth of Saccharomyces cerevisiae, an average of ~2,000 ribosomes are assembled per minute [1]. Assembly of ribosomes requires ribosomal RNA transcribed by RNA Polymerase (Pol) I, which accounts for >60% of total RNA content in a rapidly growing cell [2], as well as the RNA Pol III-transcribed 5S rRNA. Moreover, ribosomal protein (RP) gene expression accounts for >50% of the mRNAs synthesized by RNA Pol II, which also transcribes the >200 ribosome biogenesis (RiBi) genes encoding the processing factors involved in ribosome biogenesis [1,3]. Thus, for such an active process to be precise, coordination between all the events involved in synthesis of its components is required. Key to this coordination is mTORC1 (mechanistic Target of Rapamycin Complex 1), a major regulator of growth and proliferation, which is active under nutrient sufficient conditions or in the absence of any cellular stress such as that caused by DNA damage or environmental signals [4–6].

In this review, we will focus on RP gene regulation in yeast, mainly S. cerevisiae, which is the primary model system in which such regulation has been investigated. Computational analyses have pointed to a shared ancestral origin of two key transcription factors with inferred activator and repressor functions, respectively. We discuss the correlation between RP gene duplication and retention of both transcription factor paralogs, and we consider the possibility that both transcription factors function as activators.

2. mTORC1 controls cell growth and proliferation

mTORC1 contains the Ser/Thr protein kinase Tor, which is functionally conserved among all eukaryotes, from S. cerevisiae to Homo sapiens [7]. In budding yeast, mTORC1 may be assembled with Tor1p or Tor2p, although Tor1p is preferred (as evidenced by limited copurification of Tor2p and dedicated mTORC1 subunits) [8,9]. Additional mTORC1 components include Kog1p (Controller Of Growth; the homolog of mammalian Raptor), Lst8p (Lethal with Sec Thirteen; the homolog of mLST8), and Tco89p (Tor Complex One; for which there is no mammalian homolog) [10]. In contrast, Metazoans encode a single Tor protein (named mammalian Tor or mTor) [11]. All eukaryotes have two functional TOR complexes, mTORC1 and mTORC2. In higher eukaryotes, mTor is the kinase component of both TORC1 and TORC2 complexes in combination with different subunits. In budding yeast, mTORC2 contains Tor2p with its required subunits [9], and TOR2 is essential because mTORC2 is required for maintaining cell structure through actin polarization [12]. Yeast mTORC1 is maintained in an active state during amino acid sufficiency, while metazoan mTORC1 additionally responds to growth factors and mitogens (Fig. 1) [4,5]. Upstream activators such as amino acids target cytoplasmic mTORC1 localized to the lysosomal surface (the vacuole in yeast) [13], and this results in mTORC1-mediated phosphorylation of downstream targets such as regulators of translation [14–16]. Conversely, mTORC1 is inactivated as a result of stress and nutrient insufficiency [9,17]. mTORC1 thereby mediates a switch between active growth and the temporary growth cessation, which is imposed by starvation or required to prioritize recovery from DNA damage or stress. When active, mTORC1 signals to downstream effectors responsible for managing cell size and progression of the cell cycle, and it stimulates several transcriptional networks responsible for anabolic processes while simultaneously inhibiting catabolic pathways such as autophagy. In contrast, inhibition of mTORC1 promotes the catabolic state associated with starvation to allow energy to be dedicated to critical functions and survival [5,8,9,18–21].

The mTORC1 complex is characterized by its acute inhibition by the immunosuppressant rapamycin (sirolimus) [8]. Rapamycin binds FKBP (FK506 binding protein), and the complex then directly binds and inhibits mTORC1 (and indirectly inhibits mTORC2 by a mechanism thought to involve compromised complex assembly, provided chronic exposure to sufficient concentrations of this compound) [22–24]. Yeast FKBPl2, also known as Fpr1p (FK506-sensitive proline rotamase), in addition functions as a transcription factor for most RP genes [25]. However, yeast strains lacking FPR1 have no obvious phenotype, except that they are insensitive to rapamycin and FK506 [8,25].

