The conserved histone deacetylase Rpd3 and the DNA binding regulator Ume6 repress \textit{BOI1}'s meiotic transcript isoform during vegetative growth in \textit{Saccharomyces cerevisiae}

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Summary

\textit{BOI1} and \textit{BOI2} are paralogs important for the actin cytoskeleton and polar growth. \textit{BOI1} encodes a meiotic transcript isoform with an extended 5′-untranslated region predicted to impair protein translation. It is, however, unknown how the isoform is repressed during mitosis, and if Boi1 is present during sporulation. By interpreting microarray data from $\text{MAT}a$ cells, $\text{MAT}a/\alpha$ cells, a starving $\text{MAT}a/\alpha$ control, and a meiosis-impaired rrp6 mutant, we classified \textit{BOI1}'s extended isoform as early meiosis-specific. These results were confirmed by RNA-Sequencing, and extended by a 5′-RACE assay and Northern blotting, showing that meiotic cells induce the long isoform while the mitotic isoform remains detectable during meiosis. We provide evidence via motif predictions, an \textit{in vivo} binding assay and genetic experiments that the Rpd3/Sin3/Ume6 histone deacetylase complex, which represses meiotic genes during mitosis, also prevents the induction of \textit{BOI1}'s 5′-extended isoform by direct binding of Ume6 to its URS1 target. Finally, we find that Boi1 protein levels decline when cells switch from fermentation to respiration and sporulation. The histone deacetylase Rpd3 is conserved, and eukaryotic genes frequently encode transcripts with variable 5′-UTRs. Our findings are therefore relevant for regulatory mechanisms involved in the control of transcript isoforms in multi-cellular organisms.

Introduction

\textit{BOI1} and \textit{BOI2} are partially functionally redundant paralogs, which encode proteins important for establishing cell polarity and bud formation; cells lacking both genes cannot grow at 30°C, show an aberrant morphology and grow very poorly at 20°C (Bender et al., 1996; Matsui et al., 1996; Cole et al., 2009; Liao et al., 2013). A detailed cytological analysis in budding yeast revealed a role for Boi1 and Boi2 in a pathway preventing chromosomes from being broken apart during late stages of mitosis in anaphase and telophase, when sister chromatids are separated and pulled to opposite poles before cells split during cytokinesis (Norden et al., 2006). Recent work suggests only Boi2, but not Boi1, to be important for spindle disassembly (Pigula et al., 2014). Direct evidence for a role in polar growth was also reported for the fission yeast Boi1/2 homolog Pob1p (Toya et al., 1999). For single mutants, no meiotic defect has been reported in genome-wide functional genomics studies (Deutschbauer et al., 2002; Enyenihi and Saunders, 2003).

\textit{BOI1} and \textit{BOI2} transcripts are ubiquitously expressed during growth and starvation in all haploid and diploid cell types, while the proteins are controlled at the level of cell cycle stage-specific localisation (Primig et al., 2000; Norden et al., 2006; Granovskaia et al., 2010). Earlier microarray profiling analyses revealed that both genes continue to be transcribed when diploid cells undergo meiotic development (Chu et al., 1998; Primig et al., 2000). More recently, \textit{BOI1} was found to encode a meiotic transcript isoform with an extended 5′-untranslated region (UTR) in a recent high-throughput experiment, which combined transcript profiling by RNA-Sequencing (RNA-Seq) and ribosome profiling. The meiotic 5′-untranslated region (5′-UTR) contains a so-called AUG upstream open reading frame (uORF), which is predicted to exert a negative effect on the translation of the protein encoded by the down-
stream ORF (Brar et al., 2012). uORFs located within 5′-UTRs are involved in a well studied mechanism controlling mRNA translation, for example in the case of GCN4, which is a major regulator of genes involved in amino acid biosynthesis (for review, see (Hinnebusch, 2005)). No direct evidence for declining Boi1 levels during meiosis is available, however, and nothing is known about the transcriptional mechanism controlling the gene’s meiotic isoform.

