Eukaryotic Cell

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Eukaryotic Cell **2013, 12(8):1142. DOI: 10.1128/EC.00123-13.**
Published Ahead of Print 21 June 2013.

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Mutual Cross Talk between the Regulators Hac1 of the Unfolded Protein Response and Gcn4 of the General Amino Acid Control of *Saccharomyces cerevisiae*

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Hac1 is the activator of the cellular response to the accumulation of unfolded proteins in the endoplasmic reticulum. Hac1 function requires the activity of Gcn4, which mainly acts as a regulator of the general amino acid control network providing *Saccharomyces cerevisiae* cells with amino acids. Here, we demonstrate novel functions of Hac1 and describe a mutual connection between Hac1 and Gcn4. Hac1 is required for induction of Gcn4-responsive promoter elements in haploid as well as diploid cells and therefore participates in the cellular amino acid supply. Furthermore, Hac1 and Gcn4 mutually influence their mRNA expression levels. Hac1 is also involved in *FLO11* expression and adhesion upon amino acid starvation. Hac1 and Gcn4 act through the same promoter regions of the *FLO11* flocculin. The results indicate an indirect effect of both transcription factors on *FLO11* expression. Our data suggest a complex mutual cross talk between the Hac1- and Gcn4-controlled networks.

The baker's yeast *Saccharomyces cerevisiae* executes two well-established pathways, the general amino acid control (GAAC) and the unfolded protein response (UPR). The GAAC regulatory network is induced not only by amino acid starvation or imbalances but also by other environmental stimuli, including supply of glucose (1), purines (2), and tRNA synthetases (3). A variety of physiological or environmental stress conditions such as calcium depletion, glucose deprivation, hypoxia, or misfolded proteins lead to an accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER) lumen, which results in the induction of the UPR (4–7). These pathways are conserved in mammals, where they are essential.

The bZIP transcription factor Gcn4 represents the global key activator of the GAAC (8) and regulates transcription of numerous metabolic genes of amino acid or purine biosynthesis in response to amino acid starvation (9–12). In contrast to its mammalian homologues, yeast Gcn4 can bind only as a homodimer to a specific 9-bp palindromic nucleotide sequence (5′-ATGA[C/G]TCAT-3′) (termed Gcn4 protein recognition element [GCRE]) (13, 14). These GCREs are located upstream of many genes induced by amino acid starvation. Gcn4 can also bind to naturally occurring variants of this sequence (TGATTCA, TGACTCT, TGACTGA, TGACTAT, and ATGACTCT), and therefore, using a computer algorithm, this consensus site was generalized to RRRWGASTCA (R = purine, W = T or A, and S = G or C) (9). Gcn4 also binds to GCRE half-sites with high affinity *in vitro* (15, 16).

Gcn4 not only acts as a metabolic regulator but also has a developmental function. In response to nutrient starvation, Gcn4 is involved in the regulation of *FLO11* expression (17, 18). The cell surface flocculin Flo11, also named Muc1, is required for diploid pseudohypha formation and for adhesion upon nutrient starvation (19–22).

Hac1 plays a central role in the yeast UPR system and represents a bZIP transcription factor, like Gcn4 (23, 24). Conserved from yeast to mammals is the sensing and response pathway that is transduced by Ire1, leading to an upregulation of transcription levels of approximately 400 genes, i.e., 7% to 8% of the yeast genome (25–30). In *S. cerevisiae*, Ire1 senses the stress and mediates a signaling cascade to upregulate responsive genes through unconventional splicing of *HAC1* mRNA. Ire1 encodes a bifunctional transmembrane kinase/endoribonuclease consisting of an unfolded protein sensor domain in the ER lumen, a transmembrane domain, and a cytosolic effector domain, which contains an intrinsic serine/threonine kinase as well as an endoribonuclease in its C terminus (26, 27, 31–33). An accumulation of misfolded proteins in response to ER stress leads to oligomerization and *trans*-autophosphorylation of Ire1 (34, 35). This in turn results in an activated cytosolic endonuclease effector domain (33, 36). Ire1 recognizes two "loop" structures in the *HAC1* mRNA. The transcript is constitutively synthesized as a precursor bearing a 252-nucleotide intron that blocks translation, and the endonuclease effector domain of Ire1 splices the *HAC1* mRNA (37–39). Subsequently, the tRNA ligase Rlg1 religates, causing exons to produce the mature, efficiently translated *HAC1* mRNA (33, 38). As the level of Hac1 rises in the cell, the genes that harbor unfolded protein response elements (UPREs) within their promoters are induced at the transcriptional level (40).

The synthesis of Hac1 in response to ER stress is regulated not only at its translational level but also by mechanisms that regulate the rate of turnover of Hac1. A similar mechanism had been described previously for the bZIP transcription factor Gcn4 (41–43). Like Gcn4, Hac1 is ubiquitinated by the SCF^{Cdc4} E3 ligase complex, resulting in degradation by the 26S proteasome. Furthermore, phosphorylation by the cyclin-dependent kinase (CDK)

Received 23 May 2013 **Accepted** 19 June 2013
Published ahead of print 21 June 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00123-13.

doi:10.1128/EC.00123-13

Srb10 marks Hac1 for ubiquitination, similarly to Gcn4, whereas phosphorylation by the CDK Pho85 was not observed so far. Hac1 also contains a PEST region, which is typical for rapid turnover of transcription factors (44).

At least 381 UPR target genes were identified in yeast and encode functions ranging from protein folding, protein translocation, and protein transport to protein degradation within the secretory pathway. Whereas the predicted UPRE-1 consensus sequence (CAGNGTG) was absent in most of them (25), Patil and coworkers identified two further UPREs, which are recognized by Hac1 (UPRE-2, consensus sequence TACGTG; UPRE-3, consensus sequence AGGAACAAC) (45). Apart from its role as a transcriptional activator of the GAAC, Gcn4 and its activator Gcn2 are required for induction of a majority of UPR target genes upon ER stress. A direct binding of Gcn4 could be demonstrated for UPRE-1 and UPRE-2, while binding to UPRE-1 was Hac1 dependent. In contrast, Gcn4 is not necessary for the regulation of genes without a recognizable UPRE, which represent half of all UPR targets. Both Hac1 and Gcn4 are bZIP transcription factors. Heterodimer formation of Gcn4/Hac1 or its mammalian counterparts ATF4/XBP1 is an attractive hypothesis to explain the mechanism (45) but could not be verified yet. Recently, Fordyce and coworkers discovered that Hac1 binds only to UPRE-2 but not to the 7-bp UPRE-1 sequence, hereafter termed core UPRE-1 (cUPRE-1) (46). However, they could demonstrate that an extended core UPRE-1 (xcUPRE-1) containing flanking sequences is important for Hac1 binding. Therefore, the 7-bp cUPRE-1 consensus sequence can be extended to a 12-bp UPRE-1-like motif (GGACAGCGTGTC).

In this study, we identified novel functions of Hac1 in metabolic and developmental processes regulated by Gcn4. We demonstrate not only that Gcn4 is able to activate Hac1-specific target genes but also that Hac1 is involved in Gcn4-specific target gene regulation and *FLO11* expression in response to amino acid starvation.

MATERIALS AND METHODS

Yeast strains and growth conditions. All yeast strains used in this study are listed in Table 1. They are derivates of the *S. cerevisiae* strain background Σ1278b unless otherwise stated. Transformations were carried out using the lithium acetate method (47).

Yeast strains RH3351, RH3352, RH3402, and RH3403 containing a *hac1* deletion were constructed by amplification of the kanamycin resistance cassette from the Euroscarf strain collection (48) containing sequence homologous to the up- and downstream regions of the relevant gene. Integration of deletion cassettes was obtained by homologous recombination in yeast strains RH2676, RH2816, RH2819, and RH3401. Positive transformants could be selected on yeast extract-peptone-dextrose (YEPD) medium supplemented with 200 μg/ml Geneticin G418 sulfate (Carl Roth GmbH, Karlsruhe, Germany). For genetic crosses, the kanamycin resistance cassette of RH3352 and RH3403 was replaced by a nourseothricin resistance cassette, which was amplified from plasmid pAG25. Transformants (RH3404 and RH3405) could be selected on YEPD medium containing 100 μg/ml nourseothricin (clonNAT; Werner BioAgents, Jena, Germany). Homo- and heterozygous diploid strains RH3412 to RH3416 were obtained by crossing strain RH2676, RH3351, or RH3402 with strain RH2819, RH3404, or RH3405. Haploid yeast strains RH3360, RH3406, RH3407, and RH3408 were obtained by introducing the *FLO11*::*lacZ*::*URA3* cassette using ApaI-linearized plasmid pME2213 into the *URA3* locus of yeast strains RH2676, RH2816, RH3351, and RH3402. The *GCRE6*::*lacZ*::*URA3* reporter gene cassette was introduced in the same four haploid yeast strains by transformation with StuI-linearized *GCRE6*::*lacZ* reporter construct pME1112 to obtain yeast strains RH3363, RH3409, RH3410, and RH3411. The haploid *FLO11*- and *GCRE6*::*lacZ*-containing *MAT***a** strains were crossed with *MAT*α strain RH2819, RH3352, RH3404, or RH3405 to produce diploid strains RH3349, RH3350, RH3362, and RH3417 to RH3425. All gene deletions, integrations, or replacements were confirmed by PCR and Southern blot analysis (49).