2.1. Nuclear functions of mTORC1

mTORC1 also has nuclear functions under nutrient replete conditions, where its main targets include genes encoding proteins involved in ribosome biogenesis (Fig. 2). In both yeast and mammalian cells, Tor1p/mTor binds the promoters of both Pol I- and Pol III-transcribed rRNA genes, whereas rapamycin treatment or nutrient deprivation causes Tor1p/mTor to leave the nucleus, conditions under which transcription is repressed. In addition, RNA Pol III transcription of 5S rDNA is negatively controlled by Maf1p, and this negative regulation is alleviated when Maf1p is phosphorylated by active mTORC1 [26–31].

RNA Pol II is responsible for transcription of RP and RiBi genes. RPs are integral to the ribosome, whereas RiBi genes encode factors, which do not become part of the mature ribosome, but are required for its assembly. Examples include factors required for rRNA modification and ribosome assembly as well as enzymes involved in nucleotide metabolism and translation [32]. RiBi genes have been primarily characterized by the presence of promoter ele-
ments that recruit transcriptional repressors such as Dot6p and Tod6p, which in turn assemble the Rpd3L histone deacetylase to downregulate transcription during stress [33]. Thus, RiBi gene promoters were originally characterized by the presence of repressive promoter elements. More recently, RiBi gene promoters have also been reported to bind General Regulatory Factors (GRFs), which participate both in maintaining robust expression in rich media and in mediating reduced expression in response to nutrient limitation [34]. RP genes have distinct promoter architectures, and they have been sub-categorized based on specific promoter elements and the assortment of transcription factors that bind to these genes, as discussed in more detail below.

Notably, many of these transcription factors are regulated by mTORC1 [34–38]. Thus, mTORC1 controls ribosome biogenesis by coordinate controlling RNA Pol I-, II-, and III- transcribed genes. A lack of coordination causes a disturbance in ribosome assembly, leading to physiological conditions in humans categorized as ribosomopathies, one consequence of which is proteotoxic stress [39,40]. Patients with ribosomopathies generally present with tissue-specific abnormalities. One example is the congenital bone marrow failure syndrome known as Diamond-Blackfan anemia, which is most often characterized by mutations in specific RPs. In addition, many ribosomopathy patients are at greater risk of developing cancers, either because mis-assembled ribosomes may favor translation of oncogenic products, because of affected RPs moonlighting in other contexts, or due to general effects on cellular metabolism (for a recent review, see [41]). As a central regulator of cell proliferation, mTORC1 has been linked to several human cancers, as mutations in its upstream activators lead to hyper-activation. Such mTORC1 hyper-activation in turn supports the proliferative needs of tumor cells [42].

3. RP gene regulation in yeast

Regulation of RP gene transcription is key to maintaining the proper stoichiometry and assembly of ribosomal components. RPs are functionally conserved, and their numbers are also fairly constant between the three domains of life. In comparison to prokaryotes, which have 55 RPs, eukaryotes have 78–80. However, the total number of genes coding for these proteins varies between different kingdoms. Among eukaryotes, fungi have 138 RP genes, plants have about 217 RP genes, and animals have 79 RP genes, in all cases encoding 79 RPs, while protists transcribe 79 RP genes coding for 78 RPs [43,44]. In addition, yeast Asc1p and its mammalian homolog RACK1 (receptor for activated C-kinase 1) are components of the 40S ribosomal subunit, which link signal transduction pathways to the ribosome [45,46]. Although genome sizes of fungi and animals vary greatly, the number of RP genes is greater in fungi despite their overall smaller genomes. Thus, the number of RP genes is independent of the genome size and rather show a directly proportional relationship with the occurrence of genome duplication events [43,47,48].

