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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 (Hac1^{i}p) 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 (*EMG*s) by Ume6p, and the Ume6p DNA-binding site URS1 in the promoters of *EMG*s are required for nitrogen-mediated negative regulation of *EMG*s and meiosis by Hac1^{i}p. Co-immunoprecipitation experiments demonstrated that Hac1^{i}p 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 Hac1^{i}p and association of Hac1^{i}p with the HDAC are physiological events in the regulation of *EMG*s 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* (*HAC1^i*) mRNA is efficiently translated. Hac1^{i}p 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; Calfon *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 (*EMG*s). 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 *EMG*s 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).

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In addition to signaling the UPR in yeast, we identified Hac1^{i}p as a negative regulator of the *EMG*s in yeast (Schröder *et al*, 2000). Overexpression of Hac1^{i}p reduced activation of *EMG*s, and deletion of *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 *EMG*s in an *IRE1*-dependent manner (Schröder *et al*, 2000). However, *IME1* mRNA levels were not affected by overexpression of Hac1^{i}p or a *HAC1* deletion (Schröder *et al*, 2000). In addition, splicing of *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 *et al*, 2000). Thus, synthesis of Hac1^{i}p is limited to nitrogen-rich conditions, in which it is a negative regulator of *EMG*s.

To understand the mechanism of negative regulation of *EMG*s by Hac1^{i}p, we identified the upstream repression site 1 (URS1, 5′-TCGGCGGCT-3′) as the promoter element that is sufficient and required to mediate negative regulation of *EMG*s by Hac1^{i}p. We then investigated the involvement of transcriptional repressors recruited to URS1 in negative regulation of transcription by Hac1^{i}p. We show that Hac1^{i}p 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^{i}p. 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

The EMG promoter element URS1 is sufficient to mediate negative regulation of transcription by Hac1^{i}p

Three regulatory elements are common to the promoters of early meiotic genes: an enhancer on nonfermentable carbon sources, called T_4C (Bowdish and Mitchell, 1993), UAS_H, the binding site for the transcription factor Abf1p (Gailus-Durner *et al*, 1996), and URS1. URS1 is a repressing site in vegetative growth under nitrogen-rich conditions, and an activating site in nitrogen starvation (Kupiec *et al*, 1997). Comparison of the promoters of three *EMG*s, *IME2*, *HOP1*, and *SPO13*, that were negatively regulated by Hac1^{i}p (Schröder *et al*, 2000) suggested that URS1 is the site of Hac1^{i}p action (Figure 1A).

To test this hypothesis, we studied the effect of Hac1^{i}p on the activation of a minimal *CYC1* promoter fused to a T_4C enhancer, URS1 elements, or a combination of both by

