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Article

Gcn5 and Sirtuins Regulate Acetylation of the Ribosomal Protein Transcription Factor Ifh1

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Summary

Background: In eukaryotes, ribosome biosynthesis involves the coordination of ribosomal RNA and ribosomal protein (RP) production. In *S. cerevisiae*, the regulation of ribosome biosynthesis occurs largely at the level of transcription. The transcription factor Ifh1 binds at RP genes and promotes their transcription when growth conditions are favorable. Although Ifh1 recruitment to RP genes has been characterized, little is known about the regulation of promoter-bound Ifh1.

Results: We used a novel whole-cell-extract screening approach to identify Spt7, a member of the SAGA transcription complex, and the RP transactivator Ifh1 as highly acetylated nonhistone species. We report that Ifh1 is modified by acetylation specifically in an N-terminal domain. These acetylations require the Gcn5 histone acetyltransferase and are reversed by the sirtuin deacetylases Hst1 and Sir2. Ifh1 acetylation is regulated by rapamycin treatment and stress and limits the ability of Ifh1 to act as a transactivator at RP genes.

Conclusions: Our data suggest a novel mechanism of regulation whereby Gcn5 functions to titrate the activity of Ifh1 following its recruitment to RP promoters to provide more than an all-or-nothing mode of transcriptional regulation. We provide insights into how the action of histone acetylation machineries converges with nutrient-sensing pathways to regulate important aspects of cell growth.

Introduction

Growth and cell division are tightly coupled such that cells must reach a size threshold prior to irreversible commitment to a new cell cycle [1]. Growth potential, in turn, depends largely on the ability of a cell to increase its translational capacity by synthesizing new ribosomes. In budding yeast, a group of over 200 coregulated genes, termed the ribosome biogenesis cluster, must coordinate the assembly of four ribosomal RNA (rRNA) molecules transcribed by RNA pol I and RNA pol III with 79 ribosomal proteins (RPs) whose messenger RNAs (mRNAs) are transcribed by RNA pol II from 138 open reading frames (ORFs) scattered throughout the genome. This complex process of ribosome production is coupled to nutrient availability and is downregulated at multiple levels both during starvation and under conditions of cellular stress [1–4].

RP transcription accounts for up to 50% of all RNA pol II-mediated transcription and is regulated in large part by the essential transcriptional activator Ifh1 [5]. Ifh1 is recruited almost exclusively to RP promoters. This recruitment is mediated by an interaction with the fork-head-associated (FHA) domain of Fhl1, which remains constitutively bound at promoter sites [6–9]. Target of rapamycin (TOR) kinase activity promotes Ifh1 recruitment when nutrients are available [6]. In contrast, Ifh1 is not bound to RP promoters during periods of starvation or stress [6–9]. Fhl1-dependent Ifh1 recruitment is insufficient to drive transcription on its own [10], and other factors, such as promoter-bound Rap1 or the transcription factors Sfp1 and Hmo1, may function in a pathway required for Ifh1 function [3, 10, 11].

Acetylation of lysine residues in histone tails modifies chromatin structure both directly, by neutralizing the positive charge of these residues, and indirectly, by creating binding sites for acetyllysine-binding bromodomains [12]. Protein complexes recruited via acetyllysine-dependent interactions may participate in chromatin remodeling by sliding or evicting nucleosomes from DNA at promoters to provide access to site-specific regulators of transcription [12]. Two histone acetyltransferases (HATs) bind RP promoters. First, the essential HAT Esa1 positively regulates RP transcription [13, 14]. This regulation is thought to occur via acetylation of the N-terminal tails of histone H4 and is opposed by the action of the Rpd3 deacetylase [13, 15]. Esa1 recruitment to RP promoters correlates with favorable growth conditions and occurs in part through a direct interaction with Rap1, which, like Fhl1, is a constitutive resident at RP promoters [15, 16]. Chromatin immunoprecipitation studies suggest that the SAGA complex, which contains the Gcn5 HAT, also localizes to RP genes [14, 17]. In contrast to Esa1, however, SAGA recruitment to RP genes does not appear to be significantly regulated by stress in logarithmically growing cultures [18] and the relevant target(s) of Gcn5 at RP promoters are not fully understood.

Here, we provide new mechanistic insights into RP transcriptional regulation by showing that Gcn5 acetylates the Ifh1 transcription factor. Acetylation of Ifh1 occurs predominantly in an N-terminal acidic region and is negatively regulated by the sirtuin class of deacetylases. Furthermore, we find that acetylation is regulated by the TOR nutrient-sensing kinase and cellular stress. Analysis of nonacetylatable mutants suggests that Ifh1 acetylation negatively regulates its function at RP promoters. We suggest a model whereby unacetylated Ifh1 is recruited to promoters in response to nutrients and provides an initial burst of activity that is subsequently restrained by Gcn5-mediated acetylation.

Results

Ifh1 and SAGA Subunits Are Highly Acetylated Proteins in Yeast

Recent work suggests that yeast HATs may regulate cell function in part though the modification of nonhistone substrates [19, 20]. To study nonhistone acetylation in yeast, we probed

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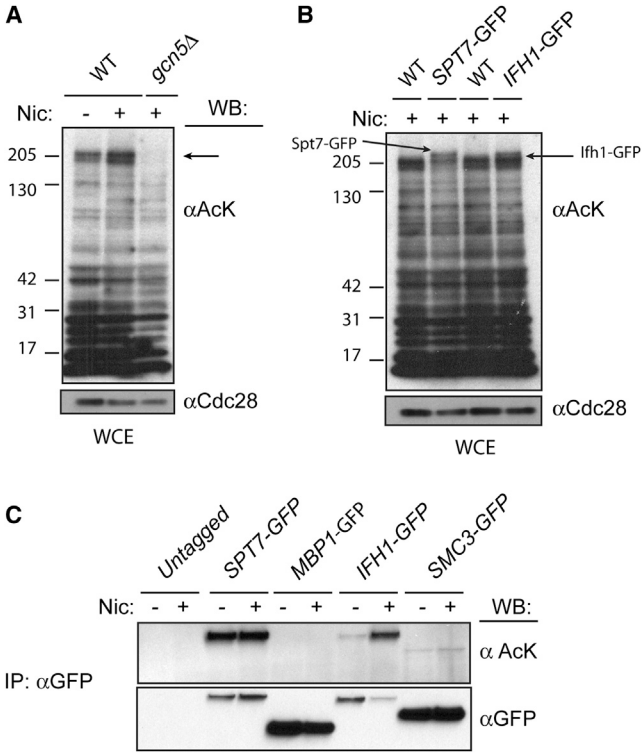

Figure 1. SAGA Subunits and Ifh1 Are Acetylated Proteins in Yeast

(A) Western blots of yeast WCEs prepared from the indicated strains were probed with anti-acetyllysine antibodies. An antibody against Cdc28 was used as a loading control. Strains were incubated with or without 20 mM nicotinamide treatment. The arrow indicates the position of an ~200 kDa species that is highly reactive with our anti-acetyllysine antibodies. Numbers indicate molecular weight markers in kDa.

(B) A Gcn5-regulated band of high molecular weight is a composite of Spt7 and Ifh1. WCEs were prepared from the indicated GFP-tagged or wild-type control strains and separated on a 4%–20% SDS-PAGE gel prior to western blotting and detection of acetylated species using an anti-acetyllysine antibody.