A whole genome duplication (WGD) event occurred in a common ancestor of several yeast genera. The WGD encompassed the entire genome of the precursor, as for example evidenced by the organization of the 16 centromeres of S. cerevisiae into eight pairs, each syntenic with non-WGD yeast species, implying that the ancestor had eight chromosomes and that the WGD event gave rise...
to a 16-chromosome descendant [49–51]. While many duplicated genes were subsequently lost, the majority of duplicated RP gene pairs (ohnologs) were retained in S. cerevisiae, with only 19 of 138 RP genes being unique. Of the duplicated genes, only 22 encode identical proteins. The duplicated RP genes are located on different chromosomes, their expression patterns have diverged, and not all duplicated RPs are functionally interchangeable [44,52,53]. As a corollary, ribosome activity may be regulated based on changes in composition and for instance optimized for translation of certain mRNAs or modified in response to stress [54,55]. This vast number of RP genes must be expressed in a coordinated fashion yet allowing for preferential expression of specific genes in response to environmental cues. Unlike the situation in bacteria, where RP genes are organized in operons, eukaryotic RP genes are expressed individually and even feature variable promoter architecture, rendering their regulation much more complex [35,37,44,56,57]. In addition to transcriptional control, it has been reported that the presence of introns in RP genes, which encode the majority of spliced introns in yeast, mediates repression in response to starvation in an mTORC1-dependent manner [58].

3.1. Three categories of yeast RP genes

RP genes in S. cerevisiae feature regulatory motifs, which are required for gene specific expression control. RP genes are classified into three categories based on the transcription factors associated with them. Most of the upstream activating sequences (UAS) in yeast RP genes are associated with Rap1p (repressor/activator protein) as seen in categories I (69 genes) and II (60 genes) [35]. Rap1p association enables the recruitment of Forkhead-like 1 (Fhl1p), which in turn recruits the activator Interacts with Fhl1p (Ifh1p) [35,59,60]. The two Rap1p-associated categories of RP genes are further sub-divided on the basis of presence or absence of the high mobility group (HMGB) protein Hmo1p [61]. Category I RP genes localize Hmo1p on their promoters, where it is required for binding of Fhl1p, while category II is devoid of Hmo1p [35,37,62,63]. Category III RP genes are independent of Rap1p, but they require ARS binding factor 1 (Abf1p) [35,37,63]. Further, RP gene promoters have also been shown to bind the nutrient-and stress-sensitive split finger protein (Sfp1p) and Fpr1p. For category I and II promoters, Sfp1p binding depends on Ifh1p, while other promoters, including those of Ribi genes, are characterized by binding of Sfp1p directly to chromatin at a gAAAAWTTTTC motif (with lower case letters indicating a less conserved position and W representing A or T) [25,37,64–67].

3.2. The role of Hmo1p

S. cerevisiae Hmo1p has been shown to communicate mTORC1 signaling to downstream target genes [62,63,68–73]. Genomewide, Hmo1p binding to target sites is variable, with particularly enhanced binding to Pol I-transcribed rDNA, category I RP genes, and its own promoter [62]. On these genes, absence of Hmo1p results in an attenuated response to mTORC1 inhibition, and another consistent feature is that Hmo1p is released during prolonged mTORC1 inhibition [62,70,72,74]. The function of Hmo1p at these genes differs. On rDNA, Hmo1p promotes transcription, perhaps in part by limiting nucleosome occupancy [74,75]. On category I RP genes and on the HMO1 promoter, Hmo1p binding facilitates binding of Fhl1p (and in turn Ifh1p) as evidenced by reduced binding of Fhl1p in hmo1Δ yeast strains. Conversely, absence of Hfl1p reduces occupancy of Hmo1p on target genes [35,62,69,71]. While deletion of IFH1 is lethal, and deletion of FHL1 confers a severe growth defect, transcription of RP genes is not markedly affected by absence of Hmo1p, despite reduced Fhl1p/Ifh1p complex assembly [62,70,76,77]. It was recently shown that Fpr1p likewise promotes Fhl1p binding to certain RP genes, and it is therefore likely that the synthetic lethality or synthetic growth defect (depending on strain background) associated with deletion of both FPR1 and HMO1 may relate to failure to recruit Fhl1p to target genes, specifically RPL25, which is markedly downregulated in hmo1Δfpr1Δ cells [25,70,78]. On RP genes, deletion of HMO1 does impair recruitment of transcription factor TFIID, however, Hmo1p may facilitate accurate start site selection, likely by masking a nucleosome-free region and preventing non-productive preinitiation complex assembly [79,80].

