The unfolded protein response represses differentiation through the RPD3-SIN3 histone deacetylase

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In Saccharomyces cerevisiae, splicing of HAC1 mRNA is initiated in response to the accumulation of unfolded proteins in the endoplasmic reticulum by the transmembrane kinase-endoribonuclease Ire1p. Spliced Hac1p (Hac1ip) is a negative regulator of differentiation responses to nitrogen starvation, pseudohyphal growth, and meiosis. Here we show that the RPD3-SIN3 histone deacetylase complex (HDAC), its catalytic activity, recruitment of the HDAC to the promoters of early meiotic genes (EMGs) by Ume6p, and the Ume6p DNA-binding site URS1 in the promoters of EMGs are required for nitrogen-mediated negative regulation of EMGs and meiosis by Hac1ip. Co-immunoprecipitation experiments demonstrated that Hac1ip can interact with the HDAC in vivo. Systematic analysis of double deletion strains revealed that HAC1 is a peripheral component of the HDAC. In summary, nitrogen-induced synthesis of Hac1ip and association of Hac1ip with the HDAC are physiological events in the regulation of EMGs by nutrients. These data also define for the first time a gene class that is under negative control by the UPR, and provide the framework for a novel mechanism through which bZIP proteins repress transcription.

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Introduction

Several discrete and complementary pathways constitute the unfolded protein response (UPR), a signaling network from the endoplasmic reticulum (ER) to the nucleus, in eucaryotic cells (Harding et al., 2002; Kaufman et al., 2002). The UPR induces ER-resident molecular chaperones, for example, GRP78/KAR2/Bip (Mori et al., 1992, 1996, 1998; Cox and Walter, 1996), upregulation of ER-associated protein degradation (Casagrande et al., 2000; Friedlander et al., 2000; Travers et al., 2000), and a global remodeling of the secretory pathway to deal with the accumulation of unfolded proteins in the ER (Ng et al., 2000; Travers et al., 2000). Of these pathways, only the one originating at the transmembrane kinase–endoribonuclease Ire1p is conserved from yeast to mammals. The accumulation of unfolded proteins in the ER activates Ire1p (Bertolotti et al., 2000; Liu et al., 2000). Activated Ire1p initiates splicing of HAC1 mRNA and removal of a translational attenuator in its intron in Saccharomyces cerevisiae (Chapman and Walter, 1997; Kawahara et al., 1997, 1998; Sidrauski and Walter, 1997; Rüegsegger et al., 2001). Therefore, only spliced HAC1 (HAC1ip) mRNA is efficiently translated. Hac1ip then binds to the UPR element (UPRE) in the promoters of ER chaperone genes and activates their transcription. Recent studies have shown that this branch of the metazoan UPR is activated in, and required for, differentiation into cell types specialized in secretion, for example, plasma cell differentiation (Reimold et al., 2001; Callon et al., 2002). However, whether an activated UPR is a driving force for differentiation or maintenance of a differentiated state has not been demonstrated. Alternatively, activation of the UPR during these differentiation processes may simply result from an increased protein load of the ER and may not be mechanistically related to the regulation of differentiation programs.

Upon encountering severe starvation for nitrogen and fermentable carbon sources, diploid S. cerevisiae initiate a transcriptional cascade that governs the execution of meiosis, also called sporulation, and formation of an ascus containing four spores. This cascade can be divided into several discrete steps of gene expression: very early, early, middle, mid–late, and late phases (Chu et al., 1998; Primig et al., 2000). One of the first genes induced in meiosis is IME1, which then activates a set of genes called the early meiotic genes (EMGs). Entry into meiosis is subject to genetic and epigenetic control. The mating type locus restricts expression of RME1, a transcriptional repressor of IME1, to haploid and non-a/α diploid cells. This genetic control ensures that meiosis is only executed in a/α diploid cells (Herskowitz, 1988). Deletion of RME1 is sufficient to allow for expression of IME1 and EMGs in haploid cells upon starvation for nitrogen and fermentable carbon sources (Su and Mitchell, 1993). At least three steps in meiosis are under epigenetic control by nutrients: expression of IME1, activation of the early genes, and the ‘commitment to meiosis’ point (reviewed in Honigberg and Purnapatre, 2003). Starvation and subsequent arrest in G1 are required to enter meiosis. The mechanisms that govern nutritional regulation of meiosis, especially by nitrogen, remain poorly understood. Current models for regulation of meiosis by nitrogen proposed that nitrogen starvation promotes meiosis largely indirectly through induction of a G1 arrest (Honigberg and Purnapatre, 2003).
In addition to signaling the UPR in yeast, we identified Hac1\textsuperscript{ip} as a negative regulator of the EMGs in yeast (Schröder \textit{et al}, 2000). Overexpression of Hac1\textsuperscript{ip} reduced activation of EMGs, and deletion of \textit{HAC1} resulted in increased EMG mRNA levels. Further, tunicamycin, an inhibitor of N-linked glycosylation of nascent polypeptides in the ER (Hubbard and Ivatt, 1981), inhibited ascus formation (Weinstock and Ballou, 1987) and transcriptional activation of EMGs in an \textit{IRE1}-dependent manner (Schröder \textit{et al}, 2000). However, \textit{IME1} mRNA levels were not affected by overexpression of Hac1\textsuperscript{ip} or a \textit{HAC1} deletion (Schröder \textit{et al}, 2000). In addition, splicing of \textit{HAC1} mRNA occurred in nitrogen-rich conditions in exponentially growing cells. Splicing was shut off upon nitrogen starvation and restored after the addition of nitrogen sources to nitrogen-starved cells (Schröder \textit{et al}, 2000). Thus, synthesis of Hac1\textsuperscript{ip} is limited to nitrogen-rich conditions, in which it is a negative regulator of EMGs.