(C) Western blotting was used to determine the acetylation status of GFP-tagged proteins following their immunoprecipitation from cultures grown with or without nicotinamide (20 mM). Smc3, a known acetylated protein, is used as a control. Mbp1, an unrelated transcription factor, showed no acetylation in this assay.

See also Figure S1.

western blots of yeast whole-cell extracts (WCEs) derived from strains mutated for various histone acetyltransferases with anti-acetyllysine antibodies. This analysis revealed a reproducible pattern of reactive species that was similar for WCEs from wild-type cells and most HAT mutants (Figure S1 available online). In contrast, extracts from *gcn5*Δ mutant cells showed a striking absence of a number of highly reactive species while having a total protein profile identical to that of wild-type cells (Figures 1A, S1A, and S1B). We focused our attention on a large (approximately 200 kDa molecular weight) band that was more reactive with our anti-acetyllysine antibodies following nicotinamide treatment, which inhibits all five members of the sirtuin family of deacetylases ([21]; Figure 1A).

We devised a simple method to identify the protein(s) contributing to this sirtuin-regulated species. We reasoned that anti-acetyllysine antibody immunoblotting of WCE prepared from a strain in which a large epitope tag was fused to the immunoreactive protein would result in a banding pattern distinct from that of a wild-type strain in two ways. First, we would observe a new acetylated species of increased apparent molecular weight. Second, we would observe the loss of an acetylated species corresponding to the untagged protein's size. We took advantage of a set of yeast strains in which each ORF is expressed individually as a GFP-fusion protein [22]. We carried out our WCE analysis for a subset of these strains in which GFP was fused to ORFs with a predicted molecular weight of 120 kDa or higher. We found that expression of GFP fusions with either Spt7 or Ifh1 caused a change in the migration of the ~200 kDa species on our blots, suggesting that acetylation of both proteins contributes to the overall signal observed in this single band (Figures 1B and S1C). While the predicted molecular weight of each protein is less than 200 kDa, both migrate anomalously on SDS-PAGE gels for reasons that are not clear. To confirm that Spt7 and Ifh1 are acetylated, we immunoprecipitated them from asynchronously growing cultures in both the presence and absence of the sirtuin inhibitor nicotinamide (Figure 1C). As a positive control, we examined acetylation of the cohesin subunit Smc3, a target of the Eco1 HAT [23–25]. Both Ifh1 and Spt7 were highly reactive with anti-acetyllysine antibodies when judged according to the amount of protein loaded (via anti-GFP signal) and our Smc3 control (Figure 1C). In contrast, no acetylation was observed on the transcription factor Mbp1 [26], a negative control, despite recovery of a significant amount of target protein (Figure 1C). The acetylation of Ifh1, but not that of Spt7 or Smc3, was dramatically increased in the presence of nicotinamide, suggesting that Ifh1 might be targeted by sirtuins (Figure 1C).

Since the acetylation of SAGA subunits by Gcn5 has recently been reported [17, 27], we focus here on the regulation and function of Ifh1 acetylation. Since Ifh1 is a transcription factor, we wondered whether other yeast transcription factors were acetylated to the same degree following nicotinamide treatment. Surprisingly, Ifh1 stood out in its strong reactivity with anti-acetyllysine antibodies among more than 36 transcription factors tested in immunoprecipitation (IP)-western experiments (Figure 2; data not shown), suggesting that Ifh1 may represent a particularly important nonhistone target for HATs.

Ifh1 Acetylation Is Regulated by a Subset of Sirtuins

S. cerevisiae encodes five sirtuins: Sir2 and Hst1–Hst4 [28]. Sir2, the eponymous founding member of the sirtuin class, has roles in transcriptional silencing at subtelomeric regions, the *HM* mating locus, and rDNA repeats in the nucleolus [12, 28–31]. Hst1 also functions in transcriptional regulation and may functionally overlap with Sir2 in some contexts [28, 32]. Hst2 is the only cytoplasmic sirtuin, and its deletion causes increased resistance to the translational inhibitor cycloheximide [33]. Finally, Hst3 and Hst4 act redundantly in deacetylating histone H3 K56 following DNA replication [34, 35]. As shown in Figure 3A, we found that Ifh1 was highly acetylated in an *hst1*Δ *hst2*Δ *sir2*Δ triple mutant compared to either wild-type cells or cells treated with nicotinamide. Examination of Ifh1 acetylation in strains lacking individual sirtuins suggested that deacetylation is likely to be mediated mostly by Sir2 and Hst1 (Figure 3B). The observation that Ifh1 acetylation was greater in an *hst1*Δ *hst2*Δ *sir2*Δ strain than a wild-type stain treated with nicotinamide suggested that sirtuins might retain limited activity toward some substrates, even in high concentrations of this drug. To determine if sirtuins act on Ifh1 directly, we purified acetylated Ifh1 from *hst1*Δ *hst2*Δ *sir2*Δ yeast and carried out in vitro deacetylation assays using

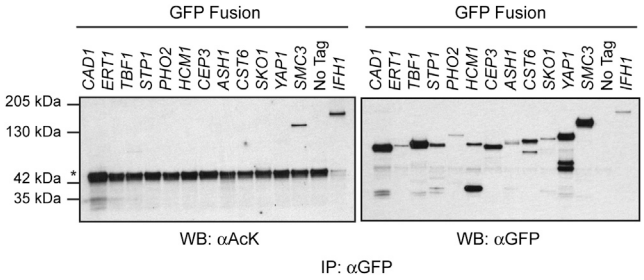

Figure 2. Nicotinamide Does Not Induce Global Acetylation of Transcription Factors

The indicated GFP-tagged transcription factors were immunopurified from log-phase cultures treated with nicotinamide (20 mM) using an αGFP antibody, and reactivity of recovered proteins with anti-acetyllysine antibodies was tested following separation on 4%–20% SDS-PAGE gradient gels and western blotting. Even when Ifh1 levels are adjusted to that of the lowest abundant protein recovered, its acetylation stood out among all candidates tested, which showed only background reactivity with our antibodies. The asterisk indicates immunoglobulin G bands from immunoprecipitations.

Sir2. Sir2 readily deacetylated Ifh1 in the presence of its cofactor nicotinamide adenine dinucleotide (NAD+), and this deacetylation was inhibited by inclusion of nicotinamide in the reaction (Figure 3C). Together, our data suggest that Ifh1 is acetylated in vivo and that its acetylations are reversed by a subset of sirtuins.

The Acetylation of Ifh1 Is Mediated by Gcn5

Esa1 has been previously implicated in the transcription of RP genes through the regulation of histone H4 acetylation [13, 15]. To test for a role of Esa1 in Ifh1 acetylation, we immunoprecipitated Ifh1 from strains carrying the temperature-sensitive *esa1-414* allele [36] and examined acetylation of the recovered protein. While global H4 acetylation was lost when *esa1-414* strains were incubated at the restrictive temperature for 2 hr, Ifh1 acetylation remained largely unaffected (Figure S2A). These data suggest that Esa1 does not have a direct role in acetylating Ifh1 at RP promoters. We also found that the deletion of *GCN4*, which has been shown to inhibit Esa1 recruitment to RP promoters [16], had no impact on Ifh1 acetylation (Figure S2B).