Strains were routinely cultivated in standard yeast extract-peptone-dextrose (YEPD; 1% yeast extract, 2% peptone, 2% dextrose) or minimal yeast nitrogen base medium (YNB; 1,5 g/liter yeast nitrogen base lacking amino acids and ammonium sulfate, 5 g/liter ammonium sulfate, 2% dextrose supplemented with the appropriate amino acids). Solid media were prepared using 2% agar.

For β-galactosidase assays, strains were cultivated in liquid synthetic minimal medium (YNB) overnight at 30°C, diluted into fresh medium, and cultivated for 6 h before assaying enzymatic activities. For amino acid starvation, 3-amino-1,2,4-triazole (3AT) (Sigma-Aldrich, Steinheim, Germany) was added to fresh diluted cultures to a final concentration of 10 mM, and cells were incubated for 8 h before further assays. For nitrogen starvation, cells grown to logarithmic phase were washed twice with 2% glucose and incubated for 24 h in liquid YNB medium containing only 50 μM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. Tunicamycin (Tm) (Calbiochem/Merck KGaA, Darmstadt, Germany) was added to fresh diluted cultures to a final concentration of 1 μg/ml and incubated for 6 h to induce UPR stress. Additionally, cultures grown to log phase (4 h or instead 6 h in YNB) were treated with 1 μg/ml Tm for 15, 30, 60, 90, and 120 min.

To compare strains under different conditions in Western hybridization experiments, strains were cultivated in 250 ml liquid synthetic minimal medium (YNB) to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 at 30°C, subsequently divided into 50-ml cultures, and cultivated for a further 90 min under different conditions each.

Plasmids. All plasmids used in this study are listed in Table 2. Plasmid pME3498 expressing the *HAC1* inclusive intron under the *MET25* promoter was constructed by amplifying *HAC1* with *Pfu*Ultra HF DNA polymerase (Promega, Mannheim, Germany) from genomic DNA and introducing it as a XbaI/ClaI fragment into SpeI/ClaI-restricted p426MET25.

Protein analysis. Whole-cell extracts of *S. cerevisiae* were prepared from yeast cultures grown to exponential phase. Cells were washed in 2.5 ml ice-cold buffer B (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 20% glycerin), lysed with glass beads (diameter, 0.25 to 0.5 mm; Carl Roth GmbH, Karlsruhe, Germany) in 500 μl of B-buffer-containing protease inhibitors (Complete, EDTA-free; Roche Diagnostics GmbH, Mannheim, Germany) and 14.3 mM β-mercaptoethanol at 4°C, and centrifuged at 13,000 rpm for 12 min to remove glass beads and large pieces of cell debris. Extracts (10 μl) were removed to determine total protein concentration using the Bradford protein assay (50), and proteins were denatured in SDS loading dye by heating at 65°C for 15 min. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Hac1, the α subunit of eukaryotic initiation factor 2 (eIF2α), and eIF2α-P were detected using ECL technology (Amersham, United Kingdom). For the first incubation, polyclonal rabbit anti-Hac1 (gift from Kazutoshi Mori, Kyoto University, Japan), polyclonal rabbit anti-eIF2α (pS52) (catalog no. 44728G; Invitrogen, Darmstadt, Germany), or rabbit anti-eIF2α (gift from Thomas Dever, NIH, Bethesda, MD, USA) antibodies were used. Peroxidase-coupled goat anti-rabbit IgG was used as a secondary antibody (catalog no. G21234; MoBiTec, Göttingen, Germany).

Adhesive growth tests. Amino acid starvation-induced adhesive growth tests on solid YNB medium were performed as described previously (18, 51). For visualization of biofilms in wells of polystyrene plates, assays were performed as described in reference 19.

Growth tests. Yeast strains were precultured to the same optical densities (OD_{600} = 0.6) and then diluted 10-fold, starting with 3×10^4 cells per 20 μl. For each dilution, 20 μl was spotted onto solid YNB medium with or without 0.5 μg/ml tunicamycin for ER stress survival assays and

TABLE 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
BY4741	*MAT***a** *his3*::*hisG leu2*::*hisG met15*::*hisG ura3*::*hisG* (S288c background)	48
Y05650	*MAT***a** *his3*::*hisG leu2*::*hisG met15*::*hisG ura3*::*hisG* Δ*hac1*::*kanMX4* (S288c background)	48
RH2398	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG his3*::*hisG/HIS3 trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2*	58
RH2656	*MAT***a**/α *ura3-52/ura3-52 trp1*::*hisG/TRP1*	18
RH2658	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2*	18
RH2661	*MAT***a**/α *ura3-52/ura3-52 trp1*::*hisG/TRP1* Δ*flo11*::*kanMX4*/Δ*flo11*::*kanMX4*	18
RH2676	*MAT***a** *ura3-52 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2*	Our collection
RH2681	*MAT***a** *ura3-52 trp1*::*hisG* Δ*flo11*::*kanMX4*	Our collection
RH2694	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2*	Our collection
RH2695	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2*	Our collection
RH2816	*MAT***a** *ura3-52 trp1*::*hisG his3*::*hisG*::*HIS3*	72
RH2819	*MAT*α *ura3-52 leu2-hisG his3*::*hisG*::*HIS3*	72
RH3349	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3350	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3351	*MAT***a** *ura3-52 trp1*::*hisG* Δ*hac1*::*kanMX4*	This study
RH3352	*MAT*α *ura3-52 leu2*::*hisG* Δ*hac1*::*kanMX4*	This study
RH3360	*MAT***a** *ura3-52*::*FLO11*::*lacZ*::*URA3 trp1*::*hisG* Δ*hac1*::*kanMX4*	This study
RH3362	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*	This study
RH3363	*MAT***a** *ura3-52*::*GCRE6*::*lacZ*::*URA3 trp1*::*hisG* Δ*hac1*::*kanMX4*	This study
RH3401	*MAT*α *ura3-52 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2*	Our collection
RH3402	*MAT***a** *ura3-52 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*	This study
RH3403	*MAT*α *ura3-52 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*	This study
RH3404	*MAT*α *ura3-52 leu2*::*hisG* Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3405	*MAT*α *ura3-52 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3406	*MAT***a** *ura3-52*::*FLO11*::*lacZ*::*URA3 trp1*::*hisG*	This study
RH3407	*MAT***a** *ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2*	This study
RH3408	*MAT***a** *ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*	This study
RH3409	*MAT***a** *ura3-52*::*GCRE6*::*lacZ*::*URA3 trp1*::*hisG*	This study
RH3410	*MAT***a** *ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2*	This study
RH3411	*MAT***a** *ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG trp1*::*hisG* Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*	This study
RH3412	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3413	*MAT***a**/α *ura3152/ura3-52 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/*HAC1*	This study
RH3414	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4*	This study
RH3415	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/trp1*::*hisG* Δ*gcn4*::*LEU2*/Δ*gcn4*::*LEU2* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3416	*MAT***a**/α *ura3-52/ura3-52 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4* Δ*hac1*::*kanMX4*/*HAC1*	This study
RH3417	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1*	This study
RH3418	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/*HAC1*	This study
RH3419	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4*	This study
RH3420	*MAT***a**/α *ura3-52/ura3-52*::*FLO11*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4* Δ*hac1*::*kanMX4*/*HAC1*	This study
RH3421	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1*	This study
RH3422	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/Δ*hac1*::*kanMX4*::*natMX4*	This study
RH3423	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/LEU2 trp1*::*hisG/TRP1* Δ*hac1*::*kanMX4*/*HAC1*	This study
RH3424	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4*	This study
RH3425	*MAT***a**/α *ura3-52/ura3-52*::*GCRE6*::*lacZ*::*URA3 leu2*::*hisG/leu2*::*hisG trp1*::*hisG/TRP1* Δ*gcn4*::*LEU2*/*GCN4* Δ*hac1*::*kanMX4*/*HAC1*	This study

on selective YNB medium with or without 5 mM 3AT for resistance upon amino acid starvation. After incubation for 3 to 4 days at 30°C, plates were photographed under white light.