To understand the mechanism of negative regulation of EMGs by Hac1\textsuperscript{ip}, we identified the upstream repression site 1 (URS1, 5\textsuperscript{\prime}:'TCGCGGCT-3\textsuperscript{\prime}) as the promoter element that is sufficient and required to mediate negative regulation of EMGs by Hac1\textsuperscript{ip}. We then investigated the involvement of transcriptional repressors recruited to URS1 in negative regulation of transcription by Hac1\textsuperscript{ip}. We show that Hac1\textsuperscript{ip} genetically and biochemically interacts with the RPD3-SIN3 histone deacetylase complex (HDAC) and that this interaction is required for negative regulation of EMG transcription by Hac1\textsuperscript{ip}. These findings demonstrate a direct, mechanistic, causal, and regulatory link between nitrogen sensing by the UPR and execution of differentiation responses controlled by extracellular nitrogen in yeast.

**Results**

\textbf{The EMG promoter element URS1 is sufficient to mediate negative regulation of transcription by Hac1\textsuperscript{ip}}

Three regulatory elements are common to the promoters of early meiotic genes: an enhancer on nonfermentable carbon sources, called T\textsubscript{4}C (Bowdish and Mitchell, 1993), UAS\textsubscript{H}, the binding site for the transcription factor Abf1p (Gailus-Durner \textit{et al}, 1996), and URS1. URS1 is a repressing site in vegetative promoters, an activating site in nitrogen starvation (Kupiec \textit{et al}, 1997). Comparison of the promoters of three EMGs, \textit{IME2}, \textit{HOP1}, and \textit{SPO13}, that were negatively regulated by Hac1\textsuperscript{ip} (Schröder \textit{et al}, 2000) suggested that URS1 is the site of Hac1\textsuperscript{ip} action (Figure 1A).

To test this hypothesis, we studied the effect of Hac1\textsuperscript{ip} on the activation of a minimal \textit{CYC1} promoter fused to a T\textsubscript{4}C enhancer, URS1 elements, or a combination of both by

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**Figure 1** The URS1 element is sufficient for negative regulation of transcription by Hac1\textsuperscript{ip}. (A) Promoter elements for the early meiotic genes \textit{IME2}, \textit{SPO13}, and \textit{HOP1} (Mitchell, 1994). The effect of overexpression of \textit{HAC1} and a \textit{HAC1} null mutant (\textit{hac1\textsuperscript{A}}) on mRNA levels for each early meiotic gene are shown to the right (Schröder \textit{et al}, 2000). ‘\textsuperscript{\textminus}’ represents a decreased mRNA level compared to WT, and ‘\textsuperscript{\textplus}’ an increased mRNA level compared to WT. For comparison, the very early meiotic gene \textit{IME1}, whose expression was not affected by \textit{HAC1} (Schröder \textit{et al}, 2000), is shown on top. The sequence of the URS1 element at position –552 in the \textit{IME2} promoter is shown. Bases in upper case are homologous to the consensus URS1 (TCGCGGCT; Mitchell, 1994). (B) Expression of \textit{lacZ} reporter plasmids harboring the inserts shown on the left in an UAS less \textit{CYC1} promoter (top) before and after induction of nitrogen starvation in WT strains transformed with \textit{pRS314} or \textit{pRS314-HAC1} to overexpress Hac1\textsuperscript{ip} and \textit{hac1\textsuperscript{A}} strains. The average and standard error from four independent transformants are shown. (C, D) Negative regulation of transcription by Hac1\textsuperscript{ip} extends to nonmeiotic genes that contain URS1 elements in their promoters. Northern analysis of WT strains transformed with \textit{pRS316} or \textit{pRS316-HAC1} to overexpress Hac1\textsuperscript{ip} (C), and WT and \textit{hac1\textsuperscript{A}} strains (D) before and after induction of nitrogen starvation. Bar graphs represent the relative mRNA levels obtained by PhosphorImager analysis and standardization of the signal for each mRNA to the loading control \textit{pC4/2}. All strains carry a deletion in \textit{RME1}.
nitrogen starvation. Synthesis of Hac1p from the endogenous genomic locus is shut off in nitrogen-starved cells (Schröder et al., 2000). To express Hac1p in nitrogen-starved cells, a previously described plasmid-borne copy of HAC1 under the control of its own constitutive promoter was introduced into yeast. As expected, nitrogen starvation induced β-galactosidase expression from reporter plasmids containing URS1, but not the Tc enhancer alone (Figure 1B). In the presence of Hac1p, activation of reporters harboring URS1 elements by nitrogen starvation was dramatically blunted. In contrast, Hac1p had no negative effect on the Tc enhancer alone (Figure 1B). hac1Δ strains consistently displayed a 2−3-fold lower expression from the Tc enhancer (Figure 1B). Expression controlled by Tc and URS1 was consistently unchanged or slightly elevated in hac1Δ strains when compared to wild-type (WT) strains (Figure 1B), suggesting the disruption of a negative regulatory mechanism on the URS1 element in hac1Δ strains. In addition, URS1 mediated repression of lacZ reporters in nitrogen-rich conditions (Figure 1B, compare Tc to Tc-URS1 at the 0 h time point) and activation during nitrogen starvation (Figure 1B), thereby validating the assay system. The negative effect of Hac1p on the URS1 element was independent of the orientation of URS1 in the promoter (data not shown).