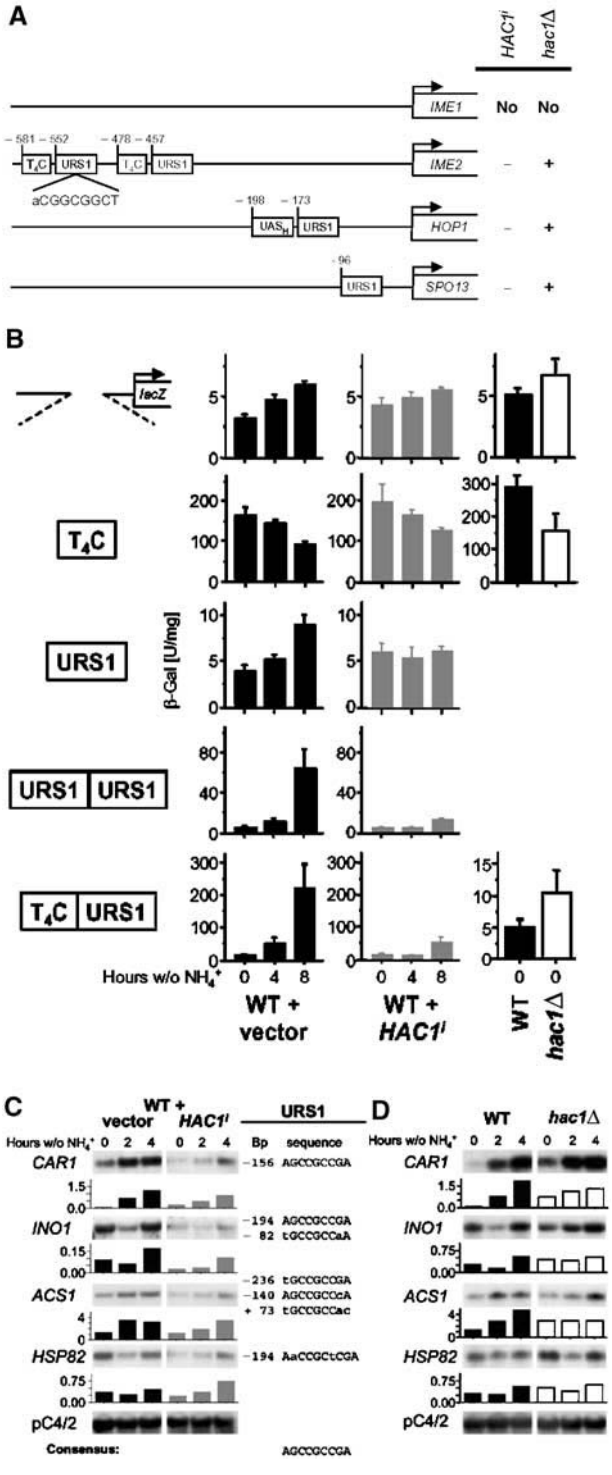


Figure 1 The URS1 element is sufficient for negative regulation of transcription by Hac1^{i}p. (**A**) Promoter elements for the early meiotic genes *IME2*, *SPO13*, and *HOP1* (Mitchell, 1994). The effect of overexpression of *HAC1^i* and a *HAC1* null mutant (*hac1*Δ) on mRNA levels for each early meiotic gene are shown to the right (Schröder *et al*, 2000). '−' represents a decreased mRNA level compared to WT, and '+' an increased mRNA level compared to WT. For comparison, the very early meiotic gene *IME1*, whose expression was not affected by *HAC1* (Schröder *et al*, 2000), is shown on top. The sequence of the URS1 element at position −552 in the *IME2* promoter is shown. Bases in upper case are homologous to the consensus URS1 (TCGGCGGCT; Mitchell 1994). (**B**) Expression of *lacZ* reporter plasmids harboring the inserts shown on the left in an UAS less *CYC1* promoter (top) before and after induction of nitrogen starvation in WT strains transformed with pRS314 or pRS314-*HAC1^i* to overexpress Hac1^{i}p and *hac1*Δ strains. The average and standard error from four independent transformants are shown. (**C, D**) Negative regulation of transcription by Hac1^{i}p extends to nonmeiotic genes that contain URS1 elements in their promoters. Northern analysis of WT strains transformed with pRS316 or pRS316-*HAC1^i* to overexpress Hac1^{i}p (C), and WT and *hac1*Δ 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 pC4/2. All strains carry a deletion in *RME1*.

nitrogen starvation. Synthesis of Hac1^{i}p from the endogenous genomic locus is shut off in nitrogen-starved cells (Schröder *et al*, 2000). To express Hac1^{i}p in nitrogen-starved cells, a previously described plasmid-borne copy of *HAC1^i* 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 T_4C enhancer alone (Figure 1B). In the presence of Hac1^{i}p, activation of reporters harboring URS1 elements by nitrogen starvation was dramatically blunted. In contrast, Hac1^{i}p had no negative effect on the T_4C enhancer alone (Figure 1B). *hac1*Δ strains consistently displayed a 2–3-fold lower expression from the T_4C enhancer (Figure 1B). Expression controlled by T_4C and URS1 was consistently unchanged or slightly elevated in *hac1*Δ strains when compared to wild-type (WT) strains (Figure 1B), suggesting the disruption of a negative regulatory mechanism on the URS1 element in *hac1*Δ strains. In addition, URS1 mediated repression of *lacZ* reporters in nitrogen-rich conditions (Figure 1B, compare T_4C to T_4C-URS1 at the 0 h time point) and activation during nitrogen starvation (Figure 1B), thereby validating the assay system. The negative effect of Hac1^{i}p 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 Hac1^{i}p, we investigated the effect of overexpression of Hac1^{i}p and deletion of *HAC1* on several URS1-controlled genes, for which expression is not limited to meiosis: *ACS1* (Kratzer and Schüller, 1997), *CAR1* (Sumrada and Cooper, 1987), *HSP82* (Szent-Gyorgyi, 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 Hac1^{i}p on transcription of URS1-controlled genes is not limited to *EMG*s. Taken together, these data show that URS1 is sufficient to mediate the negative effect of Hac1^{i}p on transcription.

These data and our earlier study (Schröder *et al*, 2000) show that nitrogen represses the *EMG*s, at least in part, through stimulation of synthesis of Hac1^{i}p. To test directly whether expression of Hac1^{i}p in nitrogen starvation interferes with spore formation, we analyzed the effect of Hac1^{i}p overexpression on ascus formation in a diploid strain. As expected, the percentage of cells that initiated meiosis was significantly lower in the Hac1^{i}p-expressing strain than in the WT strain 1 day after induction of meiosis by nitrogen starvation (Supplementary Figure 1S). We conclude that expression of Hac1^{i}p 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 Hac1^{i}p

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 *EMG*s (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 Hac1^{i}p is mediated through *UME6*, we assayed the effect of Hac1^{i}p on URS1-controlled transcription in *ume6*Δ strains. As expected, deletion of *UME6* abolished repression mediated by URS1 in vegetative cells (Figure 2C, compare T_4C to T_4C-URS1 at the 0 h time point), and activation of reporter plasmids or endogenous promoters by nitrogen starvation (Figure 2C and D, compare T_4C-URS1 in WT and *ume6*Δ strains and Figure 2E). Furthermore, any effect of either Hac1^{i}p 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 Hac1^{i}p.