β-Galactosidase assay in *S. cerevisiae*. Starting from one overnight culture, strains carrying either a *UPRE*-, a *FLO11*-, a *GCRE6*-, or a *GCN4*::*lacZ* reporter were diluted into fresh medium and further cultivated for 6 to 24 h before they were harvested. The incubation term was based on the medium (for details, see "Yeast strains and growth conditions"). Extracts were prepared and assayed for specific β-galactosidase activity as described previously (52) and normalized to the total protein (50), resulting in the specific enzyme activity ($OD_{420} \times 0.35$)/(0.0045 × protein concentration × extract volume × time). Assays were performed for at least three independent cultures.

Analysis of *FLO11* promoter elements. Rupp et al. (53) constructed a set of 14 reporter constructs containing individual 400-bp *FLO11* promoter fragments that overlap by 200 bp and were cloned in front of a *CYC1*::*lacZ* fusion gene. Thus, after transformation of these constructs in the diploid wild-type strain as well as in Δ*hac1*/Δ*hac1* and Δ*gcn4*/Δ*gcn4*

TABLE 2 Plasmids used in this study

Plasmid	Description	Source
B3782	3-kbp *FLO11*::*lacZ* in YEp355	53
p180	*GCN4*::*lacZ* reporter construct (uORFs 1 to 4) on a centromere vector (*URA3*)	59
p227	*GCN4*::*lacZ* reporter construct (without uORFs) on a centromere vector (*URA3*)	73
pAG25	NatMX4 cassette in pFA6	74
p426MET25	pRS426 containing *MET25* promoter, *CYC1* terminator	75
p*FLO11-2/1* to p*FLO11-15/14*	440-bp sequence elements cloned into pLG669Z from bp −1 to −420, bp −180 to −620, and bp −380 to −1020 until bp −2580 to −2980	53
pLG669Z	*lacZ* shuttle vector	76
pMCZ-Y	UPRE-*CYC1-lacZ* reporter construct on multicopy vector (*URA*)	23
pME1092	2.8-kb *GCN4* fragment in pRS314	57
pME1112	Integrative *GCRE6*::*lacZ* reporter construct	57
pME2212	pLG669Z without UAS[a]	18
pME2213	Integrative *FLO11*::*lacZ* reporter construct	Our collection
pME2901	*GCN4prom-GCN4^{L267S}-GCN4-term* in pRS314	17
pME3378	*GCN4prom-GCN4^{L253G}-GCN4-term* in pRS314	17
pME3498	*MET25prom-HAC1* in p426MET25	This study
pRS314	*TRP CEN* Amp^r *ori*	77
pRS426	*URA3* 2μm Amp^r *ori*	77

[a] UAS, upstream activation sequence.

mutant strains, the influence of the transcription factors on specific regions in the *FLO11* promoter can be determined by β-galactosidase assays. A construct without an insert served for background measurements.

RNA isolation and quantitative real-time PCR (qRT-PCR). Total RNA was isolated from yeast cells that were grown in YNB in the presence (8 h) or absence (6 h) of 10 mM 3AT using the High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) to determine *FLO11* transcript levels. For analyzing *GCN4* or *HAC1* mRNA expression levels, yeast cells were grown to an OD_{600} of ~0.6 at 30°C before division into independent cultures and further cultivation for 90 min under indicated conditions. cDNA synthesis was performed in duplicate for each sample using 0.8 μg RNA and the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Twenty nanograms of cDNA was used as the template for quantitative real-time PCR (qRT-PCR) experiments, and amplification was performed in a LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) using the RealMaster SYBR Rox kit (5Prime GmbH, Hamburg, Germany). Independent PCRs were performed using the same cDNA for both genes of interest (*FLO11*, *GCN4*, and *HAC1*) and *CDC28* or *H2A* as reference. The following temperature profile was applied after an initial denaturation at 95°C for 2 min 20 s: 20-s denaturation at 95°C, 22-s hybridization at 64°C, and 22-s elongation at 70°C. The 40 cycles were followed by construction of a melting curve to determine PCR specificity, contamination, and the absence of primer dimers. Gene expression was quantified using the threshold cycle (ΔC_T) method with efficiency. qRT-PCR experiments were performed from three independent cultures for each strain and condition.

RESULTS

The high-copy-number suppressor Hac1 supports Gcn4 activity during amino acid starvation. In *S. cerevisiae*, amino acid starvation triggers the activation of the general amino acid control (GAAC) network and the cell surface flocculin gene *FLO11* by the transcriptional activator Gcn4 (9, 18, 54). The Gcn4^{L267S} variant carrying an amino acid substitution in the third of the four conserved leucines of the zipper dimerization domain results in a transcription factor which is less active than wild-type Gcn4. Indeed, Gcn4^{L267S} has a reduced activation of metabolic genes in comparison to that of wild-type Gcn4, which is, however, sufficient to fulfill metabolic processes and to permit growth under amino acid starvation. In contrast, the developmental function of Gcn4^{L267S} is diminished. Gcn4^{L253G} carrying a helix breaker substitution at Leu253 results in a highly stable but transcriptionally inactive protein (17). We searched for a high-copy-number suppressor of Gcn4^{L267S} that improves the transcriptional activity and growth during amino acid starvation. Patil et al. (45) described interplay between Gcn4 and the UPR bZIP transcription factor Hac1. We analyzed whether an overexpression of the native unspliced *HAC1* influences growth of the *gcn4* deletion strain expressing different *GCN4* variants. Amino acid starvation was induced by addition of the histidine analogue 3-amino-1,2,4-triazole (3AT) to the growth medium, which acts as a false feedback inhibitor and inhibits the histidine biosynthetic enzyme His3 (55). Due to the fact that most natural yeasts are dimorphic and diploid and that the reduction in transcriptional activation capacity of Gcn4^{L267S} is less severe in haploids, we used diploid yeast cells (17, 56). Growth of the diploid *gcn4* deletion strain expressing *GCN4^{L267S}* was enhanced during amino acid starvation when the bZIP transcription factor Hac1 was overexpressed (Fig. 1). Furthermore, growth of yeast cells expressing wild-type *GCN4* in the presence of 3AT could be strengthened while overexpressing *HAC1*. In contrast, overexpression of *HAC1* could not complement the growth defect of Δ*gcn4* cells expressing the stable but inactive *GCN4^{L253G}* upon amino acid starvation.

This suppressor analysis supports a cross talk where the transcription factor Hac1 can support Gcn4 activity in amino acid biosynthesis. Hac1 controls the unfolded protein response (UPR), which is activated due to accumulation of misfolded proteins in the endoplasmic reticulum (23). The involvement of Hac1 in the Gcn4-mediated supply of amino acids (general amino acid control [GAAC]) has not been described yet, whereas it is known *vice*

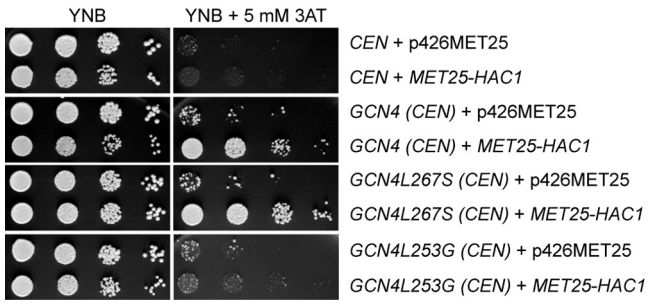

FIG 1 Hac1 enhances Gcn4 activity during amino acid starvation. The diploid *gcn4* deletion strain (RH2694) expressing wild-type *GCN4* (pME1092), the substituted *GCN4^{L267S}* (pME2901) or *GCN4^{L253G}* (pME3378) from *CEN* plasmids with *GCN4* promoter and terminator, or, as a control, the empty vector (*CEN*) (pRS314) was additionally transformed with either independently overexpressed *HAC1* (*MET25-HAC1*) (pME3498) or an empty vector (p426MET25). Strains were spotted in 10-fold dilutions either on YNB as a control or on YNB medium containing 5 mM 3AT to induce amino acid starvation. After incubation for 3 to 4 days at 30°C, plates were photographed.