To further confirm that URS1 is sufficient for negative regulation of transcription by Hac1p, we investigated the effect of overexpression of Hac1p and deletion of HAC1 on several URS1-controlled genes, for which expression is not limited to meiosis: ACS1 (Kratter and Schüller, 1997), CAR1 (Sumrada and Cooper, 1987), HSP82 (Szent-Györgyi, 1995), and INO1 (Jackson and Lopes, 1996). Northern analysis revealed negative regulation of these genes by HAC1 (Figure 1C and D). These results show that the negative effect of Hac1p on transcription of URS1-controlled genes is not limited to EMGs. Taken together, these data show that URS1 is sufficient to mediate the negative effect of Hac1p on transcription.

These data and our earlier study (Schröder et al., 2000) show that nitrogen represses the EMGs, at least in part, through stimulation of synthesis of Hac1p. To test directly whether expression of Hac1p in nitrogen starvation interferes with spore formation, we analyzed the effect of Hac1p overexpression on ascus formation in a diploid strain. As expected, the percentage of cells that initiated meiosis was significantly lower in the Hac1p-expressing strain than in the WT strain 1 day after induction of meiosis by nitrogen starvation (Supplementary Figure 1S). We conclude that expression of Hac1p is sufficient to mimic a nitrogen-rich state and to interfere with ascus formation.

The transcriptional regulator UME6 is required for negative regulation of EMG transcription by Hac1p

URS1 is the binding site for the transcriptional regulator Ume6p (Kadosh and Struhl, 1997). Ume6p recruits two repression complexes, the ISW2 chromatin remodeling complex (Goldmark et al., 2000) and the RPD3-SIN3 HDAC (Kadosh and Struhl, 1997), to URS1 (Figure 2A). Nitrogen starvation induces transcription of the transcriptional activator Ime1. Ime1p then binds to Ume6p and the Ime1p–Ume6p complex activates transcription of EMGs (Figure 2B; Bowdish et al., 1995; Rubin-Bejerano et al., 1996). However, Sin3p and Ume6p can be co-immunoprecipitated with Rpd3p in nitrogen-starved cells, suggesting that the Ume6p/Sin3p/Rpd3p complex is present on the promoter in nitrogen starvation (Figure 2B; Lamb and Mitchell, 2001).

To investigate if negative regulation of transcription by Hac1p is mediated through UME6, we assayed the effect of Hac1p on URS1-controlled transcription in ume6ΔA strains. As expected, deletion of UME6 abolished repression mediated by URS1 in vegetative cells (Figure 2C, compare Tc to Tc-URS1 at the 0 h time point), and activation of reporter plasmids or endogenous promoters by nitrogen starvation (Figure 2C and D, compare Tc-C-URS1 in WT and ume6ΔA strains and Figure 2E). Furthermore, any effect of either Hac1p or a hac1 null mutation on transcription was also abolished (Figure 2C). We confirmed this result by Northern analysis (Figure 2E). These data show that UME6 is required for negative regulation of transcription by Hac1p.