Our initial screen suggested that Gcn5 may be required for Ifh1 acetylation (Figure S1A). Indeed, Gcn5, with the SAGA complex, localizes to RP promoters [14, 17]. In contrast to protein from wild-type cells, Ifh1 from cells lacking *GCN5* did not react with anti-acetyllysine antibodies (Figure 3D). Gcn5 was also required for the increased acetylation of Ifh1 observed following treatment of cells with nicotinamide (Figure 3E). Gcn5 purified from bacteria, but not Gcn5-E173Q, carrying a mutation in its catalytic domain [37], was able to acetylate Ifh1 in vitro (Figure 3F), suggesting that Gcn5 acts on Ifh1 directly. In vivo, however, Ifh1 acetylation required the SAGA structural component Spt7, suggesting that SAGA rather than free Gcn5 mediates Ifh1 acetylation in cells (Figure 3G).

Ifh1 Acetylation Is Regulated by Nutrient Levels and Temperature Stress

Since RP genes are regulated by stress and nutrient status [2], we examined whether Ifh1 acetylation is also regulated under such circumstances. We first tested the effect of rapamycin, a TOR inhibitor that results in a rapid downregulation of RP gene transcription [38]. As an independent measure of the efficacy of drug treatment, we monitored the status of Gln3-GFP phosphorylation, which is dependent on TOR function and is lost following rapamycin treatment [39]. Twenty minutes after rapamycin treatment, Gln3 hyperphosphorylation was decreased relative to a control treatment (Figure 4A, inputs). The level of Ifh1 protein remained constant for both treated and control cells for the course of the experiment (Figure 4A). However, while Ifh1 acetylation also remained constant in control cells, TOR inhibition immediately reduced this acetylation (Figure 4A). We also measured Ifh1 acetylation during a mild temperature shock, which is associated with a temporary downregulation of RP mRNA levels [40]. While Ifh1 levels remained constant during a heat shock from 23°C to 37°C, its acetylation rapidly decreased and remained low for 10–20 min before eventually recovering by 60 min (Figure 4B). To determine if loss of Ifh1 acetylation after stress is due to the action of sirtuins, we analyzed Ifh1 from cells treated simultaneously with both rapamycin and nicotinamide. Nicotinamide had no effect on the loss of TOR-dependent Gln3 phosphorylation after rapamycin treatment (Figure 4C, inputs). However, nicotinamide treatment prevented the rapid deacetylation of Ifh1 observed in cells treated with rapamycin alone (Figure 4C), suggesting that sirtuins are required for deacetylation of Ifh1 after stress.

The interaction of Ifh1 with promoter-bound Fhl1 is thought to be a critical step in regulating the transcription of RP genes and is assumed to be inhibited in growth conditions that allow for only minimal RP transcription. Indeed, as reported previously [9], we found that the interaction of Ifh1 with Fhl1 was abolished following treatment with rapamycin (Figure 4D). Surprisingly, however, only limited reduction of the Fhl1-Ifh1 interaction was observed upon transfer of cells either to rich media lacking any source of carbon or to water (Figures S2C). We also found that the amount of Ifh1 bound to Fhl1 was virtually unchanged following the addition of glucose to cells growing in a poor carbon source (Figure 4E). However, in all cases, acetylation of Ifh1 was increased upon a return to rich media (Figures 4E and S2C). These results suggest that acetylation of promoter-bound Ifh1-Fhl1 molecules may play a role in regulating RP transcription during a recovery from stress.

Ifh1 is thought to be essential, due to both its importance in activating RP transcription directly and its role in blocking *FHL1* repression of RP genes, such that an *fhl1*Δ is viable and epistatic to *ifh1*Δ [41]. Rudra et al. previously reported that Ifh1 could still interact with Rap1 in an *fhl1* mutant strain [42], despite the fact that Fhl1 seems to be required for localization of Ifh1 to RP promoters by chromatin immunoprecipitation and for transcription from these promoters [9]. Accordingly, we found that Ifh1 was still acetylated in an *fhl1*Δ strain, although this acetylation remained sensitive to rapamycin and nicotinamide treatments (Figures 4F and S2D). These data are consistent with a model wherein Ifh1 is loosely bound at RP promoters in *fhl1*Δ strains in a manner that is nonpermissive for DNA crosslinking while remaining amendable to acetylation by SAGA. They further suggest that additional protein contacts are required for Ifh1 recruitment and function at RP promoters.

Ifh1 Is Acetylated at Multiple Lysine Residues in an N-Terminal Domain

To identify the sites of Ifh1 acetylation in vivo, we purified flag-tagged Ifh1 from *sir2*Δ *hst1*Δ *hst2*Δ cells (Figure 5A, top panel) and identified acetylated lysine residues in peptides generated