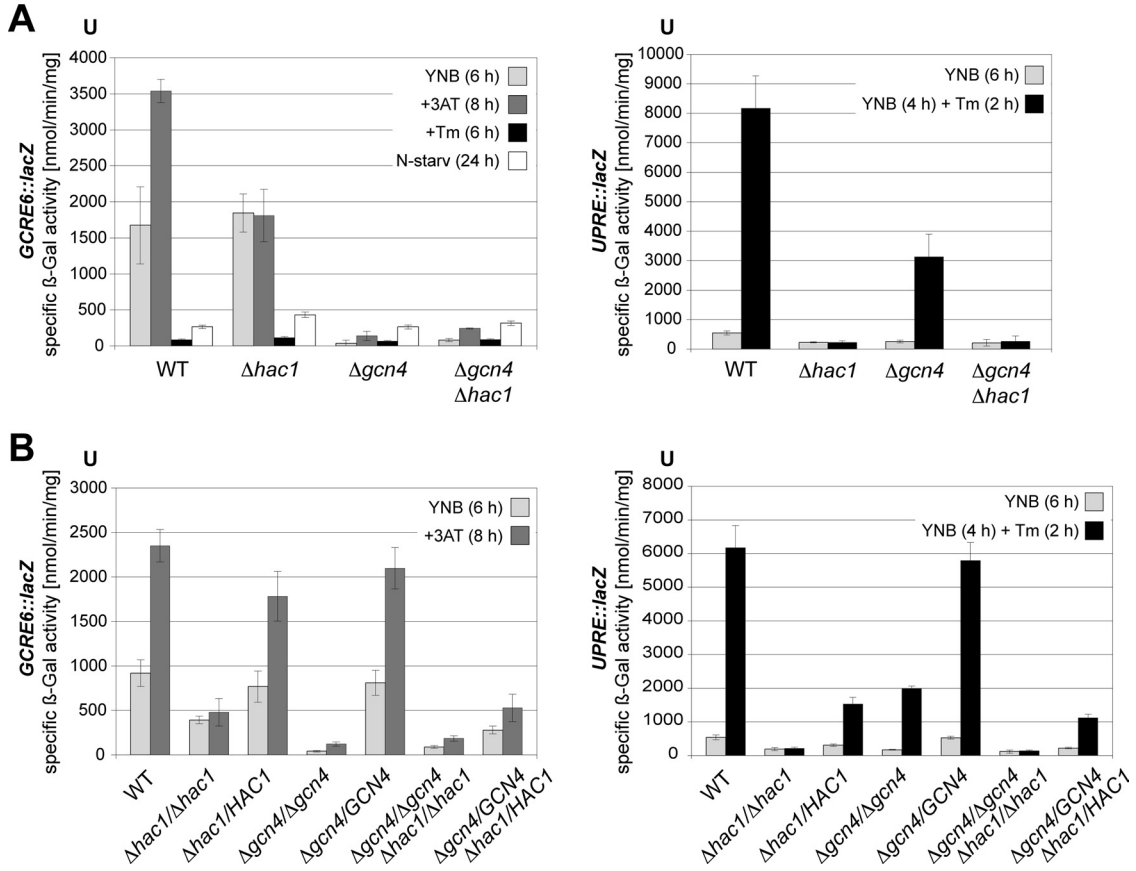

FIG 2 Hac1 is required to induce GCREs. (A) Expression of *GCRE6*::*lacZ* was determined in the haploid Σ1278b wild-type strain (WT) (RH3409) as well as in Δ*hac1* (RH3363), Δ*gcn4* (RH3410), and Δ*gcn4* Δ*hac1* (RH3411) mutant strains each carrying a chromosomally integrated *GCRE6*::*lacZ* reporter. Expression was measured under different nutritional conditions. Starting from one overnight culture, strains were diluted into fresh medium and further cultivated for 6 to 24 h in the respective media before specific β-galactosidase activities were assayed. Cultures were grown to log phase in YNB under nonstarvation conditions [light gray bars, YNB (6 h)]. Amino acid starvation and ER stress conditions were induced by addition of either 10 mM 3AT [dark gray bars, +3AT (8 h)] or 1 μg/ml tunicamycin [black bars, +Tm (6 h)]. For nitrogen starvation (N-starv), yeast cells were washed twice with 2% glucose before incubation for 24 h in minimal medium containing only 50 μM ammonium sulfate as the sole nitrogen source [white bars, N-starv (24 h)]. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. The bars represent the mean values of at least three independent measurements. As additional control, the haploid Σ1278b wild-type strain (WT) (RH2816), as well as Δ*hac1* (RH3351), Δ*gcn4* (RH2676), and Δ*gcn4* Δ*hac1* (RH3402) mutant strains, was transformed with the UPRE-*CYC1*-*lacZ* reporter gene carried on a multicopy vector (pMCZ-Y). Starting from one overnight culture, strains were diluted into fresh medium and further cultivated for 6 h in the respective media before specific β-galactosidase activities were assayed. Either cultures were grown to log phase in YNB medium [light gray bars, YNB (6 h)], or ER stress conditions were induced by the addition of 1 μg/ml tunicamycin for 2 h [black bars, YNB (4 h) + Tm (2 h)]. Units of specific β-galactosidase activities are shown in nanomoles per minutes per milligram. The bars represent the mean values based on quadruplicate determinations of at least three independent transformants. (B) The diploid wild-type yeast strain (WT) (RH3421), as well as homo- and heterozygous Δ*hac1*/Δ*hac1* (RH3422), Δ*hac1*/*HAC1* (RH3423), Δ*gcn4*/Δ*gcn4* (RH2398), Δ*gcn4*/*GCN4* (RH3424), Δ*gcn4*/Δ*gcn4* Δ*hac1*/Δ*hac1* (RH3350), and Δ*gcn4*/*GCN4* Δ*hac1*/*HAC1* (RH3425) mutant strains each carrying a chromosomally integrated *GCRE6*::*lacZ* reporter, was grown to log phase in YNB in the absence (light gray bars, YNB) or presence (dark gray bars, +3AT) of 10 mM 3AT before specific β-galactosidase activities were assayed. As a further control, the diploid yeast strains indicated on the abscissa were transformed with the UPRE-*CYC1*-*lacZ* reporter gene (pMCZ-Y) and transformants were grown in the presence or absence of tunicamycin before being used for β-galactosidase assays.

versa that Gcn4 is involved in the unfolded protein response (UPR) (45).

Hac1 is required for inducing GCREs in haploid and diploid yeast cells. Subsequently, we examined whether Hac1 is required for activating the canonical Gcn4 promoter elements (GCREs). The influence of the *hac1* deletion in comparison to the wild type was analyzed with a Gcn4-specific reporter which contains six GCRE binding sites upstream of the *CYC1*::*lacZ* minimal promoter. This construct was chromosomally integrated into the *URA3* locus (57). Basal expression of the *GCRE6*::*lacZ* reporter was almost identical in wild-type and Δ*hac1* cells under nonstarvation conditions. However, Δ*hac1* cells were unable to induce the GCREs in response to amino acid starvation (3AT) and remained at the basal level as in sated wild-type cells without amino acid limitation (Fig. 2A). As expected, strains containing a *gcn4* deletion were unable to activate *GCRE6*::*lacZ* expression in response to amino acid starvation. We compared the effect of Hac1 on the Gcn4 element GCRE with the effect of Gcn4 on the Hac1-dependent unfolded protein response element (UPRE). The reported *UPRE*::*CYC1*::*lacZ*, which is induced by tunicamycin-mediated ER stress, was tested (23). The strong induction of the *UPRE*::*CYC1*::*lacZ* reporter in the wild type is abolished when the transcription factor Hac1 is absent (Δ*hac1*). A defective *GCN4* gene (Δ*gcn4*) results in partial induction. The double mutation Δ*gcn4*