Since UME6 controls expression of several hundred genes (Williams et al., 2002), pleiotropic effects may be associated with a ume6 null mutation. To test the specificity of the results obtained from ume6ΔA strains, we employed a triple base pair substitution in the URS1 element (Figure 2D), which abolished binding of Ume6p to URS1 in vitro (Goldmark et al., 2000). The negative effect of Hac1p on expression of lacZ reporters was nearly completely abolished by the mutation in the URS1 element (Figure 2D, compare WT URS1 with the mutated URS1). However, in the presence of Hac1p, expression controlled by the mutated URS1 was still approximately two-fold lower than in the WT. This may reflect that binding of Ume6p to this site is not completely abolished in vivo. The derepression of URS1-controlled lacZ reporters seen in hac1Δ strains was completely abolished by the mutation in the URS1 element (Figure 2D).

Furthermore, we mutated the URS1 element in the genomic promoters of the EMGs DMC1 and REC104 (Figure 2F) and investigated the effect of HAC1 on these promoters by Northern analysis. Mutation of the URS1 element resulted in derepression of DMC1 and REC104 mRNA levels and unresponsiveness of the promoter to nitrogen starvation (Figure 2G and data not shown). The mutated DMC1 and REC104 promoters were unaffected by expression of Hac1p or a HAC1 deletion (Figure 2G and data not shown). In summary, these data demonstrate that negative regulation of transcription by Hac1p requires the URS1 element UME6, and binding of Ume6p to the URS1 element. Further, these data also provide a mechanistic explanation for co-regulation of all EMGs by Hac1p and why negative regulation of transcription by Hac1p is restricted to URS1-controlled genes.

The RPD3-SIN3 HDAC and its catalytic activity are required for negative regulation of transcription by Hac1p

Next, we asked which of the two repression complexes recruited to URS1 by Ume6p (Figure 2A) is required for negative regulation of transcription by Hac1p. To address this question, we assayed the effect of Hac1p on expression of URS1-controlled lacZ reporters in strains deleted for components of the ISW2 chromatin remodeling complex or the RPD3-SIN3 HDAC. Deletion of either ISW2 or ITC1, another component of the chromatin remodeling complex (Goldmark et al., 2000), resulted in partial derepression of URS1-controlled lacZ expression, but had no effect on negative regulation of transcription by Hac1p (Figure 3A). However,
deletion of either \textit{SIN3} or \textit{RPD3} relieved the negative effect of Hac1\textsuperscript{ip} on transcription (Figure 3B). We confirmed this result by Northern analysis for the \textit{EMG}s \textit{IME2}, \textit{HOP1}, and \textit{SPO13} in \textit{sin3A}/\textit{sin3A} strains and diploid strains deleted for \textit{SDS3}, an integral component of the \textit{RPD3-SIN3} HDAC (Lechner et al., 2000). The negative effect of Hac1\textsuperscript{ip} on transcription was completely abolished in both strains (Figure 3C). These findings demonstrate that the ISW2 chromatin remodeling
complex is dispensable and that the RPD3-SIN3 HDAC is required for negative regulation of transcription by Hac1p.

The RPD3-SIN3 HDAC represses transcription through deacetylation of lysine side chains in the N-terminal tails of the histones (Kurdistani and Grunstein, 2003), resulting in a net increase in the positive charge of the histones and tighter binding to DNA. To investigate whether the catalytic activity of the RPD3-SIN3 HDAC is required for negative regulation of transcription by Hac1p, or whether the HDAC simply serves as a docking site for a repressor induced by Hac1p, we employed H150A, H151A, and H188A point mutant alleles of RPD3. These point mutations in RPD3 lack detectable histone deacetylase activity in vitro and are defective in repression of target promoters in vivo (Kadosh and Struhl, 1998). However, interaction of Rpd3p with Sin3p is not altered by these point mutations in vivo (Kadosh and Struhl, 1998). Negative regulation of URS1-controlled transcription by Hac1p was abolished in strains carrying integrated point mutant RPD3 alleles (Figure 4A and B). Furthermore, the modest loss in repression observed in hac1D strains was lost in sin3D hac1D, rpd3D hac1D, and hac1D strains carrying the H150A, H151A, or H188A RPD3 alleles (Figure 4C and D). Strains carrying point mutations in RPD3 displayed strong, but when compared to rpd3D strains only partial, derepression of URS1-controlled transcription (Figure 4D). This observation is consistent with previous results (Kadosh and Struhl, 1998) and suggests that the RPD3-SIN3 HDAC possesses additional repressing functions in addition to its histone deacetylase activity. However, deacetylase activity-independent repression of transcription by the HDAC is not required for negative regulation of transcription by Hac1p, since deacetylase-deficient point mutations in RPD3 were sufficient to relieve completely the negative effect of Hac1p on transcription. Taken together, these data demonstrate that Hac1p requires the catalytic activity of the RPD3-SIN3 HDAC to negatively regulate transcription on the URS1 element.