Since *UME6* controls expression of several hundred genes (Williams *et al*, 2002), pleiotrophic effects may be associated with a *ume6* null mutation. To test the specificity of the results obtained from *ume6*Δ 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 Hac1^{i}p 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 Hac1^{i}p, 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 *hac1*Δ 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 *EMG*s *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 Hac1^{i}p or a *HAC1* deletion (Figure 2G and data not shown). In summary, these data demonstrate that negative regulation of transcription by Hac1^{i}p 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 *EMG*s by Hac1^{i}p and why negative regulation of transcription by Hac1^{i}p is restricted to URS1-controlled genes.

The RPD3-SIN3 HDAC and its catalytic activity are required for negative regulation of transcription by Hac1^{i}p

Next, we asked which of the two repression complexes recruited to URS1 by Ume6p (Figure 2A) is required for negative regulation of transcription by Hac1^{i}p. To address this question, we assayed the effect of Hac1^{i}p on expression of URS1-controlled *lacZ* reporters in strains deleted for components of the *ISW*2 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 Hac1^{i}p (Figure 3A). However,

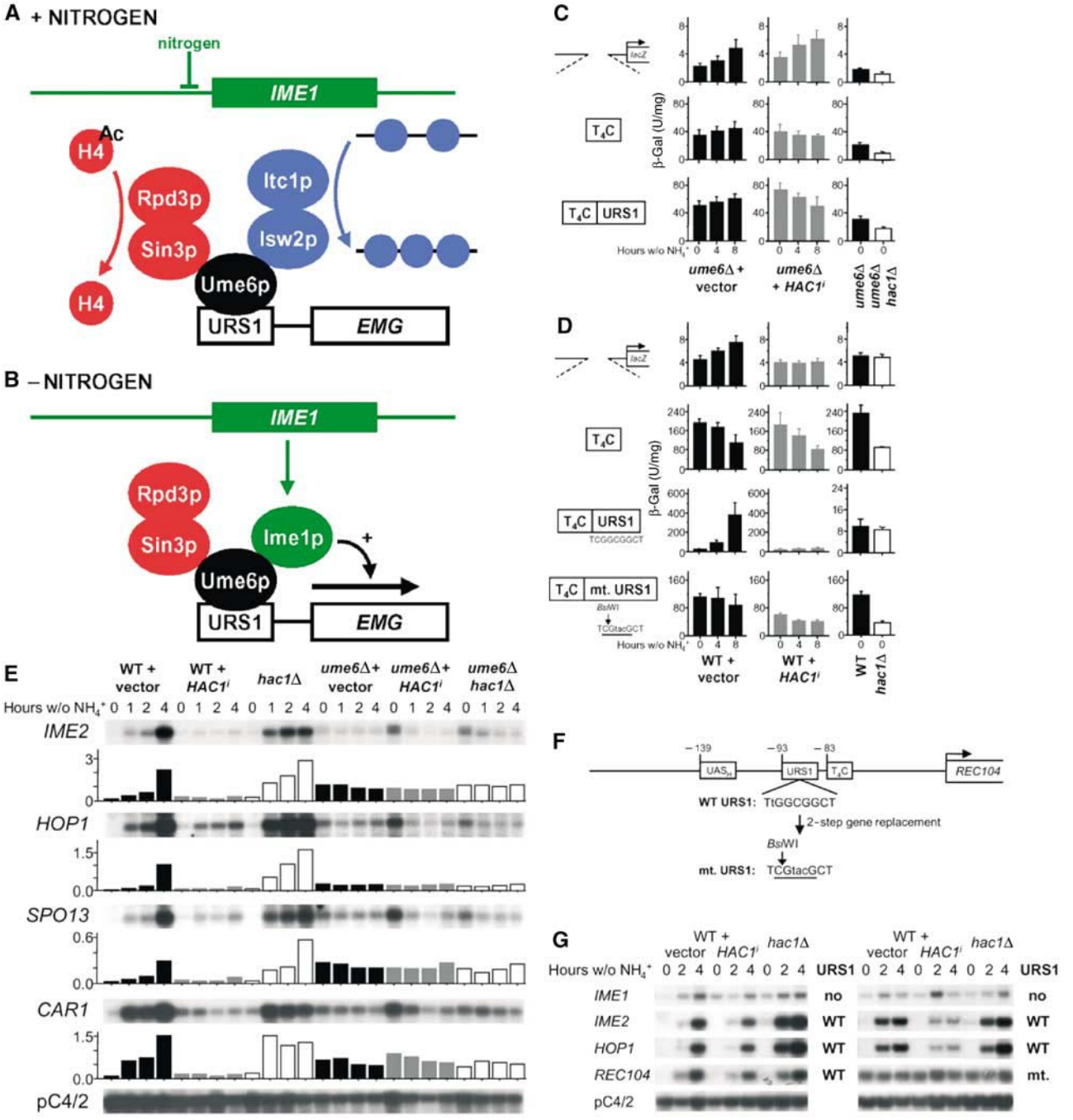
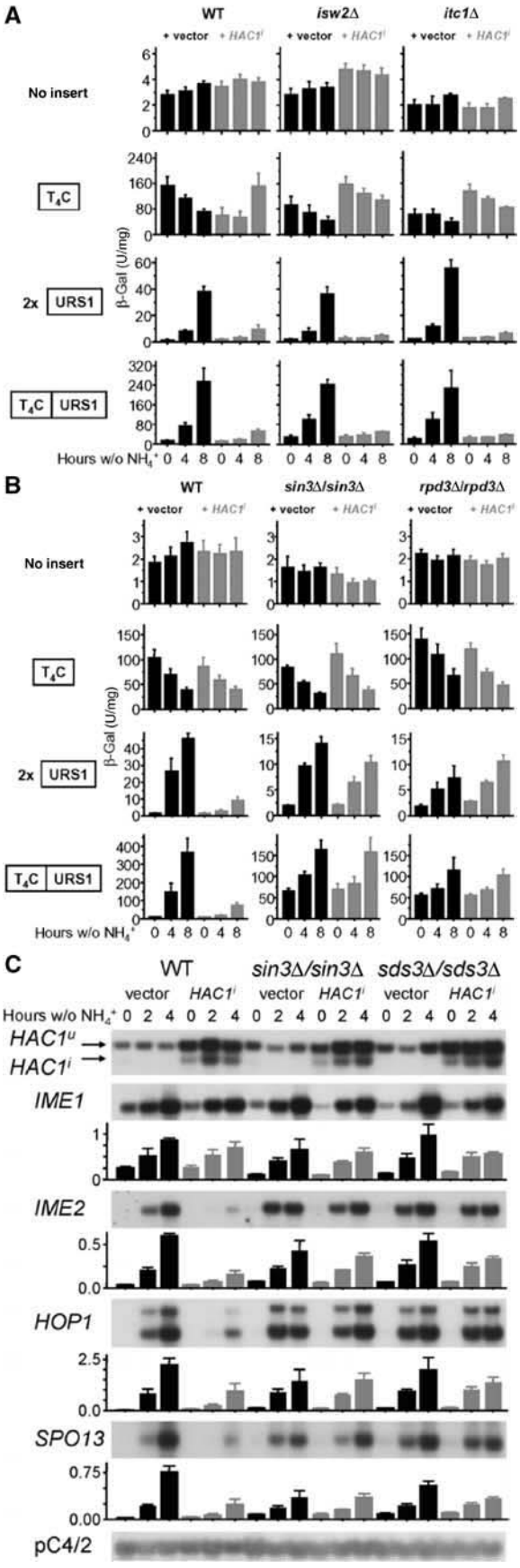