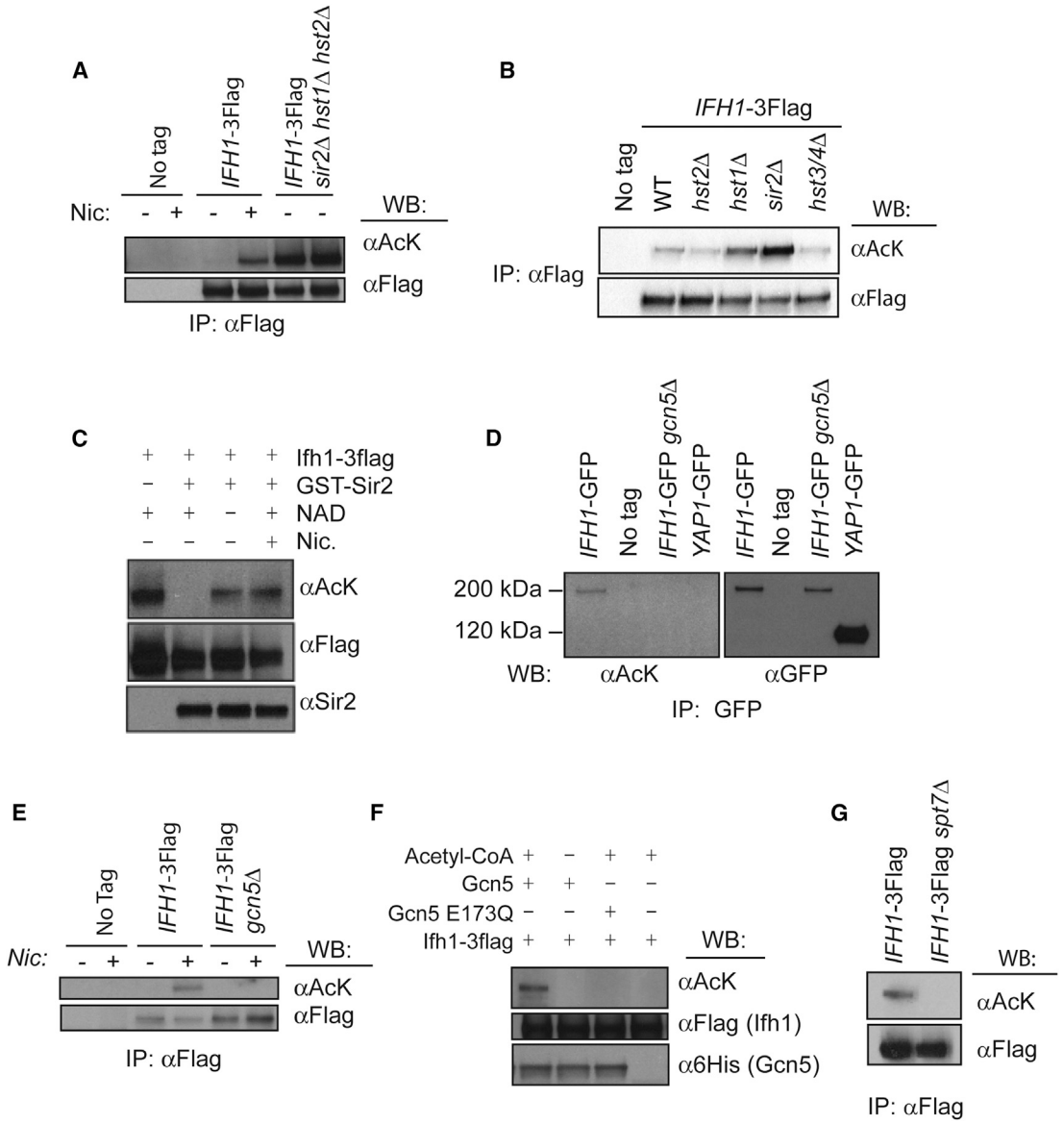

Figure 3. Regulation of Ifh1 Acetylation

(A) Flag-tagged Ifh1 was immunoprecipitated from asynchronously growing cultures of the indicated genotypes and analyzed with an antibody directed toward acetylated lysine or the flag epitope. Two isolates of the sirtuin triple mutant are shown.

(B) Flag-tagged Ifh1 was immunoprecipitated from asynchronously growing cultures of the indicated genotypes and analyzed as in (A).

(C) In vitro deacetylation of Ifh1. Partially purified GST-Sir2 was incubated with acetylated Ifh1 purified from *sir2⊿ hst1⊿ hst2⊿* cells under the reaction conditions indicated for 1 hr at 30°C. Reactions were stopped with the addition of 3× SDS-PAGE sample buffer and separated on a 4%–20% gradient gel prior to western blotting and detection of acetyllysine using an anti-acetyllysine antibody. Nic, inclusion of 5 mM nicotinamide in the reaction as a sirtuin inhibitor.

(D) Gcn5 regulation of Ifh1 acetylation was examined using an IP-western protocol. The abundant Yap1 transcription factor was used to gauge general cross-reactivity of the αAcK antibody.

(E) Gcn5 is required for the increased acetylation observed in nicotinamide-treated samples. Samples were processed as in (D), except that 3× more IPed material was loaded from slow-growing *gcn5⊿* strains relative to wild-type control strains to properly judge the level of acetylation.

(F) Gcn5 acetylation of Ifh1 in vitro. Bacterially purified Gcn5 or catalytic-dead mutant Gcn5 (Gcn5 E173Q) was incubated with Ifh1 purified from *gcn5⊿* yeast under the reaction conditions specified for 1 hr at 30°C. Reactions were stopped with the addition of 3× SDS-PAGE loading buffer and were separated on a 4%–20% gradient gel prior to western blotting and detection of reaction products with the indicated antibodies.

(G) Acetylation levels of Ifh1 IPed from wild-type or *spt7*Δ strains were compared using an anti-acetyllysine antibody following SDS-PAGE and western blotting. See also Figure S2.

by trypsin or chymotrypsin cleavage (see the Supplemental Information). We identified seven acetylation sites of medium or high confidence as judged by spectra quality and peptide abundance (Figure 5A, bottom). Intriguingly, all seven of these sites map to an acidic region in the N-terminal half of Ifh1 (Figure 5B). We tested the relative contribution of these sites to overall Ifh1 acetylation by expressing mutant versions of these proteins, in which identified sites are mutated to arginine, which maintains the charge of a lysine residue but cannot be acetylated, or to glutamine, which structurally mimics an acetylated lysine residue. We found that mutation of lysines 180 and 254 to arginine severely diminished the level of Ifh1

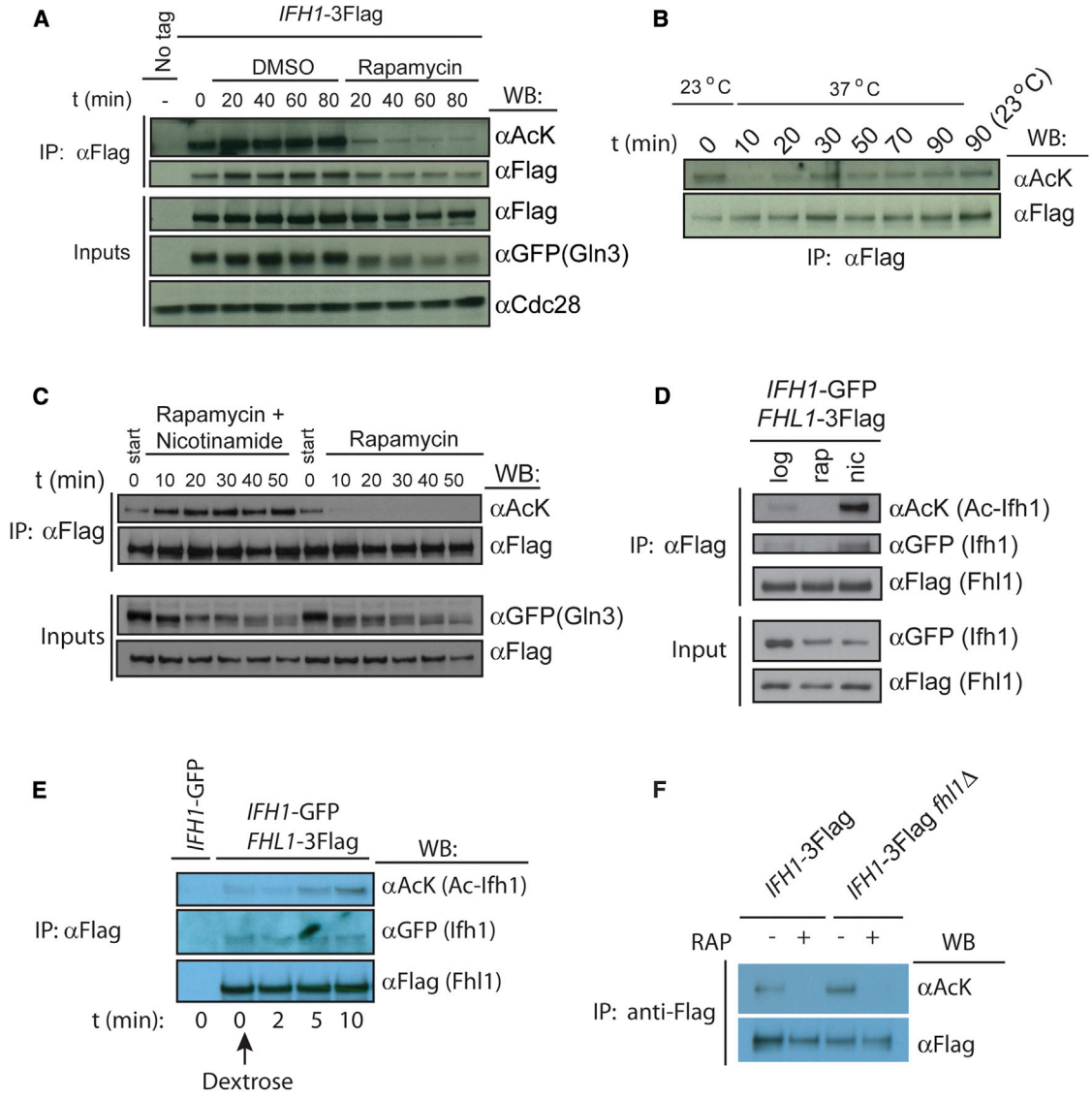

Figure 4. Ifh1 Is Deacetylated following Cellular Stress

(A) Ifh1 acetylation was examined in asynchronously growing cultures treated with 200 ng/ml rapamycin or with vehicle control at the time points indicated. The efficacy of rapamycin treatment was measured by examining loss of TOR-dependent Gln3-GFP phosphorylation in input material.