**Hac1p can associate physically with the RPD3-SIN3 HDAC in vivo**

Next, we used co-immunoprecipitation experiments to investigate if the genetic interaction between Hac1p and the RPD3-SIN3 HDAC reflects a direct physical interaction. The level of HAC1 mRNA splicing in vegetatively growing cells was too low to detect Hac1p by Western blotting or immunoprecipitation (data not shown). To express sufficient amounts of HAC1u represents the unspliced, untranslated HAC1 mRNA, and HAC1i the spliced, translated HAC1 mRNA. Two mRNAs for HOP1 and SPO13 were detected (Schroeder et al., 2000).

Figure 3 The ISW2 chromatin remodeling complex is dispensable (A) and the RPD3-SIN3 HDAC is required for negative regulation of transcription by Hac1p (B, C). Expression of lacZ reporter plasmids harboring the inserts shown on the left in the strains indicated on top before and after induction of nitrogen starvation (A, B). The average and standard error from four independent transformants are shown in panels A and B. The strains in (A, B) carry a deletion in RME1. (C) Northern and PhosphorImager analysis (bar graphs) of WT (RME1/RME1), sin3A/sin3A, and sds3A/sds3A strains transformed with pRS314 or pRS314-HAC1 before and after induction of nitrogen starvation. The bar graphs represent the average and standard error from three independent transformants. One representative Northern blot is shown. HAC1u represents the unspliced, untranslated HAC1 mRNA, and HAC1i the spliced, translated HAC1 mRNA. Two mRNAs for HOP1 and SPO13 were detected (Schroeder et al., 2000).
Hac1p for biochemical studies, we used an inducible expression system, in which expression of Hac1p is under the control of three glucocorticoid response elements and induced with the steroid deoxycorticosterone (DOC) in yeast expressing the rat glucocorticoid receptor (Schena et al., 1991). In uninduced cells, no HAC1 mRNA or Hac1p were detected by Northern (data not shown) or Western analysis (Figure 5A). At 1 h after induction with 50 μM DOC, Hac1p was readily detected by Western blotting (Figure 5A). Expression of Hac1p had no effect on the expression levels of Rpd3p or Sin3p (Supplementary Figure 2S). Immunoprecipitation with antibodies directed against the 19 N- and C-terminal amino acids of Rpd3p co-immunoprecipitated HA-tagged Hac1p (Figure 5B). Immunoprecipitations from induced cells carrying empty vector or from uninduced cells did not precipitate proteins crossreacting with the anti-HA antibody. Immunoprecipitation with protein A–sepharose and normal goat IgG did not bring down any detectable amounts of HA-Hac1p (Figure 5C and data not shown), thus further confirming the interaction between Hac1p and the HDAC. This interaction was not observed in sin3Δ strains and inhibited by the addition of excess competing peptide to the immunoprecipitation with the Sap30p antibody (data not shown). Taken together, these experiments show that Hac1p can associate with the RPD3-SIN3 HDAC in vivo.

**HAC1 is not an integral component of the RPD3-SIN3 HDAC**

The ability of Hac1p to co-immunoprecipitate with Rpd3p raised the possibility that Hac1p is an integral component of the HDAC required for its function. However, hac1Δ strains showed only a partial loss of repression when compared to sin3Δ or rpd3Δ strains (Figure 4C and D). This observation indicates that HAC1, in contrast to SIN3 or RPD3, is not absolutely required for HDAC function. To further address this question, we constructed a series of double knockout strains between the RPD3-SIN3 HDAC and the ISW2 chromatin remodeling complex and introduced a HAC1 deletion into strains deleted for the ISW2 chromatin remodeling complex.
We then assayed expression of lacZ reporters harboring a T4C enhancer or a T4C enhancer and a URS1 element in exponentially growing cells. Deletion of both the RPD3-SIN3 HDAC and the ISW2 chromatin remodeling complex resulted in complete derepression (Figure 5D and E). This result is consistent with complete derepression in ume6Δ strains or strains carrying mutations in URS1 elements (Goldmark et al., 2000; Figure 2). However, derepression on the URS1 element in isw2Δ hac1Δ and itc1Δ hac1Δ strains was very similar to the level of derepression seen in isw2Δ and itc1Δ strains (Figure 5D and E). This result shows that the RPD3-SIN3 HDAC complex is largely functional in the absence of HAC1. Taken together, these data suggest that Hac1p is a peripheral component of the HDAC. We propose that upon binding of Hac1p to the HDAC, a signal is transduced through the HDAC to inhibit transcription of EMGs in a nitrogen-rich environment.