Figure 2 URS1, *UME6*, and binding of Ume6p to URS1 are required for negative regulation of transcription by Hac1^{i}p. (**A, B**) Regulation of *EMG* transcription in nitrogen-rich conditions (A) and in nitrogen starvation (B). Please refer to the text for discussion. Blue circles represent nucleosomes. (**C**) Deletion of *UME6* abolished all effects of Hac1^{i}p on URS1-controlled transcription. (**D**) A three base pair mutation in the URS1 element abolished negative regulation of URS1-controlled transcription by Hac1^{i}p. Expression of *lacZ* reporter plasmids harboring the inserts shown on the left (C, D) in *ume6*Δ strains (C) and WT strains (D) transformed with pRS314 or pRS314-*HAC1^{i}* and *ume6*Δ *hac1*Δ strains (C) and *hac1*Δ strains (D) before and after induction of nitrogen starvation. The average and standard error from four independent transformants are shown (C and D). (**E**) Northern analysis of some of the strains in (C, D). Bar graphs represent relative mRNA levels obtained by PhosphorImager analysis and standardization of the signal for each mRNA to the loading control pC4/2. (**F**) Mutation of URS1 elements in the genomic promoters of the early meiotic genes *DMC1* and *REC104* by two-step gene replacement. The *Bsi*WI site introduced with the mutated URS1 elements is underlined. (**G**) Northern analysis of WT strains (transformed with pRS314), WT strains overexpressing Hac1^{i}p, and *hac1*Δ strains containing either a WT or mutated URS1 element in the genomic promoter of *REC104*. Cells were grown to mid-log phase on synthetic acetate medium and shifted to C-SPO medium for the indicated times. All strains carry a deletion in *RME1*.

deletion of either *SIN3* or *RPD3* relieved the negative effect of Hac1^{i}p on transcription (Figure 3B). We confirmed this result by Northern analysis for the *EMGs IME2*, *HOP1*, and *SPO13* in *sin3*Δ/*sin3*Δ strains and diploid strains deleted for *SDS3*, an integral component of the *RPD3-SIN3* HDAC (Lechner *et al*, 2000). The negative effect of Hac1^{i}p 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 Hac1^{i}p.