3.3. The proposed roles of dedicated transcription factors Ifh1p and Crf1p

As alluded to above (Section 3.2), the genetic background used may affect the outcome of gene deletions. An hmo1Δfpr1Δ strain was reported to be inviable in the Y388 strain background [81], whereas the hmo1Δfpr1Δ double deletion confers a synthetic growth phenotype in S288C cells [25,78]. The basis for this difference is unknown, but it could possibly relate to relative expression levels of RPL25. S288C is a common laboratory strain, produced by a deliberate sequence of crosses to optimize its use for analysis of biochemical mutants, and it also serves as the reference strain [82]. However, S288C is missing a number of genes present in other laboratory strains. Strain W303 was derived from S288C by introduction of several markers, hence it is a particularly popular laboratory strain, but ~15% of its genetic material is from other sources, and it has distinct phenotypes [83]. Another relevant strain, which contains some genetic material from S288C is J9K-3d (and its derivative TB50); TB50 was used for early experiments pertaining to Ifh1p and Crf1p function [59]. As specified further below (Section 3.5), a direct comparison of experiments performed with different yeast strains should consider the possibility of strain background substantially affecting experimental outcomes.

Regulation of the majority, if not all of yeast RP genes depends on Ifh1p, which binds actively expressing Category I and II RP genes and a few Ribi genes, as determined by Chromatin Immunoprecipitation (ChIP). While inhibition of mTORC1 has little effect on Fhl1p occupancy, Ifh1p dissociates rapidly upon exposure to stress [59,60,63,76,84–87]. Ifh1p has also been proposed to play a role on Category III RP genes, as it may be detected on these genes by the more sensitive Chromatin Endogenous Cleavage (CHEC) approach in which the protein of interest is fused to micrococcocal nuclease [37]. Notably, anchor-away experiments have shown that depletion of Ifh1p from the nucleus is sufficient to repress transcription [37]. Anchor-away is a technique by which the function of essential (or near-essential) nuclear proteins may be assessed, and it involves the conditional tethering of the target protein to an abundant cytoplasmic protein, thereby depleting the target from the nucleus [88].

As noted above, deletion of IFH1 is lethal, consistent with a key role for Ifh1p in activating RP gene activity. However, the double deletion of IFH1 and FHL1 is viable (albeit sick), indicating that the activator function of Ifh1p is essential only in cells expressing Fhl1p [77,89]. The original interpretation of this observation was that Fhl1p functions as a repressor in absence of Ifh1p [77]. This interpretation would also be consistent with the observation that both Hmo1p and Fpr1p promote Fhl1p binding (and in turn Ifh1p), yet the absence of either Hmo1p or Fpr1p has little effect on RP gene transcription, perhaps because of reduced binding of the repressor, Fhl1p, and in turn reduced dependence on the activator, Ifh1p [25,62]. On the contrary, absence of Hmo1p leads to increased activity of the HMO1 promoter, but only when the so-called IFHL site is present; this site was previously shown to correlate with binding of Fhl1p and Ifh1p to RP genes [60,71].
Several mechanisms have been proposed to explain how mTORC1 activity is communicated to RP genes. One response is based on the subcellular localization of protein kinase A (PKA) and the PKA-regulated kinase Yak1p. Using TB50 yeast cells, the TOR-PKA pathway was suggested to regulate RP gene transcription in a Fhl1p-dependent process, by controlling assembly of either of the two co-factors Ifh1p or Co-repressor with Fhl1p (Crf1p) [59]. According to this model, Crf1p is primarily cytoplasmic when mTORC1 is active, whereas inactivation of mTORC1 leads to activation of Yak1p, which phosphorylates Crf1p to facilitate its nuclear import. Once in the nucleus, Crf1p competes with Ifh1p for binding to Fhl1p and represses transcription of RP genes [59]. As detailed below, this model appears to be strain-specific and unlikely to apply generally, and it is difficult to reconcile with more recent reports of Crf1p functioning as an activator.