(B) Ifh1 acetylation during temperature shock. Logarithmically growing cultures were shifted from 23°C to 37°C, and acetylation of Ifh1 was assayed following immunoprecipitation and western blotting.

(C) Sirtuin control of Ifh1 deacetylation following rapamycin treatment. Acetylation of Ifh1 was measured using an IP-western protocol from a strain treated with 200 ng/ml rapamycin either with or without nicotinamide. Nicotinamide was added at the same time as rapamycin at a concentration of 65 mM.

(D) Ifh1 is acetylated when bound to Fhl1. Fhl1-3Flag was immunoprecipitated from log phase cells, cells treated with rapamycin (200 ng/ml for 40 min), or with nicotinamide (65 mM for 40 min), and the recovered material was analyzed with the indicated antibodies following western blotting.

(E) Acetylation of Ifh1 associated with Fhl1 increases during recovery from carbon stress. Fhl1-3Flag was immunoprecipitated from cells after glucose addition to strains growing in glycerol-lactate media, and the recovered material was analyzed with the indicated antibodies after western blotting.

(F) Ifh1 acetylation was analyzed in wild-type or *fhl1*Δ strains with or without 200 ng/ml rapamycin treatment for 60 min.

acetylation observed in IP-western experiments (Figure 5C). The observed signal was completely eliminated in a mutant Ifh1 protein with all seven mapped lysine residues mutated, whether they were changed to arginine or to glutamine (Figure 5C). A second independently generated antibody gave almost identical results to the first, confirming that our mapped sites account for the majority of Ifh1 acetylation observed in vivo (Figure 5C). Neither *ifh1-7k-r* nor *ifh1-7k-q* mutants displayed an obvious growth defect (data not shown), suggesting that Ifh1 acetylation serves a regulatory role.

Acetylation of Ifh1 Affects Its Activity as a Transactivator

Although Ifh1 is essential, simultaneous deletion of the gene encoding its binding partner, Fhl1, rescues this lethality, presumably by alleviating a basal level of repression at RP promoters [41]. Surprisingly, the level of RP transcripts compared to other cellular mRNAs remained relatively unchanged in the *ifh1*Δ *fhl1*Δ double mutant [9]. These data suggested the existence of feedback mechanisms that adjust total transcriptional output when RP transcription is compromised [9]. This and the fact that RP transcription is regulated by many overlapping

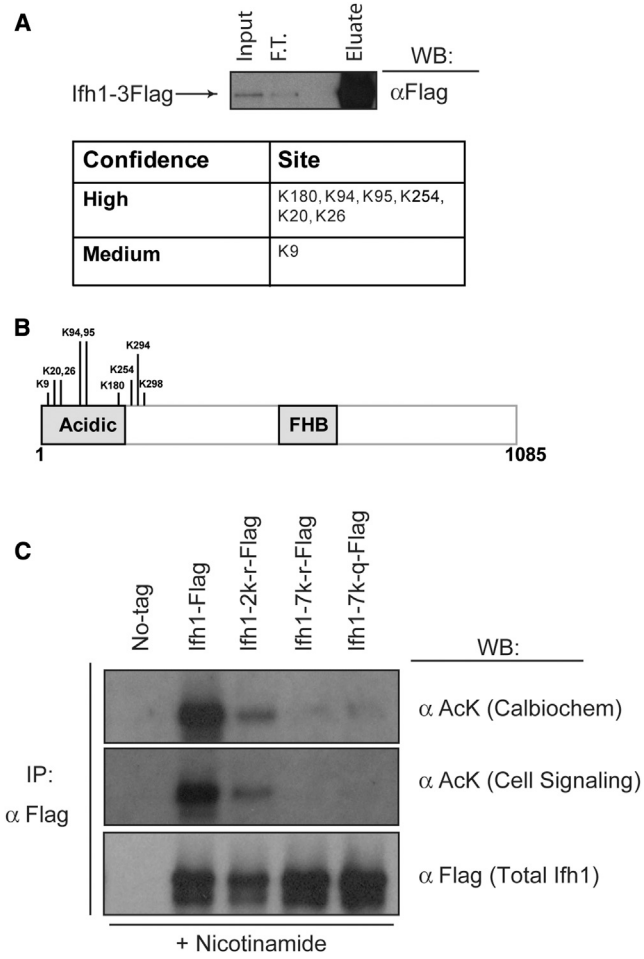

Figure 5. Acetylation of Ifh1 Occurs in an N-Terminal Domain

(A) Flag-tagged Ifh1 was immunopurified from *sir2Δ hst1Δ hst2Δ* triple mutant strains (top), and the recovered protein was trichloroacetic acid precipitated and further purified using SDS-PAGE gel electrophoresis prior to cleavage with trypsin or chymotrypsin and analysis by mass spectrometry. Potential Ifh1 acetylations were manually classified as high-, medium-, or low-confidence sites based on spectra quality and abundance, as determined by spectral counting (bottom).

(B) All high- and medium-confidence sites mapped to the N-terminal acidic region of Ifh1.

(C) In vivo acetylation status of WT Ifh1, Ifh1 with lysines K180 and K254 mutated to arginine (*ifh1-2k-r*), or with lysines 9, 20, 26, 94, 95, 180, and 254 mutated to arginine or glutamine (*ifh1-7k-r* and *ifh1-7k-q*, respectively) was determined following immunoprecipitation from asynchronously growing cultures.

pathways makes it difficult to assess the function of Ifh1 post-translational modification at native RP promoters. Therefore, we first made use of a system in which the contribution of acetylation to Ifh1 transactivator function could be measured in isolation.

We used a one-hybrid system, in which the Gal4-DNA-binding domain (GBD) was fused to the C terminus of Ifh1, Ifh1-7k-r, or Ifh1-7k-q. In these strains, the *GAL1* upstream activating sequence (UAS) has the potential to drive expression of the *HIS3* gene, in addition to *GAL1* itself, by recruiting Gal4-fusion proteins ([43]; Figure 6A). In contrast to other reporter assays used to study Ifh1 function [6–8, 44], our assay does not employ overexpression of Ifh1 fusion proteins but instead relies on the expression of such constructs from the native *IFH1* promoter. Ifh1-, Ifh1-7k-r-, and Ifh1-7k-q-GBD constructs were expressed at equal levels (Figure 6B), and strains in which these fusions are expressed as the only source of Ifh1 grew similarly on synthetic complete media (Figure 6C, left panel). In contrast, strains expressing GBD fusions with nonacetylatable Ifh1 (7k-r) showed significantly better growth than those expressing fusions with wild-type Ifh1 or the 7k-q mutant on plates lacking histidine, where expression of *HIS3* was required for growth. To confirm these results, we measured the mRNA produced from the *GAL1* gene in our one-hybrid system. In this assay, strains expressing the nonacetylatable Ifh1 mutant showed a significant increase in *GAL1* transcripts compared to either wild-type or acetyl-mimic Ifh1 fusion proteins (Figure 6D). When we expressed our Ifh1-GBD constructs from centromere/autonomously replicating sequence plasmids, we observed that all three constructs allowed for some growth in liquid media lacking histidine. Under these conditions, we observed a clear trend, with wild-type Ifh1-GBD constructs allowing for an intermediate level of growth relative to Ifh1-7k-r and Ifh1-7k-q fusions, which conferred faster and slower growth, respectively (Figure 6E). These data suggest that acetylation inhibits Ifh1 function.