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 Hac1^{i}p, or whether the HDAC simply serves as a docking site for a repressor induced by Hac1^{i}p, 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 Hac1^{i}p was abolished in strains carrying integrated point mutant *RPD3* alleles (Figure 4A and B). Furthermore, the modest loss in repression observed in *hac1*Δ strains was lost in *sin3*Δ *hac1*Δ, *rpd3*Δ *hac1*Δ, and *hac1*Δ 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 *rpd3*Δ 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 Hac1^{i}p, since deacetylase-deficient point mutations in *RPD3* were sufficient to relieve completely the negative effect of Hac1^{i}p on transcription. Taken together, these data demonstrate that Hac1^{i}p requires the catalytic activity of the *RPD3-SIN3* HDAC to negatively regulate transcription on the URS1 element.

Hac1^{i}p can associate physically with the RPD3-SIN3 HDAC in vivo

Next, we used co-immunoprecipitation experiments to investigate if the genetic interaction between Hac1^{i}p 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 Hac1^{i}p by Western blotting or immunoprecipitation (data not shown). To express sufficient amounts of

Figure 3 The *ISW2* chromatin remodeling complex is dispensable (**A**) and the *RPD3-SIN3* HDAC is required for negative regulation of transcription by Hac1^{i}p (**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*), *sin3*Δ/*sin3*Δ, and *sds3*Δ/*sds3*Δ strains transformed with pRS314 or pRS314-*HAC1^{i}* 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. *HAC1^{u}* represents the unspliced, untranslated *HAC1* mRNA, and *HAC1^{i}* the spliced, translated *HAC1* mRNA. Two mRNAs for *HOP1* and *SPO13* were detected (Schröder *et al*, 2000).