Ifh1p shows a dynamic response to environmental stress or nutrition deprivation, regardless of strain background, as communicated by mTORC1 inhibition [59,60,63,76,84–87]. According to one mechanism for removing Ifh1p from RP genes, which was illustrated using the W303 strain background, Ifh1p dissociates from RP gene promoters and associates with the UTP-C 90S pre-ribosome complex localized in the nucleolus. The UTP-C 90S pre-ribosome complex is involved in processing pre-ribosomal RNA, and it is a multi-subunit protein complex containing Casein Kinase 2 (CK2) and two RiBi proteins, U three protein-22 (Utp22p) and Ribosomal RNA Processing-7 (Rrp7p). These proteins form the CURI complex upon association with Ifh1p (with CURI named for the components CK2, Utp22p, Rrp7p, Ifh1p), and this complex formation is crucial for titrating away Ifh1p from RP genes while simultaneously sequestering proteins required for pre-rRNA processing [90–93]. The rapid removal of Ifh1p has also been suggested to depend on its entrapment within aggregates of RP proteins, which accumulate as a consequence of ribosome assembly stress [85]. This expression control could possibly be reinforced by activation of Yak1p on mTORC1 inhibition, by phosphorylating Crf1p and enabling its movement from the cytoplasm to the nucleus and binding target gene promoters abandoned by Ifh1p.

Inhibition of mTORC1 also affects post-translational modifications of Ifh1p. For example, the histone acetyl transferase Gcn5 has been implicated in acetylating Ifh1p, particularly in its N-terminal domain [94,95]. Cellular stress, as imposed for example by addition of rapamycin, results in reduced levels of acetylation, and acetylation is increased upon recovery from stress. One notable consequence of reduced Ifh1p acetylation appears to be reduced protein stability [95]. Interestingly, hypoacetylated, promoter-bound Ifh1p was inferred to support a rapid increase in RP gene transcription on recovery from stress or starvation, whereas acetylation of Ifh1p appears to limit this initial burst of activity [94].

3.4. The evolutionary relationship between Ifh1p and Crf1p

**IFH1** and **CRF1** appear to be the result of an ancient WGD event [47]. This gene pair evolved from a common ancestor, which is still found, for example, in *Kluyveromyces lactis*, a pre-WGD relative of *S. cerevisiae* (Fig. 3A). While pre-WGD orthologs are similar to Ifh1p, Crf1p lacks the acidic N-terminal trans-activation domain, and it features a greater rate of amino acid substitutions compared to Ifh1p (Fig. 3B). These observations suggest that Ifh1p represents the pre-WGD ancestor, whereas the function of Crf1p was derived following the WGD event [47]. Based on the previously suggested activator and repressor functions of *S. cerevisiae* Ifh1p and Crf1p, respectively [59], the Ifh1p/Crf1p ancestor was proposed to have both activator and repressor functions as evidenced by conserved and coordinated repression of RP gene expression during stress in pre-WGD species such as *K. lactis* [47].

Ifh1p and Crf1p were not retained in all post-WGD species, rather the *Candida glabrata* genome reflects a loss of Crf1p [47,96]. Two possible theories could explain the evolution of Crf1p; with two functional copies of the ancestral protein, each of which experiencing mutations, an alteration in a functional domain in one of the copies may have been backed by the paralog to retain the overall functionality, ultimately leading to specialized functions of each paralog [97,98]. Alternatively, managing the

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**Fig. 3.** Conservation of transcription factors. A. Phylogenetic tree of select ascomycetes. Species identified with solid lines diverged after the WGD event, and species identified with dashed lines diverged prior to the WGD. Adapted from [47]. B. *S. cerevisiae* Ifh1p and Crf1p. Both proteins feature a conserved forkhead-binding (FHB) domain. Crf1p lacks N- and C-terminal extensions compared to Ifh1p. C. Alignment of Fhl1p, Ifh1p, and Utp22p from *S. cerevisiae* (blue), *C. glabrata* (orange), and *K. lactis* (red) using NCBI Blastp. Identity and coverage relative to the corresponding *S. cerevisiae* ortholog is indicated at the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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increased dosage of RP gene products post duplication may have been favored by the evolution of dedicated regulators [99,100]. C. glabrata, which lost Crf1p, has 85 RP genes, which is comparable to the pre-WGD relatives, e.g., K. lactis (81 RP genes) and C. albicans (84 RP genes). By contrast, S. cerevisiae and S. castellii, in which Crf1p is retained, have 138 RP genes. One interpretation is that the need for a specialized regulator is related to the requirement for more intricate transcription control in strains with a higher number of RP genes. For example, recent analyses of duplicated gene pairs in mammalian genomes suggested that tandem duplicates tend to be co-regulated, unlike duplicate genes located on separate chromosomes [101]. As noted above, duplicated RP genes in Saccharomyces are located on different chromosomes, and compared to single copy RP genes, duplicate RP genes in S. cerevisiae are expressed at lower levels [102].