Acetylation of Ifh1 Limits RP Transcription Immediately after a Change in Carbon Source

To address the role of Ifh1 acetylation at native RP promoters, we examined the mRNA levels of four RP genes following addition of glucose to cells growing in acetate and glycerol—a nonfermentable carbon source that allows for only a slow rate of growth. By 5 min after glucose addition, wild-type cells had increased RP mRNA levels by 30% (Figure 7A). Consistent with the increased transactivator activity of *ifh1-7k-r* mutants observed in our reporter assays (Figure 6), the levels of RP mRNA increased by 70% in *ifh1-7k-r* cells at this same 5 min time point ($p = 0.002$ for wild-type [WT] versus mutant at $t = 5$ min; Figure 7A). Intriguingly, mRNA appeared to largely equalize in the two strains toward the end of the experiment (Figure 7A). These observations suggest that strains eventually compensate for the initial increase in transcription observed in *ifh1-7k-r* cells (see the Discussion). In contrast, strains expressing the acetyl-mimic—*ifh1-7k-q*—allele showed no significant increase in mRNA levels over wild-type cells but instead showed a decrease in mRNA levels at the 10 min time point (Figure S3A). Treatment of cells with nicotinamide or deletion of *GCN5* both reduced RP transcription after a change in carbon source (Figure S3B), likely due to their pleiotropic effects on a large number of genes. Indeed, this result highlights the importance of examining the specific effects of these regulators on individual targets. Our analysis of Ifh1 mutants suggests that Gcn5 acetylation of Ifh1 in particular functions to restrict the initial increase in RP transcription that accompanies a switch to a more efficient carbon source and a faster rate of growth.

Dynamics of Ifh1 Acetylation

In addition to its binding to Fhl1 at promoters, Ifh1 is a member of the CURI complex, containing Rrp7, Utp22, and Casein kinase II subunits [42]. CURI has been proposed to function as a link between rRNA processing and RP transcription, with free Ifh1 functioning to bind to the Rrp7 and Utp22 rRNA processing factors to inhibit their activity [42]. As such, CURI

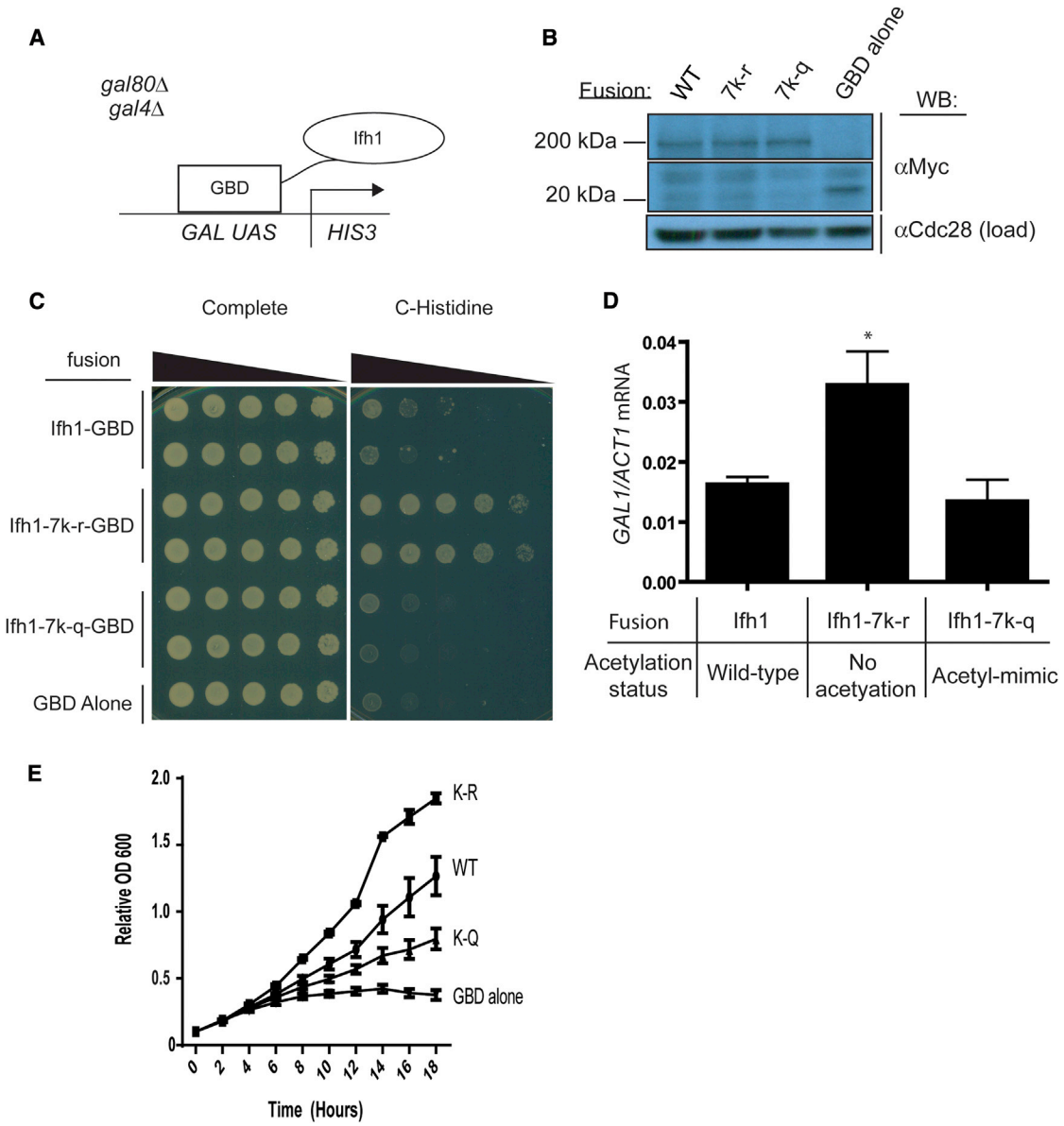

Figure 6. Ifh1 Acetylation Inhibits Its Transactivator Activity

(A) Experimental design. Ifh1 or the indicated mutants were expressed under the Ifh1 promoter as fusion proteins with the GBD in a strain in which the *GAL1* UAS drives transcription of the *HIS3* gene in addition to endogenous *GAL* genes.

(B) Expression of Ifh1 fusion proteins in the strains described in (A). Myc-tagged GBD fusions are the only source of Ifh1 in these strains.