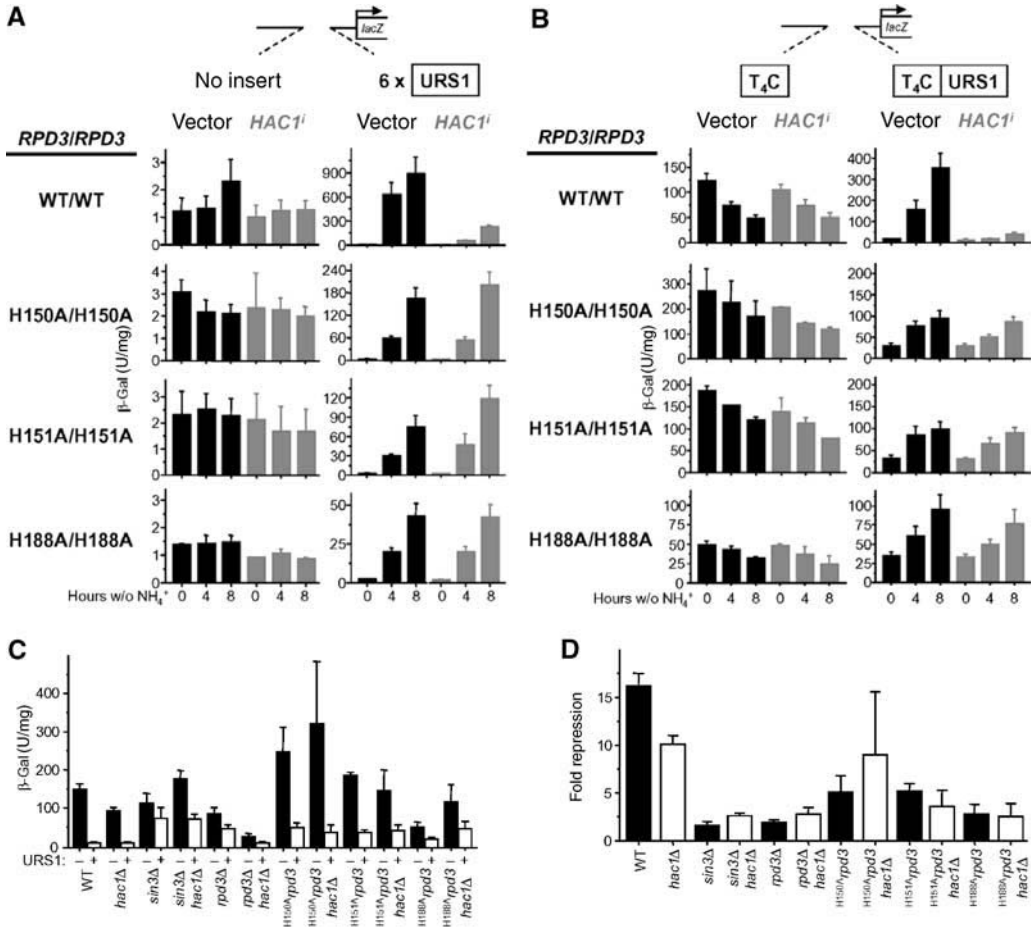


Figure 4 Catalytic activity of the *RPD3-SIN3* HDAC is required for negative regulation of transcription by Hac1^{i}p. (**A, B**) Expression of *lacZ* reporter plasmids harboring the inserts indicated on top in homozygous diploid mutant *RPD3* strains (left) transformed with pRS314 or pRS314-*HAC1^i* before and after induction of nitrogen starvation. (**C**) Expression of *lacZ* reporter plasmids harboring a T_4C enhancer (labeled '−') and a T_4C enhancer and a URS1 element (labeled '+') in mid-log phase on synthetic acetate medium. The average and standard error from 20 (WT and *hac1*Δ strains), 10 (all *rpd3*Δ strains), six (all *sin3*Δ strains), or four (all *RPD3* point mutant strains) independent transformants are shown. (**D**) Fold repression mediated by the URS1 element. The fold repression was defined as the ratio of β-galactosidase expression from the plasmid without URS1 element ('−', panel C) to the amount of β-galactosidase expressed from the plasmid containing a URS1 element ('+', panel C). All strains carry a deletion in *RME1*.

Hac1^{i}p for biochemical studies, we used an inducible expression system, in which expression of Hac1^{i}p 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^i* mRNA or Hac1^{i}p were detected by Northern (data not shown) or Western analysis (Figure 5A). At 1 h after induction with 50 μM DOC, Hac1^{i}p was readily detected by Western blotting (Figure 5A). Expression of Hac1^{i}p 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 Hac1^{i}p (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-Hac1^{i}p (Figure 5C). Antibodies directed against other components of the *RPD3-SIN3* HDAC, Sin3p and Sap30p (Zhang *et al*, 1998) also immunoprecipitated HA-HAc1^{i}p (Figure 5C and data not shown), thus further confirming the interaction between Hac1^{i}p and the HDAC. This interaction was not observed in *sin3*Δ 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 Hac1^{i}p can associate with the *RPD3-SIN3* HDAC *in vivo*.

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

The ability of Hac1^{i}p to co-immunoprecipitate with Rpd3p raised the possibility that Hac1^{i}p is an integral component of the HDAC required for its function. However, *hac1*Δ strains showed only a partial loss of repression when compared to *sin3*Δ or *rpd3*Δ 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.