A group of meiosis-specific genes is repressed during vegetative growth by a tripartite complex comprising the histone deacetylase Rpd3 and the co-repressor Sin3 that are recruited to DNA by Ume6, which binds the upstream regulatory site 1 (URS1) (Strich et al., 1994; Rundlett et al., 1998). Target gene repression is progressively relieved during respiration and sporulation when Ume6 is degraded via a multi-step mechanism involving the Spt-Ada-Gcn5 Acetyltransferase (SAGA) complex, the anaphase promoting complex/cyclosome (APC/C) and the inductor of meiosis 1 (Ime1) (Mallory et al., 2007; Law et al., 2014). Ume6 was shown to bind the upstream region of BOI1 in vivo in a large-scale chromatin immunoprecipitation assay analysing mitotic cells, but since BOI1 is expressed in vegetatively growing cells the functional significance of this finding remained unclear (Harbison et al., 2004). Predicting biologically active regulatory motifs has become more reliable due to large-scale in vivo binding data, which are now available for nearly all known yeast transcription factors (TFs) (Harbison et al., 2004; Xie et al., 2011). The TRANSFAC database provides position weight matrices (PWMs) for TFs. A PWM is generated by aligning the DNA sequences the target TF binds to, and log transforming the number of observations of each base at each position in the matrix (Wingender, 2008; Spivak and Stormo, 2012).

In this study we report a detailed analysis of BOI1 isoform expression in fermenting, respiring, starving and sporulating cells using tiling array data obtained with wild-type strains and an rrp6 mutant (Lardenois et al., 2011; Stuparevic and Liu et al., in preparation) and RNA-Seq data (Becker et al., in preparation). We validate and extend the high-throughput data by RT-PCR, 5′-RACE assays and Northern blotting. We combine published Ume6 in vivo binding data with predicted URS1 motifs, a chromatin immunoprecipitation (ChiP) assay and genetic experiments, to demonstrate that Rpd3 and Ume6 are needed to repress BOI1’s meiotic isoform in cells undergoing rapid mitotic growth. Finally, we report that increasing levels of the 5′-extended BOI1 transcript correlate with declining levels of Boi1 protein as cells transit from fermentation to respiration and sporulation. These results provide insight how Boi1’s mitotic mechanism of regulation is altered during meiotic development, and they provide initial evidence that the HDAC Rpd3/Ume6 complex negatively regulates Boi1.

Results

BOI1 encodes a transcript isoform with an extended 5′-UTR expressed in early meiosis but not vegetative growth and starvation

We used tiling array data to determine the transcript architecture of BOI1 and BOI2 in fermenting, respiring, and sporulating diploid MATα/α cells as compared to a sporulation-deficient starving MATα/α control strain and vegetatively growing haploid MATα cells (Lardenois et al., 2011). For BOI1 we find that wild-type MATα/α cells express a transcript isoform with an extended 5′-UTR from meiotic pro-phase onwards, while starving control cells and synchronously growing haploid cells do not express this long transcript at any point during prolonged nutrient deprivation or the mitotic cell cycle. The extended isoform entirely covers the ARS202 origin of replication (Fig. 1A). We were unable to investigate BOI2 because the gene is juxtaposed to SPR6, which is highly expressed during growth and starvation, and encodes a transcript strongly
induced during sporulation that covers the \textit{BOI2} upstream region (Fig. S1).

\textbf{Cells express two BOI1 isoforms when progressing from mitotic growth to meiotic differentiation}

Neither tiling array data nor RNA-Seq data reveal if cells co-express the short mitotic isoform and the long meiotic isoform during sporulation, or if the 5' extended transcript becomes the dominant mRNA. This is, however, an interesting question from a functional perspective since the long isoform was predicted to inhibit protein translation (Brar \textit{et al.}, 2012). If that mechanism was solely responsible for the protein pattern observed, one might expect cells to predominantly express the extended transcript. We therefore used 5' rapid amplification of cDNA ends (5'-RACE) assays to study isoform levels and found that fermenting and respiring cells

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express the short mitotic isoform, while cells during early meiosis (SPII 4–6 hours) express both the 5′-extended and the short mitotic isoform; post-meiotic cells (SPII 8 hours) slowly start reverting back to the mitotic pattern (Fig. 1B). Unexpectedly, we observed two bands for **BOI1**. We therefore determined by DNA sequencing that the faster migrating band indeed corresponded to **BOI1** (272/273 bases identical, Fig. S2A and B), while the slower band was amplification artefact unrelated to the target gene, perhaps stemming from GC-rich sequences in the yeast genome (Fig. S2C). Next, we confirmed the 5′-RACE data by Northern blotting using probes that monitor isoforms in combination with RT-PCR assays that validate array data (Fig. 1C–D). We conclude that vegetatively growing cells express only the short mitotic isoform, while in early meiotic cells the 5′-extended isoform is transiently induced reaching peak levels at 4–6 hours.