(C) Five-fold serial dilutions of strains expressing the indicated Ifh1 fusions on complete media or complete media lacking histidine (C-histidine). Plates were imaged after 2 days' growth at 30°C.

(D) *GAL1* mRNA level (versus *ACT1* control) in strains expressing the Ifh1 fusion proteins described in (A), as determined by quantitative PCR (qPCR) analysis. Error bars represent the SEM. The asterisk indicates significantly different from WT ($p < 0.03$), with p values calculated using a two-tailed Student's t test ($n = 4$ for each strain type indicated).

(E) The indicated strains were grown to midlog phase in minimal media lacking tryptophan before being washed in water and transferred to media lacking both tryptophan and histidine. Growth, which requires expression of the *HIS3* gene, was assayed using OD 600 readings at the indicated time points. $n = 3$, with error bars indicating the SEM.

is thought to provide a mechanism through which the cell can titrate both rRNA and RP production by regulating Ifh1 availability. We wondered whether CURI might play a role in the acetylation-deacetylation cycle of Ifh1. We found that a significant fraction of the total acetylated Ifh1 in the cell is contained within the CURI complex (Figure 7B). The acetylation of CURI-bound Ifh1 was increased by nicotinamide treatment and eliminated with rapamycin, and these effects could not be explained by changes in Ifh1 binding to CURI (Figure 7B). These data suggest the possibility that Ifh1 acetylated at the promoter may subsequently accumulate in the CURI complex for some period of time after its release. Interestingly, Ifh1 expressed from the strong TEF promoter did not result in an increase in acetylated Ifh1, suggesting that overexpression results in a large free pool of Ifh1 that is not targeted by SAGA (Figure S3C).

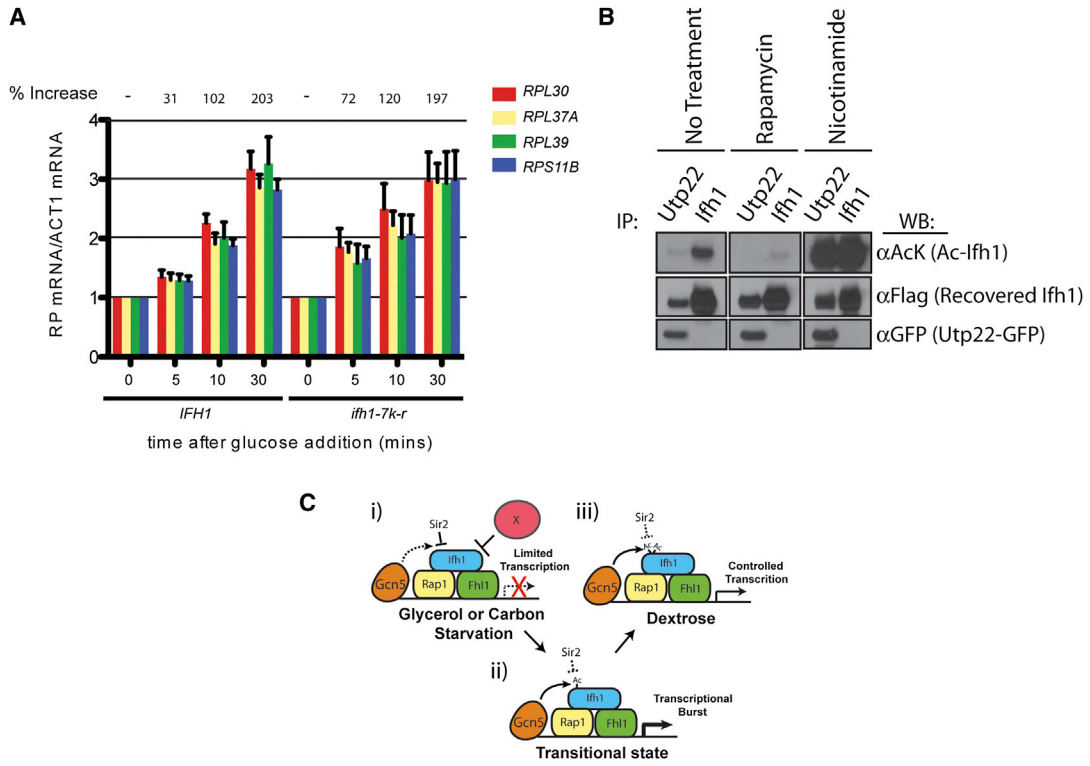

Figure 7. Ifh1 Acetylation Regulates Native RP Transcription following a Change in Carbon Source

(A) Wild-type and *ifh1-7k-r* strains were grown in acetate/glycerol prior to the addition of glucose (t = 0). Samples were collected at the indicated time points, and mRNA levels of the indicated RP genes were quantified, relative to levels of *ACT1* mRNA, using qPCR. The mean and the SEM for seven independent experiments are shown for each gene measured. The average percentage increase of RP mRNAs, relative to time zero, is shown for each strain at the indicated time points.

(B) Acetylated Ifh1 accumulates within the CURI complex. Utp22-GFP was immunoprecipitated from log phase culture or from culture treated with rapamycin (200 ng/ml) or nicotinamide (65 mM) for 40 min. Ifh1-3Flag was immunoprecipitated from a separate strain subjected to the same treatments to serve as a control. Recovered material was analyzed with the indicated antibodies following western blotting.

(C) Model of Ifh1 function at RP promoters. (i) In poor carbon sources (e.g., glycerol), Ifh1 bound at RP promoters via an interaction with Rap1, Fhl1, and possibly other proteins is inhibited directly or indirectly from promoting transcription by unknown factor(s) (protein "X") or through stress-induced changes to overall promoter architecture. Gcn5-mediated acetylation of Ifh1 is inhibited by these same factors or is countered by sirtuin activity. (ii) With the addition of glucose, repression of Ifh1 activity is relieved, resulting in increased transcription. The burst of transcription may function to jump-start ribosome biogenesis. (iii) Inhibition of sirtuin activity accompanying a switch to a better carbon source leads to an increase in Ifh1 acetylation. Acetylation inhibits Ifh1 transactivator function to control the rate with which RP transcripts accumulate to their maximal level. This regulation may serve as part of a broader mechanism to allow for a tightly controlled response to a cell's increased need for ribosome biogenesis.

See also Figure S3.

Discussion

HATs and histone deacetylases (HDACs) that bind to the promoters of genes are often presumed to regulate transcription through the modulation of histone acetylation. We have used a WCE analysis technique to identify the Ifh1 transactivator as a target of the Gcn5 HAT. We provide evidence that Gcn5 acetylation of Ifh1 inhibits its ability to act as a transcriptional activator. We also find that rapamycin and heat shock rapidly eliminate Ifh1 acetylation, likely via directed deacetylation by Sir2. As ribosomal protein gene transcripts account for half of the mRNA in the cell, even a minor disruption to RP transcription caused by alterations to this acetylation-deacetylation cycle may have significant consequences for cellular metabolism. While a role for sirtuins in metabolic regulation is well documented, to our knowledge, our work is the first to suggest a direct connection for these enzymes to the control of RP transcription.