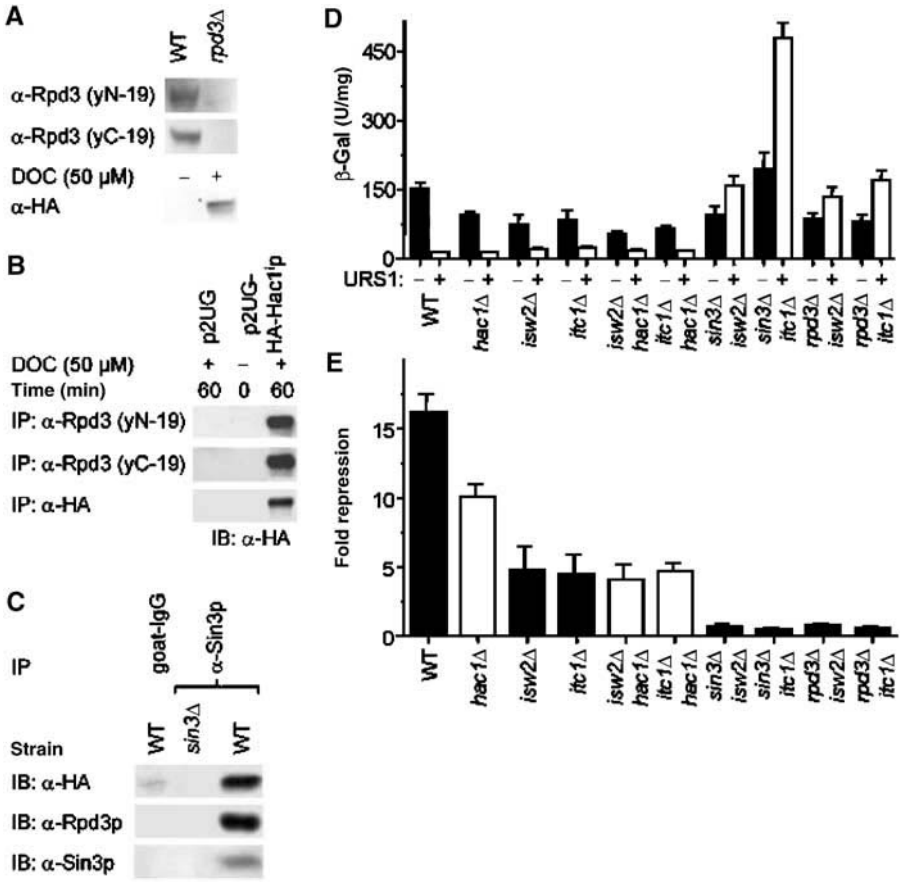


Figure 5 Hac1^{i}p associates with the *RPD3-SIN3* HDAC, but is not an integral component of the HDAC. (**A**) Detection of Rpd3p and HA-Hac1^{i}p after induction with 50 μM deoxycorticosterone (DOC) in mid-log-phase cultures by Western blotting. Co-immunoprecipitation of Hac1^{i}p with (**B**) Rpd3p and (**C**) Sin3p in a WT strain. Hac1^{i}p expression was induced with 50 μM DOC for 1 h in a culture grown to mid-log phase on synthetic acetate medium. (**D, E**) *HAC1* is not an integral component of the *RPD3-SIN3* HDAC. (D) Expression of *lacZ* reporter plasmids harboring a T_4C enhancer (labeled '−') and a T_4C enhancer and a URS1 element (labeled '+') in the mid-log phase on synthetic acetate medium. The average and standard error from 20 (WT and *hac1*Δ strains) or six (all *isw2*Δ and *itc1*Δ strains) independent transformants are shown. All strains carry a deletion in *RME1*. (E) Fold repression mediated by the URS1 element as defined in the legend to Figure 4.

We then assayed expression of *lacZ* reporters harboring a T_4C enhancer or a T_4C 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 *ume6*Δ strains or strains carrying mutations in URS1 elements (Goldmark *et al*, 2000; Figure 2). However, derepression on the URS1 element in *isw2*Δ *hac1*Δ and *itc1*Δ *hac1*Δ strains was very similar to the level of derepression seen in *isw2*Δ and *itc1*Δ 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 Hac1^{i}p is a peripheral component of the HDAC. We propose that upon binding of Hac1^{i}p to the HDAC, a signal is transduced through the HDAC to inhibit transcription of *EMG*s 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 (*EMG*s). 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 (Hac1^{i}p) and deletion of *HAC1* revealed that Hac1^{i}p is a negative regulator of *EMG*s (Schröder *et al*, 2000).

In the present study, we identified the complex that integrates the nitrogen signal transduced by Hac1^{i}p into regulation of *EMG*s. We have shown that negative regulation of *EMG*s by Hac1^{i}p requires URS1, Ume6p, the *RPD3-SIN3* HDAC, and the catalytic activity of the HDAC. Co-immunoprecipitation studies revealed that Hac1^{i}p can interact with the HDAC *in vivo*. Further, negative regulation by Hac1^{i}p was abolished when URS1 was mutated in the *REC104* promoter (Figure 2G), but retained for *EMG*s 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 *EMG*s by Hac1^{i}p. Further, the

requirement of the catalytic activity of the *RPD3-SIN3* HDAC for negative regulation of *EMG*s by Hac1^{i}p shows that simple recruitment of another transcriptional repressor to URS1 by Hac1^{i}p cannot account for negative regulation of *EMG*s by Hac1^{i}p. Therefore, we propose the following model for nitrogen-mediated regulation of *EMG*s by Hac1^{i}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^{i}p correlates very well with the degree of *HAC1* mRNA splicing, since the half-life of Hac1^{i}p is about 2 min (Kawahara *et al*, 1997). Hac1^{i}p then associates with the *RPD3-SIN3* HDAC (Figure 5B). The Hac1^{i}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 *EMG*s (Figure 2E and G) by nitrogen starvation in cells overexpressing Hac1^{i}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^{i}p is rapidly cleared from the cell. This results in loss of HDAC-dependent repression of *EMG*s. 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 *EMG*s (Bowdish *et al*, 1995; Rubin-Bejerano *et al*, 1996), the HDAC is associated with the promoters of *EMG*s in nitrogen starvation. Thus, nitrogen-induced association of Hac1^{i}p with the HDAC and the enhanced repression potential of the Hac1^{i}p–HDAC complex are physiological events in the control of *EMG*s by nutrients. On the basis of this model, we predict that association of Hac1^{i}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^{i}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