3.5. Is Crf1p really a repressor?

Ifh1p and Crf1p have a conserved forkhead-binding (FHB) domain (Fig. 3B), which binds the forkhead-associated (FHA) domain in Fhl1p [59], and phosphorylation of the FHB domains by CK2 is required for the interaction with the FHA domain of Fhl1p [91]. Structurally, both Ifh1p and Crf1p remain uncharacterized, and secondary structure predictions indicate extended regions of disorder. Such disorder is not uncommon in eukaryotic transcription factors, and it may confer a plasticity required for interaction with multiple cellular targets [103]. The N-terminal domain of Ifh1p was shown to be required for the removal of Ifh1p from RP genes on inhibition of mTORC1, and Crf1p also lacks the C-terminal region, which in Ifh1p is required for its interaction with Utp22p and sequestration into the CURI complex [90]. These features could explain why Ifh1p is key to the mTORC1-sensitive regulation of RP gene expression.

While these characteristics may explain how the activator Ifh1p responds to mTORC1 inhibition, they do not shed much light on the function of Crf1p, beyond documenting molecular mechanisms underlying its binding to RP gene promoters and Ifh1p. That Ifh1p and Crf1p have distinct functions is indubitable and it is supported by several additional lines of evidence, including the expression pattern of IFH1 and CRF1 on mTORC1 inhibition. In S. cerevisiae strains W303 and BY4741, rapamycin treatment and amino acid limitation induces Ifh1p and CRF1 on mTORC1 inhibition. In S. cerevisiae, another post-WGD strain, which encodes Crf1p [47,104]. And while deletion of IFH1 is lethal, deletion of CRF1 is not [77]. As noted above, Crf1p was reported to be required for mTORC1-mediated repression of RP gene expression to be manifest in S. cerevisiae strain TB50 [59]; in apparent support of a repressor function for Crf1p, C. glabrata, which has lost Crf1p, does not experience concerted downregulation of RP genes in response to stress [47].

Perhaps more importantly, whether Crf1p truly functions as a repressor appears to be in question. Notably, the requirement for Crf1p is not conserved among S. cerevisiae strains. The inference that Crf1p functions as a repressor in controlling of RP gene expression was based on experiments with S. cerevisiae strain TB50; by contrast, RP gene expression declines in strain W303 after mTORC1 inhibition irrespective of the presence of Crf1p [37,90,91,105,106]. These differences were inferred to derive from multiple differences, as opposed to a single gene, as suggested by the behavior of haploid segregants resulting from crosses between W303 and TB50 [106]. It has also been reported that plasmid-based overexpression of Crf1p in an ifh1Δ strain (W303 genetic background) reverses the lethality of the IFH1 deletion, suggesting that Crf1p may substitute for Ifh1p in enabling RP gene expression. This experiment also showed that repression of Category I and II RP genes in response to rapamycin treatment is compromised when Crf1p entirely replaces Ifh1p [37], consistent with absence of the N- and C-terminal protein domains in Crf1p, which have been implicated in removing Ifh1p from RP gene promoters. Furthermore, we have recently reported that endogenous Crf1p also promotes expression of select Category I and II RP genes during balanced growth as evidenced by reduced mRNA accumulation in a crf1Δ strain [105]. Taken together, these observations suggest that Crf1p functions as an activator.