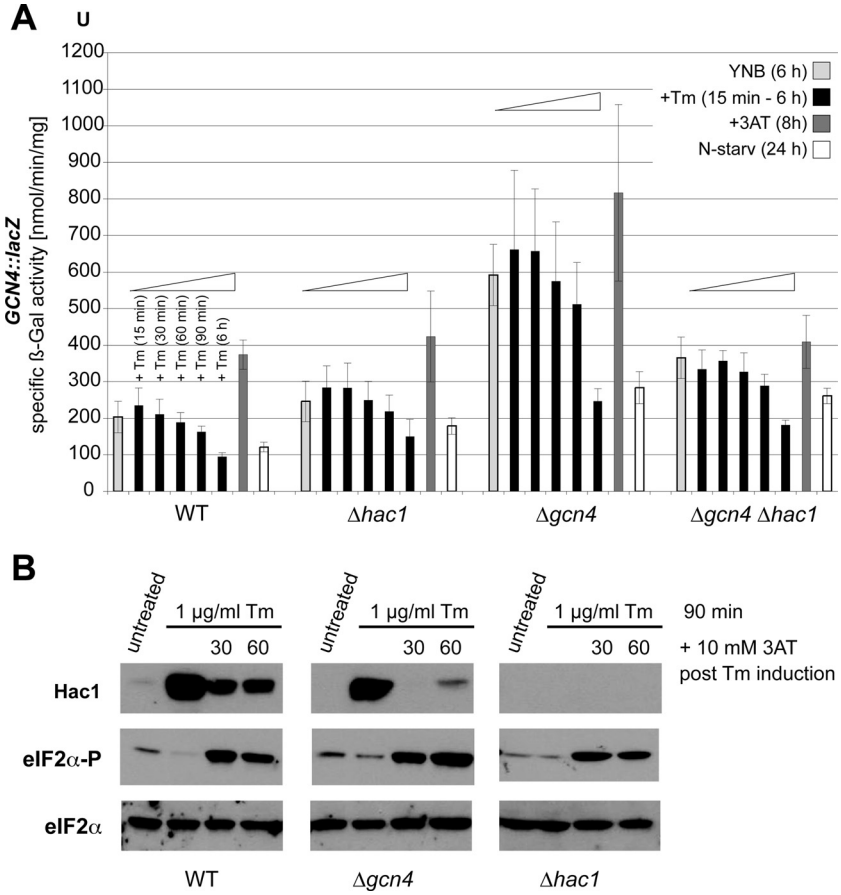

FIG 3 Tunicamycin-mediated ER stress represses *GCN4* mRNA translation by a Hac1-independent mechanism. (A) Expression of a *GCN4*::*lacZ* fusion gene (p180) was measured in a haploid Σ1278b wild-type strain (WT) (RH2816) as well as in Δ*hac1* (RH3351), Δ*gcn4* (RH2676), and Δ*gcn4* Δ*hac1* (RH3402) mutant strains under different nutritional conditions. Bars depict means of at least three independent measurements of β-galactosidase activities. (B) Crude protein extracts were prepared from haploid Σ1278b wild-type cells (WT) (RH2816) as well as from Δ*gcn4* (RH2676) and Δ*hac1* (RH3351) mutant strains, respectively. Cells were grown either under normal conditions (untreated) or in the presence of ER stress conditions induced by 1 μg/ml tunicamycin (Tm). Additional amino acid starvation was obtained by adding 10 mM 3AT. Starting from one main culture with an OD_{600} of ~0.8 at 30°C, cultures were quartered and cultivated for a further 90 min under the indicated conditions. Protein levels of Hac1 and eIF2α-P were analyzed by immunoblotting using specific antibodies. eIF2α was used as a loading control.

Δ*hac1* provides a phenotype similar to that of the Δ*hac1* mutation, further supporting the conclusion that UPRE transcription is primarily mediated by Hac1 (Fig. 2A).

For analyzing the impact of Hac1 on Gcn4 target gene expression in diploids, haploid *MAT***a** strains containing the *GCRE6*::*lacZ* reporter gene were crossed with respective *MAT*α strains to obtain homo- or heterozygous diploid strains deleted for *HAC1*, *GCN4*, or both. Heterozygous yeast strains revealed that a single intact copy of either *GCN4* or *HAC1* is sufficient to provide induction of the *GCRE6*::*lacZ* reporter upon amino acid starvation. A strain carrying only one intact *GCN4* and one intact *HAC1* copy was hardly able to induce the GCRE reporter. Induction of the GCRE reporter by 3AT was abolished when diploid homozygous strains deleted for *HAC1* or *GCN4* were analyzed (Fig. 2B). Therefore, neither haploid nor diploid Δ*hac1* cells showed intrinsic activation of GCRE upon amino acid starvation. In accordance with the data with haploids, the analysis of the corresponding activation of the UPRE-mediated unfolded protein response in diploids revealed that the homozygous Δ*gcn4* cells showed a reduced response to ER stress, whereas homozygous Δ*hac1* cells failed to activate UPRE-*lacZ* expression after tunicamycin treatment (Fig. 2B).

These data suggest that there is a cross talk in both directions between Hac1 and Gcn4. Besides the known requirement of Gcn4 for UPRE activation, there is also a Hac1 influence on the activation of Gcn4-specific target genes in response to amino acid starvation in haploids, as well as in diploids. One copy of either *GCN4* or *HAC1* is sufficient to fulfill the activation of Gcn4-specific target gene expression in diploids. The noncomplementation of the double heterozygous mutants could be a consequence of direct Gcn4 and Hac1 interactions.

ER stress inhibits *GCN4* mRNA by a Hac1-independent mechanism. Since the ER stress regulator Hac1 is required for the GCRE response during amino acid starvation, we analyzed the effect of tunicamycin-mediated ER stress on GCRE activity. ER stress represses GCRE activity independently of the presence or absence of Gcn4 or Hac1, suggesting an additional molecular mechanism (Fig. 2A). Nitrogen starvation was used as a control and was achieved by decreasing the ammonium sulfate concentration in the culture medium (from 50 mM to 50 μM). GCRE-

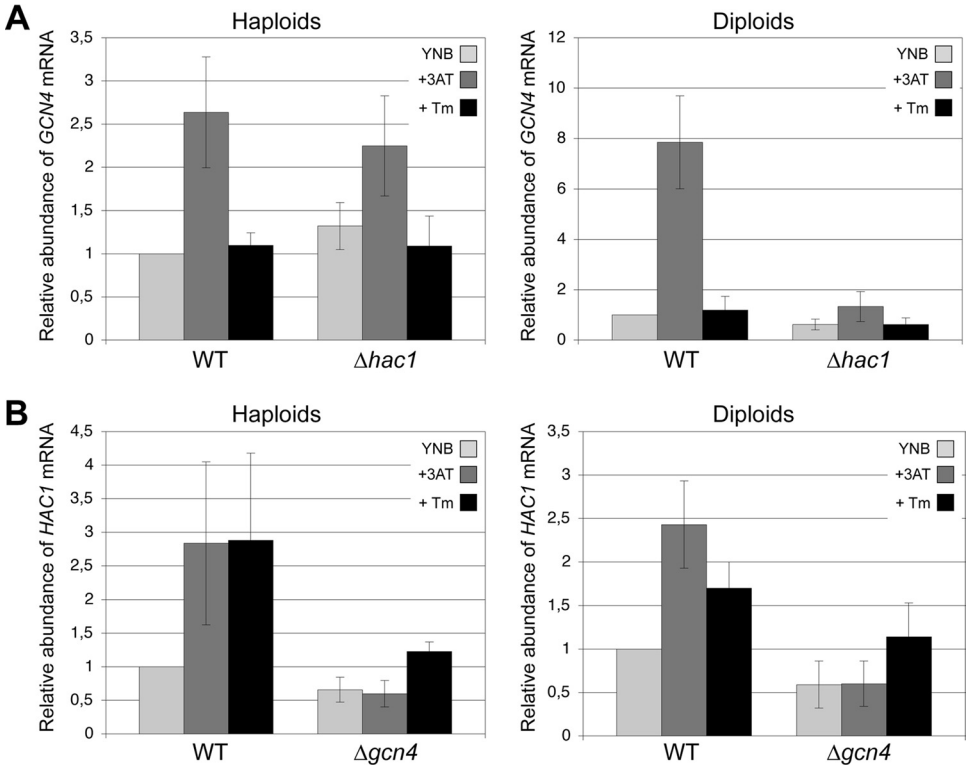

FIG 4 Hac1 and Gcn4 mutually influence their mRNA expression levels. (A) Relative *GCN4* mRNA levels of either a haploid or diploid wild-type strain (RH2816 and RH2656) and a haploid or diploid *HAC1* deletion strain (RH3351 and RH3412) were determined by qRT-PCR and normalized against *H2A*. Starting from one main culture with an OD_{600} of ~0.6 at 30°C, cultures were divided and cultivated for a further 90 min under the indicated conditions. Amino acid starvation was induced by 10 mM 3AT (dark gray bars, +3AT), ER stress conditions were achieved by 1 μg/ml tunicamycin (black bars, +Tm). Experiments were performed from three independent cultures for each strain and condition. The mRNA levels were normalized to the respective untreated (YNB) wild type. Standard deviations are indicated as error bars. (B) *HAC1* mRNA levels were analyzed similarly in either a haploid or a diploid wild-type strain (RH2816 and RH2656) and a haploid or a diploid *GCN4* deletion strain (RH2676 and RH2658).

driven gene expression was drastically reduced by ER stress, and the effect was similar to, and even more pronounced than, the described repressive effect of nitrogen starvation on GCREs (58). The kinetics of the observed repression of GCREs by ER stress suggests that it starts after about 60 min. A transient upregulation of Gcn4 protein levels 15 and 30 min after tunicamycin treatment had been described previously (45). However, it did not result in increased GCRE activation in the corresponding period (see Fig. S1 in the supplemental material).