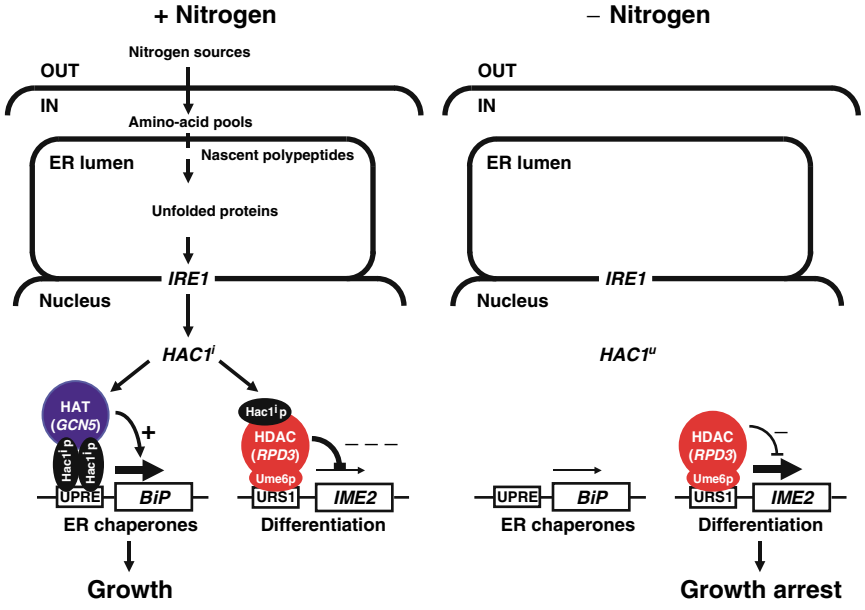


Figure 6 Model for promotion of growth and proliferation by the UPR. The UPR promotes growth through Hac1^{i}p-mediated induction of ER chaperone genes and phospholipid biosynthesis. Activation of ER chaperone genes by Hac1^{i}p requires the histone acetyltransferase *GCN5* (Welihinda *et al*, 1997, 2000). At the same time, the Ume6p/HDAC/Hac1^{i}p complex represses entry into meiosis and growth arrest associated with entry into the differentiation program. For details, see Discussion. Dashed lines indicate incompletely understood relationships. For simplicity, the Hac1^{i}p species interacting with the HDAC is depicted as a monomer. For the same reason, only events related to the UPR are shown.

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 Hac1^{i}p complements the growth-promoting activities of the UPR to promote growth as long as nutritional conditions are sufficient (Figure 6).

Our finding that Hac1^{i}p negatively regulates transcription of *EMG*s 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 eIF2α by PERK (Shi *et al*, 1998, 1999; Harding *et al*, 1999). Our study demonstrates, for the first time, that a large group of 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 Hac1^{i}p 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/EBPα and C/EBPβ (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_1 by E4BP4 (Cowell and Hurst, 1996), *SSN6-TUP1* by Sko1p (Proft 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 *EMG*s by Hac1^{i}p does not involve direct binding of Hac1^{i}p to DNA, since it is dependent on Ume6p (Figure 2), which is constitutively bound to URS1. Second, Hac1^{i}p does not recruit the HDAC to URS1 or Ume6p, since repression by the HDAC on URS1 was mostly intact in *hac1*Δ 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 (Reimold *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 (Calfon *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 Blimp-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 Blimp-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 pLGΔ312SΔSS and its derivatives is driven from a *CYC1* promoter from which all upstream activating sites were removed (Bowdish and Mitchell, 1993). In pRS316-*HAC1^i* and pRS314-*HAC1^i*, expression of Hac1^{i}p is driven from its own promoter and *HAC1* is tagged with a single HA tag that was inserted into the *Spe*I site in the *HAC1* ORF (Chapman and Walter, 1997). Expression of Hac1^{i}p from p2UG-HA-*HAC1^i* 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 Hac1^{i}p expression

Rich dextrose (YPD), rich acetate (YPAc), synthetic dextrose (SD), synthetic acetate (PSP2), and nitrogen starvation medium (C-SPO) were described before (Schröder *et al*, 2000). Cultures grown to mid-log phase (typically 0.3–0.5 OD_{600}) from 0.01 OD_{600} were washed once with water, and resuspended in C-SPO medium to induce meiosis or nitrogen starvation. To maintain plasmids in yeast strains, the strains were grown on synthetic media lacking the appropriate nutrients. For sporulation experiments, at least 200 cells were counted in each sample. Expression of Hac1^{i}p from the steroid-inducible plasmid p2UG-HA-Hac1^{i}p was induced for 1 h with 50 μM deoxycorticosterone (DOC, Sigma, St Louis, MO). Induction of Hac1^{i}p was confirmed by Northern and Western blotting.

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 (Hercules, CA) D_C protein assay.

Northern blots

Isolation of RNA, the Northern blotting protocol, and the probes for *HAC1*, *HOP1*, *IME1*, *IME2*, and *SPO13* were described previously (Schröder *et al*, 2000). Probes for *ACS1*, *CAR1*, *DMC1*, *HSP82*, *INO1*, and *REC104* were generated by PCR using genomic DNA as template. Dextran sulfate, 10% (average molecular weight 500 000, Sigma), was added to all hybridizations with *DMC1* and *REC104* probes. All mRNAs were quantitated by PhosphorImaging on a Typhoon 9400 (Amersham Biosciences, Piscataway, NJ) 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 Na_3VO_4, 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 D_C 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-Rpd3p (yN-19 or yC-19, Santa Cruz Biotechnology, Santa Cruz, CA), 2 μg anti-Sin3p (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 (0.45 μm, Schleicher & Schuell, Keene, NH; Dunn, 1986) and probed with anti-Rpd3p (1 μg/ml), anti-Sin3p (1 μg/ml), or anti-HA (0.1 μg/ml) antibodies as primary antibodies. Mouse anti-goat IgG(H + L)-peroxidase ($1{:}2 \times 10^4$, Pierce Biotechnology, Rockford, IL cat. no. 31400) or goat anti-mouse IgG(H + L)-peroxidase ($1{:}2 \times 10^4$, Pierce Biotechnology, 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.

Supplementary data

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

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