Ifh1p and Crf1p bind the same genes, and they function in overlapping conditions, features generally proposed to imply at least partial redundancy for duplicated transcription factors. A relevant comparison is the ancestral transcription factor Msn, which gave rise to S. cerevisiae Msn2 and Msn4 following the WGD event. Msn2 and Msn4 regulate the same stress response genes, and it has been proposed that they confer adaptive benefit, not through functional divergence, but by cooperation [107]. In this scenario, the advantage to having two separate factors is that stress responsiveness is optimized while adverse effects on cell growth are attenuated. The possibility that Ifh1p and Crf1p cooperate in activating RP genes is therefore not unprecedented.

3.6. An alternate explanation for the strain-specific requirements for Crf1p

In C. glabrata, which has lost Crf1p, RP gene expression is not suppressed during stress [47]. This phenotype is similar to that characteristic of S. cerevisiae TB50 deleted for CRF1 [59,91]. These observations speak to a role for Crf1p in communicating mTORC1 inhibition to target genes. However, what would be the likelihood of the repressor function of Crf1p to be entirely lost in the closely related S. cerevisiae strain W303? First of all, that CRF1 is upregulated during stress in this strain would be consistent with a specific function under such conditions [47,104]. Secondly, according to the Saccharomyces Genome Database [108], the amino acid sequences of Crf1p, Ifh1p, and Hmo1p are invariant between S. cerevisiae strain W303, in which Crf1p is dispensable for RP gene regulation during stress, and JK9-3d, the parent strain of TB50, in which Crf1p is required to maintain the repressed state. This suggests that strain-specific differences are unrelated to Crf1p per se or to its interaction with Ifh1p. Differences between W303 and TB50 in terms of the dependence on Crf1p for communication of mTORC1 inhibition were inferred to be due to multiple changes [106], also suggesting that they are not merely due to loss of a specific Crf1p function in W303.

Ifh1p is an essential activator of RP genes, and its removal from gene promoters in response to mTORC1 inhibition depends on its interaction with Utp22p, which in turn leads to the sequestration of both proteins in the CURI complex [90]. These experiments, which were performed with S. cerevisiae W303, show that in wild-type cells, Ifh1p leaves the promoter 5 min after rapamycin treatment, and Ifh1p occupancy remains low after 25 min. Notably, deletion of Utp22p does not change the rapid dissociation of Ifh1p, but it results in faster re-binding of Ifh1p to RP gene promoters, with readily detectable binding 10 min after addition of rapamycin [90]. A possible explanation for the strain-specific dependence on Crf1p is therefore that the dynamics of CURI complex assembly/disassembly varies between strains (Fig. 4). According to this model, Crf1p may be dispensable for RP gene regulation in W303 because Ifh1p remains stably sequestered in the CURI complex or within RP aggregates, causing its re-binding to RP gene promoters to be slow. This model also predicts that Ifh1p re-binding following rapamycin treatment would be faster in S. cerevisiae TB50 (and C. glabrata), unless Crf1p can take its place. According to this prediction, Ifh1p should quickly (<5 min) leave RP gene promoters on rapamycin treatment of TB50 cells, but it
Fig. 4. The dynamics of CURI complex formation may dictate dependence on Crf1p. When mTORC1 is active (left), Crf1p is predominantly cytoplasmic. Nuclear Crf1p may bind Fhl1p and promote transcription of RP genes if Ifh1p is limiting. Direct phosphorylation of Sfp1p by mTORC1 ensures its nuclear localization. The nuclear UTP-C subcomplex composed of CKII, Utp22p, and Rrp7p processes pre-rRNA. On inhibition of mTORC1 (right), Sfp1p leaves the nucleus. Crf1p is phosphorylated and translocates to the nucleus. Ifh1p interacts directly with Utp22p, and both proteins become sequestered in the CURI complex. Alternatively, Ifh1p may become trapped within newly synthesized RP aggregates. Absence of Ifh1p is sufficient for repression of RP gene transcription. We propose that Crf1p may be required to maintain reduced RP gene expression under conditions where sequestration of Ifh1p is transient and re-binding of Ifh1p to RP genes is rapid.