We analyzed how the repressive effect of ER stress on GCREs correlates with the translational control of the *GCN4* mRNA, which was monitored by a *GCN4*::*lacZ* reporter containing the wild-type leader with all four intact upstream open reading frames (uORFs) (59). Translational efficiency of *GCN4* mRNA decreased in response to tunicamycin-mediated ER stress in haploid wild-type and Δ*hac1* and Δ*gcn4* single and double mutant strains (Fig. 3A). The increased activity in the Δ*gcn4* strain corresponds to permanent limitation caused by the lack of the Gcn4 regulator, resulting in an induced translation of the *GCN4* reporter. This further suggests that ER stress activates an additional mechanism, which controls gene expression independently of Hac1. The repression of *GCN4*::*lacZ* by tunicamycin, which was measured as the control, is similar to the described effect of nitrogen repression (58) (Fig. 3A). There is no hint of a translational control of *GCN4* by Hac1 in haploids since Hac1 influenced neither *GCN4*::*lacZ* expression of a reporter containing all four uORFs nor the expression of a reporter with mutated uORFs (Fig. 3A; see also Fig. S2 in the supplemental material).

Amino acid starvation activates the general control system primarily on the level of translation. The eukaryotic initiation factor of translation eIF2α is phosphorylated (eIF2α-P) resulting in two effects: (i) the overall translation rate is reduced to save precursors of translation during amino acid limitation and (ii) *GCN4* mRNA translation is increased to produce larger amounts of this transcription factor, which then activates numerous genes for amino acid biosynthesis (11). We monitored phosphorylation of eIF2α to analyze how ER stress (tunicamycin) in addition to simultaneous amino acid limitation (3AT) affects the levels of eIF2α-P (Fig. 3B). Phosphorylation of eIF2α upon amino acid starvation is unaffected by additional ER stress. Accordingly, ER stress without amino acid starvation does not result in phosphorylation of eIF2α. We also analyzed whether Hac1 is required for phosphorylation of eIF2α. Hac1 protein levels are significantly increased as a result of ER stress. Additional amino acid limitation still shows elevated Hac1 protein levels, but due to the generally reduced translation rate as a result of the increased levels of eIF2α-P, the amounts of Hac1 are decreased (Fig. 3B). In the absence of Hac1, eIF2α phosphorylation is still functional during simultaneous amino acid starvation and ER stress. In contrast, upon additional amino acid starvation Hac1 protein levels are reduced in Δ*gcn4*

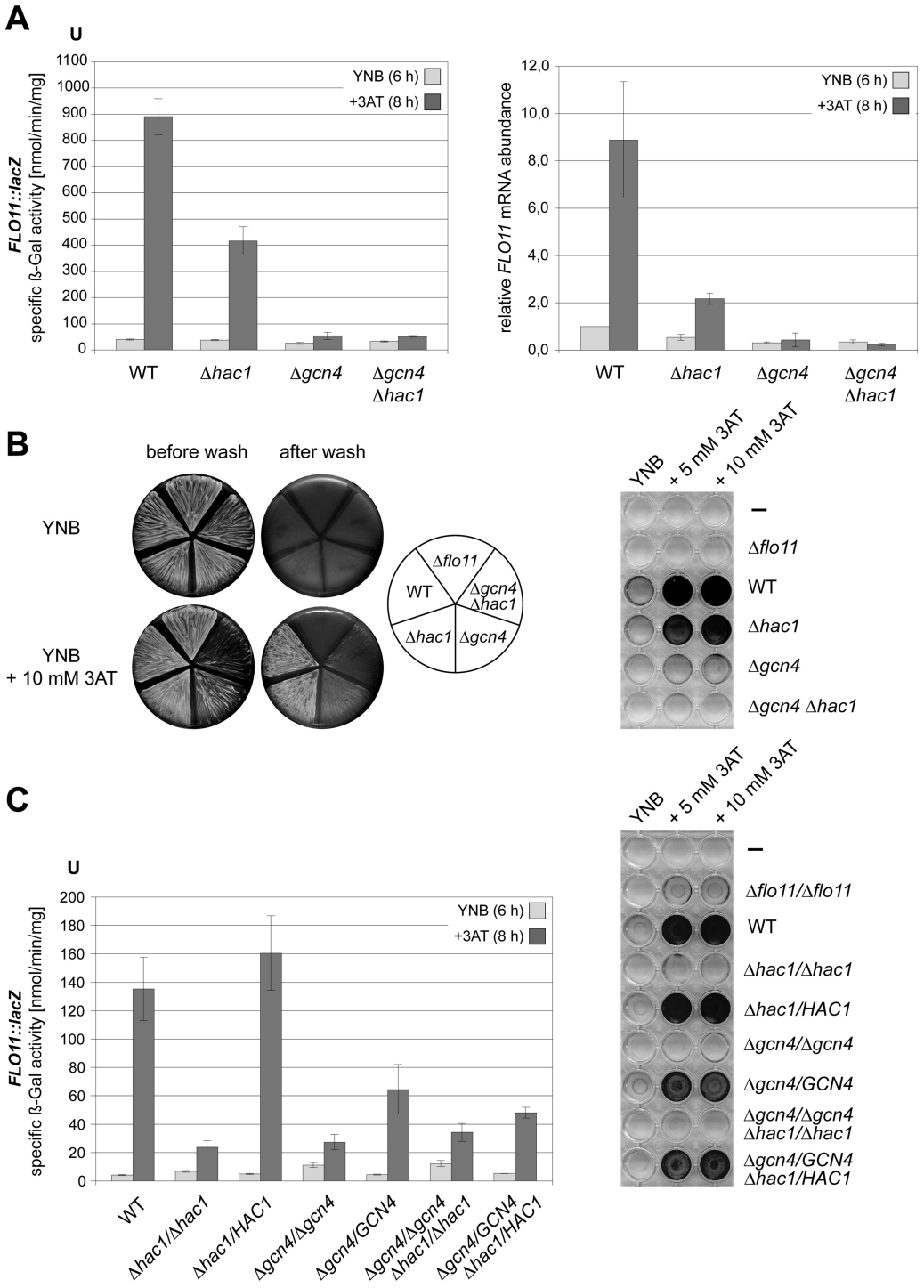

FIG 5 Hac1 supports *FLO11* expression during amino acid starvation. (A) Expression of *FLO11*::*lacZ* was assayed in the haploid Σ1278b wild-type yeast strain (WT) (RH3406) as well as in Δ*hac1* (RH3360), Δ*gcn4* (RH3407), and Δ*gcn4* Δ*hac1* (RH3408) mutant strains each carrying a chromosomally integrated *FLO11*::*lacZ* reporter. Cultures were grown to log phase in YNB in the absence (light gray bars, YNB) or presence of 10 mM 3AT (dark gray bars, +3AT) before specific β-galactosidase activities were measured. Relative *FLO11* mRNA abundances determined by qRT-PCR were measured in haploid Σ1278b wild-type (WT) (RH2816), Δ*hac1* (RH3351), Δ*gcn4* (RH2676), and Δ*gcn4* Δ*hac1* (RH3402) yeast strains and normalized against *CDC28*. Experiments were performed from three independent cultures for each strain and condition, and cultivation was accomplished as described for *FLO11*::*lacZ* expression. The ΔC_T method including efficiencies was used for quantification. Standard deviations are indicated as error bars. (B) The untransformed yeast strains described in panel A as well as a Δ*flo11* (RH2681) mutant strain were streaked out on solid YNB medium (nonstarved cells) and with 10 mM 3AT (histidine-starved cells), respectively. After incubation for 3 days at 30°C, adhesive growth was determined. Plates were photographed before (before wash) and after washing under a stream of water (after wash) to document remaining cells on the agar surface. The same yeast strains were grown in liquid YNB medium until reaching an optical density of 0.6 before 300 μl of each culture was transferred in a microtiter well. Cells were grown in the absence or presence of 5 or 10 mM 3AT to induce starvation-dependent adhesive growth. After incubation for 24 h at 30°C, sedimented cells were dyed with crystal violet and carefully washed. Finally, plates were photographed to

cells. In conclusion, these data suggest that ER stress inhibits translation of *GCN4* mRNA in a molecular mechanism, which does not affect eIF2α phosphorylation caused by amino acid starvation. This novel ER stress-induced process on *GCN4* and subsequently GCRE expression is independent of Hac1. In contrast, Gcn4 appears to influence Hac1 upon amino acid starvation, which further supports a cross talk between the two transcription factors.