**Discussion**

Repression of meiotic differentiation by extracellular nutrients, most notably glucose and nitrogen sources, in *S. cerevisiae* is a well-established phenomenon. Mechanisms for direct control of meiosis by nitrogen have not been reported (Kupiec et al., 1997; Honigberg and Purnapatre, 2003), with the exception of control of subcellular localization of Ime1p by TOR (Colomina et al., 2003). In this study, we describe a mechanism that contributes to nitrogen repression of the early meiotic genes (EMGs). In our earlier work, we demonstrated that the activity of a signaling pathway from the ER to the nucleus, the UPR, correlated with the abundance of extracellular nitrogen sources (Schröder et al., 2000). Moreover, genetic manipulations of the readout of the UPR, overexpression of spliced Hac1p (Hac1ip) and deletion of HAC1 revealed that Hac1p is a negative regulator of EMGs (Schröder et al., 2000).

In the present study, we identified the complex that integrates the nitrogen signal transduced by Hac1p into regulation of EMGs. We have shown that negative regulation of EMGs by Hac1p requires URS1, Ume6p, the RPD3-SIN3 HDAC, and the catalytic activity of the HDAC. Co-immunoprecipitation studies revealed that Hac1p can interact with the HDAC in vivo. Further, negative regulation by Hac1p was abolished when URS1 was mutated in the REC104 promoter (Figure 2G), but retained for EMGs in the same strain that still carried a WT URS1 (Figure 2G). This observation shows that recruitment of the RPD3-SIN3 HDAC to URS1 is required for negative regulation of EMGs by Hac1p. Further, the
Figure 6 Model for promotion of growth and proliferation by the UPR. The UPR promotes growth through Hac1’p-mediated induction of ER chaperone genes and phospholipid biosynthesis. Activation of ER chaperone genes by Hac1’p shows that simple recruitment of another transcriptional repressor to URS1 by Hac1’p cannot account for negative regulation of EMGs by Hac1’p. Therefore, we propose the following model for nitrogen-mediated regulation of EMGs by Hac1’p (Figure 6). In a nitrogen-rich environment, HAC1 mRNA is spliced (Schröder et al., 2000) and spliced HAC1 mRNA translated (Chapman and Walter, 1997; Kawahara et al., 1997, 1998; Sidrauski and Walter, 1997; Rüegsegger et al., 2001). The level of Hac1’p correlates very well with the degree of HAC1 mRNA splicing, since the half-life of Hac1’p is about 2 min (Kawahara et al., 1997). Hac1’p then associates with the RPD3-SIN3 HDAC (Figure 5B). The Hac1’p–HDAC complex possesses an enhanced ability to repress transcriptional activation, as illustrated by a decrease in URS1-mediated activation of reporter constructs (Figure 1B) and EMGs (Figure 2E and G) by nitrogen starvation in cells overexpressing Hac1’p. In nitrogen starvation, HAC1 mRNA splicing stops rapidly (Schröder et al., 2000) and translation of HAC1 mRNA ceases (Chapman and Walter, 1997; Kawahara et al., 1997, 1998; Sidrauski and Walter, 1997; Rüegsegger et al., 2001). Due to its short half-life, Hac1’p is rapidly cleared from the cell. This results in loss of HDAC-dependent repression of EMGs. Earlier co-immunoprecipitation experiments showed that the HDAC is still associated with Ume6p in nitrogen starvation (Lamb and Mitchell, 2001). Since Ume6p is required for activation of EMGs (Bowdish et al., 1995; Rubin-Bejerano et al., 1996), the HDAC is associated with the promoters of EMGs in nitrogen starvation. Thus, nitrogen-induced association of Hac1’p with the HDAC and the enhanced repression potential of the Hac1’p–HDAC complex are physiological events in the control of EMGs by nutrients. On the basis of this model, we predict that association of Hac1’p with the HDAC alters some aspects of HDAC function, for example, increases its specific deacetylase activity, or alters its substrate spectrum through altering the conformation or composition of the HDAC. These hypotheses are currently being addressed by more detailed studies.

The model that currently can best explain how HAC1 mRNA splicing is stimulated by extracellular nitrogen is that protein synthesis and influx rates for nascent, unfolded polypeptide chains into the ER are higher in a nitrogen-rich environment than in nitrogen starvation (Figure 6). The accumulation of unfolded proteins in the ER lumen activates Ire1p (Bertolotti et al., 2000; Liu et al., 2000). Activated Ire1p then initiates HAC1 mRNA splicing and synthesis of Hac1’p (Sidrauski and Walter, 1997, Kawahara et al., 1998). In nitrogen starvation, protein synthesis and influx rates for nascent polypeptides into the ER decrease, resulting in inactivation of Ire1p and shut-off of HAC1 mRNA splicing. In this model, the UPR monitors the influx rate for nascent, unfolded polypeptide chains into the ER as a measure for extracellular nitrogen levels, and an activated UPR then contributes to nitrogen repression of meiosis.