5. Evolution of promoter elements

As outlined above (Section 3.1), RP gene promoters have been subdivided into three categories, each of which characterized by the binding of a specific set of transcription factors. The presence of conserved promoter elements in RP genes was appreciated decades ago, with the description of the HOMOL1-box and RPG-box elements [109,110]. This inspired interest in understanding how specific promoter elements participate in the control of gene expression, particularly in strains with duplicated RP genes for which dosage compensation is required. For instance, consensus Rap1p binding sites are frequently occurring in duplicate, approximately 200–500 bp upstream of the transcriptional start site of Category I and II RP genes, and the IFH1 site was reported to correlate with Fhl1p/Ifh1p binding [60,111,112]. By contrast, Category III RP genes feature a distinct promoter architecture and the binding of the general transcription factor Abf1p in place of Rap1p. Such dependence on different transcription factors permits varied responses to different growth conditions [37].

The rewiring of gene regulatory networks can lead to different expression patterns. One scenario, which appears to characterize RP genes, comprises a generalized transcription factor, such as Rap1p or Abf1p, which in turn associates with different combinations of specialized factors to provide an adaptive response. Even for paralogous transcription factors, regulatory sequences may
have diverged due to either drift or selection, and the resulting promoters may then operate optimally under different conditions, building in a redundancy to ensure adequate production of the gene products [37]. Indeed, a comparison of paralogous RP gene pairs reveals non-identical expression patterns, rationalizing the retention of duplicated genes [53].

This type of promoter evolution described above has also occurred between pre- and post-WGD species of yeast. For example, IFHL elements and Rap1p binding sites are over-represented in RP gene promoters in both pre- and post-WGD yeast species, consistent with a regulatory mechanism that depends on Ifh1p. C. glabrata RP genes feature similar promoter elements as S. cerevisiae RP genes, except for the additional presence of an MCM1 element. However, the MCM1 site is also found in RP genes from the pre-WGD species K. lactis, whose RP genes are repressed during stress. These observations support the interpretation that lack of RP gene repression in C. glabrata is due to evolutionary changes in transcription factors, not cis-elements [47].

6. Summary and outlook

Control of ribosome biogenesis is paramount to cellular health, and the mTORC1 pathway is key to this process. Perturbations generate ribosome assembly stress, which in turn provokes a range of pathological conditions, underscoring the necessity of a complete molecular understanding of underlying mechanisms. One component of this process is the transcriptional control of RP genes. Much insight into this process has been derived from experimental analyses of control mechanisms in the model yeast S. cerevisiae, combined with computational analyses of related yeast species. Recent evidence has emphasized the dynamics of Ifh1p removal from RP genes in response to mTORC1 inhibition as key to gene regulation, either by the sequestration of Ifh1p in the CURI complex or in aggregates of newly synthesized, unassembled RPs, which accumulate as a consequence of the ribosome assembly stress response (RASTR) [85,90]. Much remains to be deciphered about these processes, including mechanisms leading to protein aggregation.

Ifh1p and its paralog Crf1p derive from a common ancestor [47]. We propose that the ancestral mode of RP gene regulation during stress involved the removal of the Ifh1p/Crf1p ancestral activator, possibly by a mechanism akin to the CURI or RASTR complex formation described for S. cerevisiae. We further suggest that Crf1p serves the same function in all S. cerevisiae and possibly all post-WGD yeast strains in which this paralog was retained. It functions as a backup activator in situations where Ifh1p is in short supply, perhaps to ensure a baseline level of RP gene expression that may increase rapidly when cells are released from mTORC1 inhibition. Such backup activation may be particularly pertinent when RP genes are duplicated, a situation that may more readily cause Ifh1p to become limiting. When mTORC1 is active, Crf1p is primarily cytoplasmic. This subcellular localization makes sense because Crf1p is a constitutive activator, which is not removed from RP genes in response to mTORC1 inhibition. Accordingly, nuclear levels are lower in rapidly growing cells to prevent it from competing too effectively with Ifh1p for binding to target promoters. This model for Crf1p function consolidates both available experimental evidence as well as computational predictions.

CRediT authorship contribution statement

Sanjay Kumar: Writing – original draft, Writing – review & editing, Visualization. Muneera Mashkoor: Writing – original draft, Writing – review & editing. Anne Grove: Writing – original draft, Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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