Hac1 and Gcn4 mutually influence their mRNA expression levels. We further analyzed the impact of Hac1 on the mRNA expression level of *GCN4* and *vice versa*. Therefore, we performed quantitative real-time PCR (qRT-PCR) experiments under amino acid starvation as well as under ER stress conditions. The levels of *GCN4* and *HAC1* mRNA were upregulated by induction of amino acid starvation (3AT) in both haploid and diploid wild-type strains (Fig. 4). Deletion of *HAC1* slightly reduced *GCN4* mRNA expression levels upon 3AT treatment in haploids, whereas this effect was very prominent in diploid deletion strains. In contrast to amino acid starvation, 90-min tunicamycin-mediated ER stress did not result in enhanced *GCN4* mRNA expression levels (Fig. 4A). The data are consistent with our results obtained from the *GCN4*::*lacZ* experiments (Fig. 3A). As expected, *HAC1* mRNA expression levels increased under ER stress conditions in both haploid and diploid wild-type cells. Deletion of *GCN4* impaired *HAC1* mRNA expression levels in response to amino acid starvation as well as under ER stress conditions. The data are consistent with our results where Hac1 protein levels were reduced in Δ*gcn4* cells when they were treated with 3AT (Fig. 3B). In contrast to *GCN4* mRNA levels, *HAC1* mRNA expression levels were similar in haploid and diploid Δ*gcn4* cells (Fig. 4B).

These findings further support our assumption that there is a cross talk between Hac1 and Gcn4. Gcn4 appears to be involved in *HAC1* transcription in both haploid and diploid cells, whereas Hac1 appears to be absolutely required for *GCN4* transcription in diploids.

Hac1 supports *FLO11* expression and adhesive growth in haploid or diploid cells during amino acid starvation. Besides the activation of target genes by binding to specific Gcn4 recognition elements in their promoter regions, Gcn4 evokes a strong adhesion of yeast cells on surfaces or on each other upon amino acid starvation. This adhesion is mediated by the flocculin Flo11. The *FLO11* promoter lacks a typical GCRE and therefore is not a typical Gcn4-dependent promoter (18). Since Hac1 is required for activation of Gcn4 promoter elements (GCREs), we analyzed Hac1-dependent *FLO11* expression in more detail in haploid and diploid cells. We investigated how Hac1 influences expression of a *FLO11*::*lacZ* reporter construct that contains 3,500 bp of the *FLO11* promoter in front of a *CYC1*::*lacZ* minimal promoter integrated into the *URA3* locus. Amino acid starvation (3AT) led to only a partial induction of β-galactosidase activity in Δ*hac1* cells in comparison to haploid wild-type cells. Haploid cells containing a *gcn4* deletion showed no induction upon amino acid starvation. These findings were confirmed by quantitative real-time PCR (qRT-PCR) (Fig. 5A).

Subsequently, the consequences of the reduced *FLO11* expression of Δ*hac1* cells on the adherence phenotype were analyzed. We investigated to what extent Δ*hac1* cells can still grow adhesively when starved for amino acids on either agar or plastic surfaces. Cells deleted for *GCN4* were not able to grow adhesively and were comparable to the Δ*flo11* strain deficient in the structural gene for the adhesin. Haploid wild-type and Δ*hac1* cells became adhesive on agar when starved for amino acids (3AT), but Δ*hac1* cells showed only a constricted adhesive growth on plastic, which reflected the halved *FLO11* expression (Fig. 5B).

We also explored Hac1-dependent *FLO11* expression in diploids and found a much more pronounced phenotype. Haploid *MAT***a** strains containing the *FLO11*::*lacZ* reporter gene were crossed with respective *MAT*α strains to obtain homo- and heterozygous diploid strains deleted for *HAC1*, *GCN4*, or both. Starvation-dependent *FLO11*::*lacZ* expression was strongly decreased in diploid homozygous Δ*hac1* and Δ*gcn4* strains, and the cells were not able to grow adhesively (Fig. 5C). A single copy of *HAC1* was sufficient in heterozygous Δ*hac1*/*HAC1* cells to restore *FLO11*::*lacZ* expression and adhesive growth, whereas heterozygous Δ*gcn4*/*GCN4* resulted only in a partial *FLO11* induction and reduced adherence.

These data support an auxiliary role of Hac1 for *FLO11* expression during amino acid starvation in haploid and even more in diploid yeasts.

Hac1 and Gcn4 act on similar promoter elements in the *FLO11* promoter. Both Hac1 and Gcn4 affect *FLO11* expression during amino acid starvation. Gcn4 action on the presumably longest promoter of *S. cerevisiae*, spanning approximately 3.5 kb, might be indirect, because no promoter binding had been detected. Gcn4 action is also complex because it includes several promoter regions, including also basal promoter elements (18). We compared the effects of the presence or absence of Hac1 and Gcn4 on a set of 14 described reporter constructs containing individual 400-bp *FLO11* promoter fragments that overlap by 200 bp in front of a *CYC1*::*lacZ* fusion gene (53). Homozygous diploid yeast cells were analyzed, because they show the most pronounced effect of Hac1 on *FLO11* expression (Fig. 5). The comparison between diploid wild-type, Δ*gcn4*, and Δ*hac1* cells shows two promoter regions, which are significantly affected by both Gcn4 and Hac1. These regions are located approximately 2 or 1 kb upstream of the AUG of the *FLO11* open reading frame, respectively (Fig. 6; see also Table S1 in the supplemental material). Already in the absence of amino acid limitation, the 1-kb upstream region requires not only Gcn4 but also Hac1 for basal expression. Activation by amino acid limitation (3AT) is reduced when Hac1 is missing and is abolished in the absence of Gcn4. This regulatory region corresponds to the major regulatory region of the *FLO11* promoter (18, 21, 60). The second upstream region at −2 kb is also affected by Hac1 and Gcn4. There are differences between the

document adhesive growth. (C) *FLO11*::*lacZ* expression was also determined in the diploid Σ1278b wild-type yeast strain (WT) (RH3417) as well as in diploid homo- and heterozygous Δ*hac1*/Δ*hac1* (RH3362), Δ*hac1*/*HAC1* (RH3418), Δ*gcn4*/Δ*gcn4* (RH2695), Δ*gcn4*/*GCN4* (RH3419), Δ*gcn4*/Δ*gcn4* Δ*hac1*/Δ*hac1* (RH3349), and Δ*gcn4*/*GCN4* Δ*hac1*/*HAC1* (RH3420) mutant yeast strains each carrying a chromosomally integrated *FLO11*::*lacZ* reporter. For testing amino acid starvation-induced adhesive growth, the diploid wild-type yeast strain (WT) (RH2656) as well as Δ*flo11*/Δ*flo11* (RH2661), Δ*hac1*/Δ*hac1* (RH3412), Δ*hac1*/*HAC1* (RH3413), Δ*gcn4*/Δ*gcn4* (RH2658), Δ*gcn4*/*GCN4* (RH3414), Δ*gcn4*/Δ*gcn4* Δ*hac1*/Δ*hac1* (RH3415), and Δ*gcn4*/*GCN4* Δ*hac1*/*HAC1* (RH3416) mutant strains was used, and the assay was performed as described for panel B.