The UPR was first identified as a signal transduction pathway responsible for induction of ER chaperones when protein folding in the ER is impaired (Harding et al., 2002; Kaufman et al., 2002). Therefore, it may be surprising that the UPR plays a role in nutrient sensing. However, several independent observations support this idea. First, major targets of the UPR are the glucose-regulated proteins (GRPs), for example, the ER-resident molecular chaperones GRP78/KAR2/Bip and GRP94, that are induced upon glucose starvation (Pouysségur et al., 1977; Lee, 1987). Second, the UPR is required for the biosynthesis of inositol and phospholipids (Nikawa and Yamashita, 1992; Cox et al., 1997; Chang et al., 2002). Third, functionally Ire1p is similar to the
AMP-activated kinase and its yeast homolog Snf1p in that its activity is regulated by the ATP to ADP ratio (Papa et al., 2003). Fourth, the activity of the ER-resident sulfhydryl oxidase ErO1p is tightly linked to the cytoplasmic FAD pool (Tu and Weissman, 2002). The stimulatory effects of the UPR on protein secretion and phospholipid biosynthesis are similar to the stimulatory effects of the TOR pathway on protein synthesis in a nutrient-rich environment (Jacinto and Hall, 2003), and constitute a growth-promoting activity (Figure 6). In this light, repression of nitrogen starvation responses, for example, meiosis, by Hac1p complements the growth-promoting activities of the UPR to promote growth as long as nutritional conditions are sufficient (Figure 6).

Our finding that Hac1p negatively regulates transcription of EMGs through URS1 constitutes a novel function for this bZIP transcription factor. One consequence of UPR activation in the fungus *Trichoderma reesei* and in the plant *Arabidopsis thaliana* is transcriptional downregulation of genes encoding secretory proteins (Martínez and Chrispeels, 2003; Pakula et al., 2003). Transcriptional downregulation of secretory proteins in these organisms compensates for lack of translational attenuation mediated by phosphorylation of elf2z by PERK (Shi et al., 1998, 1999; Harding et al., 1999). Our study demonstrates, for the first time, that a large group of our genes, as defined by the promoter element URS1 and the URS1-binding protein Ume6p, are under negative transcriptional control by the UPR. Future work will determine the extent of functional overlap between Hac1p and Ume6p in the regulation of these genes.

bZIP transcription factors repress transcription through several mechanisms. Many can form repressive homo- and heterodimers, for example, ATF3 (Hai et al., 1999; Hai and Hartman, 2001), the small Maf proteins (Motohashi et al., 2002), bach1 and bach2 (Oyake et al., 1996), and alternative translation products of C/EBPz and C/EBPb (Cornelius et al., 1994). Small Maf proteins recruit gene loci into heterochromatin (Motohashi et al., 2002), and many recruit corepressors to the promoter, for example, Dr; by E4BP4 (Cowell and Hurst, 1996), SSN6-TUP1 by Sko1p (Profitt and Struhl, 2002), and Sin3p by OpI1p (Wagner et al., 2001; Kaadige and Lopes, 2003). In addition, direct inhibition of the transactivation domain of the basic helix-loop-helix transcription factor complex Ino2p–Ino4p by association with OpI1p was proposed (Wagner et al., 2001). Repression of EMGs by Hac1p does not involve direct binding of Hac1p to DNA, since it is dependent on Ume6p (Figure 2), which is constitutively bound to URS1. Second, Hac1p does not recruit the HDAC to URS1 or Ume6p, since repression by the HDAC on URS1 was mostly intact in hac1Δ strains (Figure 5). These observations define a novel framework for negative transcriptional regulation by a bZIP transcription factor.

Recent studies revealed that XBP-1, the functional homolog for HAC1 in metazoans, is required for differentiation into cells specialized in secretion, for example, plasma cell differentiation (Reinold et al., 2001). Both XBP-1 and HAC1 belong to the family of ATF/CREB bZIP transcription factors. Splicing of XBP-1 mRNA was observed in B cells undergoing terminal differentiation (Callon et al., 2002; Iwakoshi et al., 2003). However, it has not been established that the UPR is a driving force in metazoan differentiation. Activation and requirement of the UPR in these differentiation processes may simply reflect the need for an increased handling capacity for nascent secretory proteins as these cell types undergo differentiation. Based on our observation that the UPR directly controls a differentiation program in yeast, it is interesting to speculate that XBP-1 performs similar functions in metazoans. This idea is supported by recent observations. For example, terminal differentiation of B cells requires repression of c-myc transcription (Lin et al., 2000), which in turn is dependent on recruitment of mammalian orthologs of yeast Rpd3p to the c-myc promoter by B limp-1 (Lin et al., 1997; Yu et al., 2000). In addition, the kinetics of activation of the UPR, splicing of XBP-1 mRNA, downregulation of c-myc expression, and activation of B limp-1 expression are similar (Iwakoshi et al., 2003). In summary, we have shown that UPR signalling directly contributes to transcriptional control of a differentiation program in yeast. We propose that this physiological function of the UPR is conserved in higher eucaryotes.