**Meiosis-deficient mutants fail to normally induce the long isoform of BOI1**

We then sought to complement tiling array data by RNA-Seq and confirmed that diploid **MATα/α** cells express a 5′-extended **BOI1** isoform from early meiosis onwards. Consistently, the transcript levels decrease as cells exit M-phase and enter spore formation. As expected, starving **MATα/α** cells do not express the meiotic isoform, and do not downregulate **BOI1** eight hours after having been transferred into sporulation medium (Fig. 2A; the complete dataset will be published elsewhere; Becker et al., in preparation). We next asked if a strain lacking the 3′–5′ exoribonuclease Rrp6, which fails to undergo efficient meiosis and spore formation, expresses the **BOI1** isoform when cultured in sporulation medium (Lardenois et al., 2011). Tiling array data indicate that the meiotic isoform of **BOI1** is expressed at lower levels in cells lacking Rrp6 (Fig. 2B; the complete dataset will be published elsewhere; Stuparevic and Liu et al., in preparation). We confirmed this finding by RT-PCR assays carried out with samples from wild-type cells and an **rrp6** mutant strain cultured in rich media and sporulation medium (Fig. 2C). Taken together, the results show that the 5′-extended isoform of **BOI1** is only induced to normal levels when cells undergo efficient meiotic development, and that nutrient deprivation alone is not sufficient to fully de-repress the extended isoform.

**The BOI1 upstream region contains a predicted URS1 motif**

**BOI1**'s isoform shows an expression pattern reminiscent of early meiotic genes and Ume6, a mitotic repressor of meiotic genes, was found to bind to the **BOI1** promoter in vivo in a high-throughput chromatin immunoprecipitation assay (Fig. S3; Harbison et al., 2004; see also www.genmonline.org (Lardenois et al., 2010)). Consequently, we examined the locus using PWMs (Fig. 3A) and found a predicted URS1 element just upstream of the putative meiotic transcription start site (meiTSS) for **BOI1**'s developmental-stage specific isoform (Fig. 3B). This target motif is strongly bound by Ume6 in vivo as shown by a chromatin immunoprecipitation (ChIP) assay; the **NUP85** locus was used as a negative control (Fig. 3C) (Lardenois et al., 2014). Our data are consistent with the notion that Ume6 and, by inference, its interactors Rpd3 and Sin3 are recruited to the **BOI1** promoter region during vegetative growth.

**Rpd3 and Ume6 repress the 5′-extended meiotic isoform of BOI1 in mitosis**

The long **BOI1** isoform showed the pattern of a typical Rpd3/Sin3/Ume6-dependent early meiotic gene, and Ume6 binds in vivo to the **BOI1** upstream region, which contains a predicted URS1 motif. We therefore hypothesized that Ume6 and Rpd3 repress the meiotic isoform during mitotic growth. First, we sought to further validate the tiling array data and RNA-Seq data using RT-PCR and different combinations of oligonucleotide primer pairs to analyse RNA samples from diploid wild-type SK1 and JHY222 strains (Fig. 3D). Primers located in the 5′-UTR (**mBOI1**), revealed no signal in an SK1 sample from fermenting cells (YPD), a weak signal in a sample from respiring cells (YPA), strong signals during early meiosis (SPII 2–4 h), and decreasing signals during post-meiotic spore formation (SPII 8–12 h; Fig. 3E). We then repeated the experiment using wild-type JHY222 cells, which sporulate well but not quite as fast and as efficient as SK1 cells, to assess the degree of reproducibility of our results between distinct genetic backgrounds (Lardenois et al., 2011). We find a similar pattern except for a stronger signal in respiring cells and a broader peak of induction (Fig. 3F). Then, we confirmed that **BOI1** encodes an extended isoform rather than two overlapping transcripts being transcribed on the same strand in the same direction. To this end, we carried out an RT-PCR assay with a primer pair located at the 5′ and 3′ ends of the locus, which generate a long DNA fragment (**lmBOI1**). This experiment nearly perfectly reproduced the pattern observed in SK1 (compare panels E and F). Primers located within the coding region (**BOI1**) yielded the expected homogenous pattern in all samples. We employed **ACT1** as a loading control.