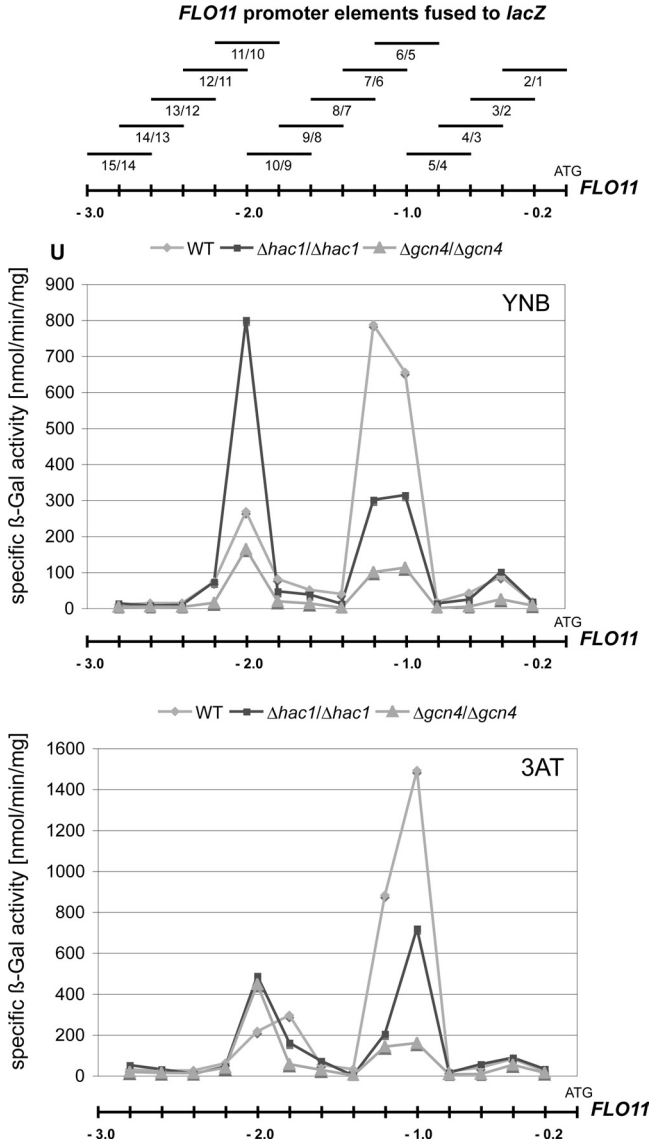

FIG 6 Effect of Hac1 and Gcn4 on *FLO11*::*lacZ* promoter elements. The β-galactosidase activity of 14 isolated 400-bp elements of the *FLO11* promoter region was assayed in diploid wild-type cells (RH2656) or in corresponding cells deleted for the transcription factor *HAC1* (RH3412) or *GCN4* (RH2658) (see Table S1 in the supplemental material). The activity was determined under nonstarvation conditions (YNB, upper graphs) or in the presence of amino acid starvation (3AT, lower graphs) induced by the addition of 10 mM 3AT. Each breakpoint represents the measured reporter construct expression less the measured expression of the construct without insert (pME2212) in the indicated genetic background. β-Galactosidase activities measured in the wild type are shown by diamonds, Δ*hac1*/Δ*hac1* breakpoints are shown by squares, and Δ*gcn4*/Δ*gcn4* breakpoints are shown by triangles.

two transcription factors, because only Hac1 seems to participate in repression of this promoter region in the absence of amino acid limitation. The effect of Hac1 and Gcn4 on the same *FLO11* promoter elements further corroborates the interplay between the two transcription factors.

DISCUSSION

XBP1 and ATF4, the mammalian homologues of Hac1 and Gcn4, represent essential genes, which are involved in a multiplicity of metabolic and developmental processes ensuring the survival of the organism. The general amino acid control (GAAC) and the unfolded protein response (UPR) of baker's yeast are, in contrast to those of mammals, not essential. Yeast has been used here to study the interplay between the two networks. Patil et al. (45) discovered a linkage between the two pathways where Gcn4 is required for induction of a majority of UPR target genes during ER stress. We demonstrate here that this is a mutual interplay, which also applies *vice versa*. The initial finding was that the survival of diploid cells expressing either wild-type *GCN4* or the partially active $GCN4^{L267S}$ (17) could be increased upon amino acid starvation when native *HAC1* was overexpressed. Hac1 can only improve Gcn4 function but is unable to complement a *gcn4* deletion strain or a strain with an inactive Gcn4 variant.

Hac1 and the general amino acid control. Gcn4 target gene expression and *GCN4* mRNA expression itself are influenced by Hac1 in both haploid and diploid cells, but effects are stronger and more prominent in diploids. In haploids, Hac1 is not involved in basal expression of Gcn4 target genes but is required for the Gcn4-mediated response to amino acid starvation. Diploid cells show the same lack of induction during starvation, but in addition, Hac1 is required for the basal expression levels. This could be a direct effect where Hac1 itself or in combination with Gcn4 activates Gcn4 targets, or an indirect effect like Hac1-dependent stabilization of Gcn4 as earlier proposed (45). Several findings indicate that the effects of Hac1 on Gcn4 are indirect: (i) Hac1 itself does not influence eIF2α phosphorylation, (ii) Hac1 does not affect *GCN4* mRNA translation in haploids, (iii) ER stress inhibits *GCN4* mRNA translation in a Hac1-independent mechanism, (iv) ER stress represses Gcn4 target gene expression, and (v) Hac1 is hardly detectable in Δ*gcn4* cells upon ER stress conditions and additional amino acid starvation. A regulation of the *HAC1* gene by Gcn4 is supported by the fact that in the absence of *GCN4* and ER stress constitutive expression of *HAC1* does not activate transcription of Hac1 target genes (45). Furthermore, Hac1 contains two Gcn4-specific consensus sequences in its promoter, which is not the case *vice versa*. Furthermore, Gcn4 has a weak but distinct RNase activity, and therefore, it might be possible that Gcn4 regulates *HAC1* mRNA stability (61).

The situation is more complicated because Hac1 is responsible for full activation of Gcn4 target gene expression upon amino acid starvation. *Vice versa*, it is described that Gcn4 directly interacts with two of the three Hac1-specific promoter elements, which share half-site similarity: three bases (GTG) are identical. Gcn4 is able to bind to half-sites (15), and the shared half-site is present in the consensus sequence which is found in the promoters of Gcn4 target genes (RRRWGASTCA, with R = purine, W = T or A, and S = G or C) (9). An attractive explanation is that both Gcn4 and Hac1 regulate the activation of the other target genes by binding to half-sites in their promoter regions. Hac1 and Gcn4 could also act together at target promoters, but we could not copurify a Gcn4/Hac1 heterodimer in our experiments. Nevertheless, a collaboration between the two factors which might depend on a stable physical interaction is supported by the findings that (i) overexpression of *HAC1* increased survival of diploid cells expressing either wild type or the partially active $GCN4^{L267S}$ during amino acid starvation and (ii) Hac1- and Gcn4-specific target gene expression is strongly decreased when the responsible main transcription factor is deleted.

Hac1 function in dimorphism. CaHac1 and HacA, the Hac1

homologues of *Candida albicans* and *Aspergillus fumigatus*, respectively, play important roles in regulating morphology, which in turn is important for virulence of these pathogenic fungi (62, 63). Furthermore, both CaGcn4 and CpcA, the Gcn4 homologue proteins, are also involved in pathogenicity (64–66). The mammalian Hac1-like XBP1 and the unfolded protein response (UPR) play important roles in tumorigenesis, and UPR suppressors are proposed as therapeutic agents (67–69). In *S. cerevisiae*, Gcn4 is required for adhesion and pseudohyphal development upon nutrient starvation (18). In this study, we identified Hac1 as an auxiliary regulator of *FLO11* expression and therefore dimorphism of *S. cerevisiae*. Gcn4 and Hac1 influence an identical *FLO11* promoter element and presumably act indirectly in combination with other transcriptional regulators because there is neither a Gcn4 nor a Hac1 predicted recognition element in the *FLO11* promoter. It is yet unclear why both Hac1 and Gcn4 are specifically required in diploids to induce *FLO11* expression. The overlapping elements, which are influenced by the transcription factors Tec1, Ste12, and Flo8, support this hypothesis (53, 70). *STE12* and *TEC1* represent potential target genes of Gcn4, since both carry at least one Gcn4 recognition element in their promoters. Furthermore, the promoter of *TEC1* actually contains five independent UPREs, whereas three of them are arranged on the complementary strand. These findings enforce our hypothesis that both Gcn4 and Hac1 are involved in Flo11 regulation *per se*, however, presumably by binding to another transcription factor, e.g., the TEA protein Tec1 as one of the major regulators of the *FLO11* promoter. However, Tec1 protein levels did not depend on the absence or presence of Gcn4 or Hac1 (data not shown). Our data suggest that the impact of Gcn4 and Hac1 on *FLO11* is complex. It includes the same regions of the *FLO11* promoter and is at least partially mediated by changes of the amount of another transcription factor, which in turn is able to bind to the *FLO11* promoter.

In summary, Hac1 not only affects the UPR to reduce misfolded or unfolded proteins but also is involved in metabolic and developmental processes generally regulated by Gcn4 in response to amino acid starvation. At least 13 bZIP transcription factors exist in *S. cerevisiae*, whereas the increasing complexity during evolution is reflected by a minimum of 51 bZIP factors in humans (71). Understanding the complex regulation of dimorphism, stability control, nuclear trafficking, and cell death pathways in fungal models might be relevant for tumor therapy in humans. The mutual cross talk between the UPR and the GAAC in yeast is much broader than supposed, and it will be interesting to analyze if such a cross talk also exists in higher eukaryotes.

ACKNOWLEDGMENTS

We thank Kai Heimel and Susanna Braus-Stromeyer for critically reading the manuscript and helpful comments. We are grateful to Agnieszka Kolka and Maria Meyer for their technical assistance during the experiments. Special thanks to Olaf Grundmann, Thomas Dever, Alan Hinnebusch, and Steffen Rupp for strains, plasmids, and antibodies.

This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Cluster of Excellence and DFG Research Center Nanoscale Microscopy and Molecular Physiology of the Brain, the Volkswagenstiftung, and the Fonds der Chemischen Industrie.

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