**Materials and methods**

**Plasmids and yeast strains**

*Escherichia coli* DH5α was used for all cloning purposes. Plasmids (Supplementary Table 1), yeast strains (Supplementary Table 2) and their construction are described in the Supplementary data online. Expression of the lacZ gene in pLG312 SASS and its derivatives is driven from a CYCl promoter from which all upstream activating sites were removed (Bowdish and Mitchell, 1993). In pRS316-HAC1 and pRS314-HAC1, expression of Hac1p is driven from its own promoter and HAC1 is tagged with a single HA tag that was inserted into the SpeI site in the HAC1 ORF (Chapman and Walter, 1997). Expression of Hac1p from p2UG-HA-HAC1 is controlled by three glucocorticoid response elements, and strictly dependent on the presence of a glucocorticoid receptor (constitutively expressed from pG-N795; Schena et al., 1991) and steroids. Standard genetic methods were used for yeast strain constructions. Yeast were transformed by the LiOAc method (Schröder et al., 2003). All genomic manipulations were confirmed by PCR and Southern blotting.

**Yeast media, growth conditions, induction of meiosis, and Hac1p expression**

Rich dextrose (YPD), rich acetate (SD), synthetic acetate (PSP2), and nitrogen starvation medium (C-SPO) were described before (Schroeder et al., 2000, 2003) and standardized to the loading control pC4/2, which hybridizes to an eucaryotes.

**β-Galactosidase assays**

β-Galactosidase activity in cell extracts was determined as described before (Schröder et al., 2000, 2003) and standardized to the intracellular protein content determined with the BioRad DC protein assay. The intracellular protein content determined with the BioRad DC protein assay and standardized to the loading control pC4/2, which hybridizes to an mRNA unaffected by starvation (Schröder et al., 2000).
Immunoprecipitation experiments
Mid-log-phase cultures were lysed by vortexing with glass beads (0.5 mm diameter) or two passages through a French Press in lysis buffer (25 mM sodium phosphate (pH 7.3), 150 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM EDTA, 100 mM NaF, 1 mM Na3VO4, 0.05% (v/v) β-mercaptoethanol, 120 μg/ml PMSF, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 10 μg/ml aprotinin or complete protease inhibitors, EDTA-free (Roche)). Cell lysates were cleared by a final centrifugation at 15 000 g and 4°C for 10 min. Protein concentrations were determined with the BioRad DC protein assay. In all, 1 mg total protein was precleared in 500 μl final volume with 25 μl of 50% protein A or G-Sepharose slurry (Amersham, Piscataway, NJ). In all, 1–2 μg goat polyclonal anti-Rpdp3p (yN-19 or yC-19, Santa Cruz Biotechnology, Santa Cruz, CA), 2 μg anti-Sap30p (yL-20, Santa Cruz), 2 μg anti-Sap30p (yN-19, Santa Cruz). 2 μg normal goat IgG (Santa Cruz sc-2028), or 1 μg anti-HA antibody (12CA5, Roche Diagnostics, Indianapolis, IN) were added and immune complexes were allowed to form overnight at 4°C. Competing peptides were used at a 250-fold molar excess. Immune complexes were collected by addition of 25 μl 50% protein A or G-Sepharose slurry and further incubation at 4°C for 1–2 h. Immunoprecipitates were washed three times with lysis buffer, dissolved in SDS-PAGE sample buffer by boiling, separated on 4–15% gradient gels (BioRad), transferred to nitrocellulose or PVDF membranes and probed with anti-Rpdp3p (1 μg/ml), anti-Sin3p (1 μg/ml), or anti-HA (0.1 μg/ml) antibodies as primary antibodies. Membranes were blocked in 5% milk powder in TBS (0.1% Tween-20, 50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Triton X-100 for 1 h at room temperature, washed three times with TBS-Tween (0.1% Tween-20, 50 mM Tris, 150 mM NaCl, pH 7.5) and incubated for 1 h at room temperature with 1 μg/ml goat anti-mouse IgG (Amersham) and 0.1% β-mercaptoethanol for 1 h at 70°C.

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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References
Chu S, DeRisi J, Eisen M, Mulholland J, Botstein D, Brown PO, Ploegh H, Rockford, IL cat. no. 31400) or goat anti-mouse IgG(H+L)-peroxidase (1:2 × 104, Pierce Biotechnology, Rockford, IL). Cat. no. 31432) were used as directed by the manufacturer as secondary antibodies. Blots were developed by ECL (SuperSignal West Pico reagent, Pierce Biotechnology or ECL + , Amersham Biosciences) and exposed to BioMax MR (Eastman Kodak, Rochester, NY, USA) or Hyperfilm ECL (Amersham Biosciences). For reprobing, blots were stripped with 2% (w/v) SDS and 0.1 M β-mercaptoethanol for 1 h at 70°C.