Importantly, we found that SK1 mutant **ume6** and **rrp3** cells and JHY2223 mutant **ume6** cells express the extended transcript during vegetative growth in the presence of glucose or acetate. Moreover, the mutant cells
Fig. 2. Lack of normal mBOI1 induction in sporulation-impaired cells.
A. Histograms depicting RNA-Seq data (not DNA strand specific) obtained with SK1 strains are shown. RNA was analysed from fermenting (YPD), respiring (YPA) and sporulating (SPII 4, 6, 8 hours) cells as indicated. Mitotic and meiotic samples from the wild-type strain are given in blue and green, respectively. Corresponding samples from the control strain are shown in red and orange. Thin dotted blue lines represent the ORFs, and a red dotted line represents the extended 5’-UTR as determined by DNA strand specific tiling arrays.
B. A heatmap (left panel) is shown for tiling array data obtained with wild-type and rrp6 mutant cells cultured in growth media and sporulation medium as indicated. The strains are indicated to the right. A color-coded bar diagram (right panel) quantifies log-transformed tiling array signals for the segments that correspond to the ORF (BOI1) or the extended UTR (BOI1 5’UTR; y-axis) in wild-type cells (blue) versus rrp6 mutant cells (red) that were cultured in growth media (YPD, YPA) and sporulation medium (SPII) at three time points as indicated.
C. To the left, the output of RT-PCR assays is shown for samples indicated at the top from wild-type versus rrp6 mutant strains indicated to the left. Data are given for the mitotic isoform (BOI1), and the meiotic isoform (mBOI1). ACT1 was used as a loading control. To the right, two bar diagrams summarize the RT-PCR band’s relative signal intensities (y-axis) for each sample from wild-type cells (WT in blue) and mutant cells (rrp6 in red; x-axis). Signals corresponding to the extended 5’-UTR (mBOI1) and the ORF (BOI1) are shown. The samples were harvested after incubation in rich media (YPD, YPA) and sporulation medium (SPII) at the time points given in hours (x-axis).
also failed to induce the isoform when cultured in sporulation medium (Fig. 3G and H). Finally, we complemented the RT-PCR data by a Northern blot showing that both short and long isoforms are present in *ume6* mutant cells cultured in YPD and YPA; we note that the long isoform is weaker in respiring SK1 *ume6* cells (compare panels G and I). Taken together, the data imply that the HDAC repressor complex Rpd3/Sin3/Ume6 represses the meiotic *BOI1* isoform during mitotic growth.

The *Boi1* protein rapidly declines when cells switch from fermentation to respiration and sporulation

The 5′-extension of *BOI1*’s isoform harbours a uORF, which was predicted to downregulate *Boi1* protein translation during meiosis and spore formation (Brar *et al.*, 2012). To further study this question, we tagged *Boi1* with a C-terminal myc epitope. The diploid *Boi1*<sup>myc</sup> strain showed no discernible growth phenotype, we therefore conclude
that the protein is functional within the limits of our assay. An RT-PCR assay of samples from this strain revealed constitutive expression of Boi1 during growth and development, moderate expression of mBOI1 in respiring cells (YPA), and the expected induction pattern during sporulation (SPII; Fig. 4A, top panel). The corresponding time-course experiment revealed an approximately 3.5-fold decrease of Boi1 when cells change from glucose metabolism to acetate as the sole non-fermentable carbon source. Furthermore, the protein is undetectable by Western blot in protein extracts from cells cultured in sporulation medium for two hours, and it remains absent until spore formation is initiated at 10 hours (in the W303 background; Fig. 4A, bottom panel). We then compared this pattern of protein levels to the one reported for Ume6, by including a late time point at 24 hours covering spore maturation and found that Boi1 did not re-accumulate at late stages of yeast gametogenesis like Ume6 (Fig. 4B; Mallory et al., 2007). Pgk1 was used as a loading control. Our results show that the Boi1 protein is indeed downregulated at the post-transcriptional level in respiring cells and differentiating cells until late stages of spore maturation, which is consistent with the prediction by Brar et al. that a uORF present in the long isoform inhibits protein translation.