Previous work has demonstrated that the TOR-dependent interaction between Ifh1 and Fhl1 is of critical importance for RP transcription [6–9]. However, we found that the amount of Ifh1 interacting with Fhl1 was unchanged following the addition of glucose to cells growing slowly in glycerol-lactate medium, despite the observation that RP transcription increased substantially under these same conditions (Figures 4E and 7A). Moreover, the Ifh1-Fhl1 interaction was only moderately reduced when cells growing in rich medium were transferred to medium lacking any source of carbon or to water (Figure S2C). Alternative mechanism(s) must therefore exist to prevent RP transcription under some stress conditions, and these mechanisms must be overcome to allow for the burst of RP transcription that accompanies a return to normal growth once such stress is alleviated. Known RP regulators, such as Sfp1, Hmo1, Rpd3, and Esa1, may be involved in regulating the architecture of RP promoters—either to promote or to inhibit transcription—during these transitional states, independently of the Ifh1-Fhl1 interaction.

Our results suggest that Ifh1 acetylation increases as cells recover from stress. Surprisingly, however, cells expressing mutant Ifh1 that cannot be acetylated show increased

mRNA production during recovery from carbon starvation, suggesting an inhibitory role for Ifh1 acetylation. We propose a model wherein promoter-bound and hypoacetylated Ifh1 could act as a strong transactivator to provide an initial burst of RP transcription following recovery from stress or starvation (Figure 7C). This rapid increase to transcriptional output could jump-start ribosome biogenesis in the first minutes following nutrient addition. Our data suggest that SAGA-mediated acetylation of Ifh1 may normally function to limit the strength of this burst of RP transcription. We found that RP mRNA levels increased 70% 5 min after glucose addition in the *ifh1-7k-r* mutant, as opposed to 30% in wild-type cells (Figure 7A). Given that RP mRNAs account for up to half of all RNA pol II-derived messages in the cell [5], this difference amounts to a very large increase in total cellular mRNA transcripts.

Despite a greater increase in RP transcription in *ifh1-7k-r* mutants relative to wild-type controls early during carbon-shift experiments, the levels of RP mRNAs were equalized in wild-type and mutant *ifh1* strains after 30 min (Figure 7A). This equalization may involve a negative feedback mechanism triggered by the initial burst of RP transcription and may function by affecting RP promoter architecture. Controlling the strength and timing of the initial response to the addition of nutrients may help cells to coordinate RP transcription with rRNA processing and other growth-related processes.

How acetylation of Ifh1's acidic domain might inhibit its transactivator function is unclear. The transactivator domain(s) of Ifh1 have been proposed to reside in the C terminus of the protein, although it is not obvious how these domains function to promote RNA polymerase II recruitment and/or activity [44]. Paradoxically, it has both been reported that deletion of the N terminus of Ifh1 can dramatically increase [44] or slightly decrease [6] transactivator activity of Ifh1 in one-hybrid assays. The difference between these assays appears to be whether Ifh1 was recruited to reporter genes directly or indirectly via a GBD fusion with the Fhl1 FHA domain. Moreover, a third study found that full-length Fhl1 fused to a DNA-binding domain was incapable of stimulating transcription, even though this construct promoted Ifh1 recruitment [10]. These data suggest that Fhl1 may have a regulatory role in RP transcription, in addition to its role in initial Ifh1 recruitment. Acetylation in Ifh1's acidic domain may regulate this function. Acetylation may also alter Ifh1's interaction with other promoter-bound proteins, such as Rap1. In this context, it is noteworthy that overexpression of a construct containing an N-terminal Ifh1 fragment disrupts Sir2-mediated telomere silencing, which also requires the Rap1 protein [45].

Ifh1 within the CURI complex and Ifh1 bound to Fhl1 at RP promoters are deacetylated after stress, and this deacetylation requires the action of sirtuins. While Sir2 and Hst2 have been previously implicated in the deacetylation of two nonhistone proteins, Pck1 [19] and Snf2 [46], respectively, Ifh1 is, to our knowledge, the first such protein known to be regulated redundantly by multiple yeast sirtuins. Purified Hst1 was not active against acetylated Ifh1 in our in vitro reactions, although also it appears to act as a poor enzyme in vitro on histone substrates [47]. Hst1-mediated Ifh1 deacetylation may be facilitated in vivo by additional factors, or Hst1 may regulate Ifh1 acetylation indirectly.

During carbon starvation, most Ifh1 localizes to the nucleolus [3], where Sir2 and Hst1 have been shown to function [28]. Since the bulk of Ifh1 appears to be bound within the CURI complex [42], it is tempting to speculate that relocalization of CURI to the nucleolus may facilitate Ifh1 deacetylation. The accumulation of hypoacetylated Ifh1 at RP promoters in particular may also be facilitated by the inhibition of Gcn5 activity toward Ifh1. Although significant changes in the recruitment of the SAGA complex to RP promoters have not been described for logarithmically growing cells, even following stresses, such as heat shock [18], the inhibition of Gcn5's action on Ifh1 could result from the same mechanisms that prevent transcription in the presence of Fhl1-bound Ifh1 molecules. Deacetylation of Ifh1 may reset its ability to act as a strong activator once starvation or stress conditions are relieved.

Experimental Procedures

Details regarding specific experiments are contained in the Supplemental Information.

Yeast Strains, Plasmids, and Growth Conditions

Yeast strains and plasmids were generated using standard techniques and are described in Tables S1 and S2 and the Supplemental Experimental Procedures.

Immunoprecipitations

Cells were lysed using a bead-beating protocol, and WCEs were clarified via centrifugation at 4°C (see the Supplemental Experimental Procedures for specific conditions). Immunoprecipitations were carried out in volumes of 500 μl with 0.5 μl of AB290 anti-GFP antibody for 2 hr. Proteins were then recovered with 20 μl Protein A beads (Dynabeads, Invitrogen) for 40 min. For anti-Flag purifications, magnetic anti-Flag M2 beads (Sigma) were used for 2 hr. Beads were washed with lysis buffer three times and protein complexes were eluted in 60 μl SDS-PAGE sample buffer with 0.1 M dithiothreitol (DTT) at 65°C for 10 min. Eluates were boiled prior to SDS-PAGE.

HAT Assays

HAT assays were carried out in a final volume of 50 μl with 3 μl of 6His-TRX-Gcn5 or 6His-TRX-Gcn5-E173Q (approximately 3 μg), 800 μM acetyl coenzyme A, and 25 μl 2X HAT buffer (100 mM NaCl, 10% glycerol, 100 mM Tris-HCl pH 8.0, and 0.2 mM EDTA supplemented with 2 mM sodium butyrate and 2 mM DTT). Ifh1-3flag protein was purified from *gcn5*Δ yeast. Reactions were carried out at 30°C for 1 hr, stopped with the addition of 3× SDS-PAGE sample buffer containing 0.1M DTT, and boiled prior to SDS-PAGE.

HDAC Assays

Ifh1 purified from *hst1*Δ *hst2*Δ *sir2*Δ cells was used in a final volume of 25 μl with 5 μl 5X HDAC reaction buffer (250 mM Tris HCl, pH 8.0, 2.5 mM DTT, one Roche Protease inhibitor tablet without EDTA per 10 ml), 10 μl GST-Sir2 (approximately 0.5 μg total), and 100 μM NAD. Nicotinamide was used at a final concentration of 5 mM. Reactions were incubated for 1 hr at 30°C. Reactions were stopped with the addition of SDS-PAGE sample buffer with 0.1 M DTT and boiled to remove Ifh1 from beads.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.050.

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