The Boi1 level decreases and the protein is altered in fermenting ume6 mutant cells

Our RT-PCR data and protein data in JHY222 negatively correlate the induction of BOI1’s meiotic isoform with Boi1 protein levels in a dose dependent manner (in YPD, YPA and SPII 2 h). If the long isoform indeed inhibited protein translation, mutant cells that de-repress the meiotic isoform in mitosis should contain little or no Boi1 protein. To test this idea, we myc-tagged Boi1 in a ume6 deletion strain, and monitored BOI1 transcript isoforms and Boi1 protein levels in rich medium with glucose (YPD). The meiotic isoform is de-repressed in ume6 cells cultured in YPD also in the W303 background (Fig. 4C left panel). Furthermore, we observe in three independent experiments that the concentration of Boi1 protein is indeed reduced approximately 10-fold in fermenting cells that lack Ume6 (Fig. 4C right panel). In addition, we find that Boi1 appears to be physically altered such that it migrates slower in the gel; no such change in migration was observed in the case of Pgk1 (Fig. 4C, right panel). Our finding that fermenting ume6 mutant cells contain very little Boi1 provides further evidence that the long isoform is negatively correlated with protein levels.

We then asked if the extended 5′-UTR contained an in-frame ATG start codon indicating that the long isoform might be translated into a larger protein with an N-terminal extension, but found several stop codons in frame with BOI1 (Fig. S4). Furthermore, we predict one uORF encoding a peptide of 45 amino acids 5 bases downstream of the mTSS (Fig. 4D), which is in keeping with previous observations (Brar et al., 2012). We conclude that an increased level of mBOI1 in vegetatively growing mutant ume6 cells correlates with a very low level of Boi1, and that the altered migration properties of Boi1 in fermenting cells lacking Ume6 are not due to an extended N-terminus.

Discussion

In this study we classify BOI1’s extended transcript as early meiosis-specific in the context of a broader expression program we have recently discovered using DNA strand specific tiling arrays (Lardenois et al., 2011; 2014). BOI1 was initially not identified as a candidate for the expression of 5′-extended isoforms by the segmentation algorithm we employed, because of the convoluted expression signals associated with its 5′-leader sequence. However, additional information from RNA-sequencing data (Becker et al., in preparation), RT-PCR and 5′-RACE assays and a Northern blot experiment enabled us to clarify the issue. We note that in the course of this work Brar et al. reported that BOI1 (but not BOI2) encodes a transcript containing a
meiotic 5′-extension, which was predicted to negatively regulate protein translation in sporulating cells via a uORF (Brar et al., 2012).

**BOI1 is a prototype locus for studying the regulation and function of developmental stage specific transcript isoforms**

Following a yeast genome duplication event, identical gene pairs – called paralogs – evolved in different ways. Either one of the loci was lost, or each paralog acquired different roles for example in mitosis or meiosis, or both genes fulfill partially redundant functions, which is the case for **BOI1** and **BOI2** (Dahmann and Futcher, 1995; Dietrich et al., 2004; Kellis et al., 2004). The genes share no synteny, as opposed to other genome regions such as those containing for example **VTH1** and **VTH2**, for which the orientation of neighboring genes located up-stream and down-stream is conserved (see the Saccharomyces Genome Database (SGD) at www.yeastgenome.org and the Saccharomyces Genomics Viewer (SGV) at www.germonline.org; Lardenois et al., 2010)). The intergenic region between **BOI1** and the upstream ORF **YBL086C** is relatively large (1274 bp in S288C), while **BOI2**'s upstream region comprises 580 bp and appears to be mostly covered by the mRNA encoded by **SPR6** (see Fig. S1). The distinct pattern of synteny is not relevant for Boi1/Boi2 protein function during mitosis, since they are regulated at the post-translational level, and no role has been found in meiosis (Bender et al., 1996; Matsui et al., 1996; Norden et al., 2006). However, our results mark out **BOI1** as a useful model locus to study the regulation and, ultimately,
the possible function of 5′-extended isoforms in the control of protein translation during growth and development.

How is Boi1 downregulated at the post-transcriptional level during meiosis and spore formation?

Based on a recent ribosome profiling study and our Western blot data in wild-type cells and a *ume6* mutant strain, we speculate that *BOI1* may at least in part be negatively controlled at the level of translation when cells induce the developmentally regulated isoform harbouring an upstream open reading frame (uORF; Brar *et al.*, 2012). Such uORFs are present in UTRs throughout the yeast genome (Zhang and Dietrich, 2005). For the well-studied *GCN4* locus, it was shown that uORFs act as competitive inhibitors preventing the translation of the down-stream ORF via a mechanism called reinitiation (Hinnebusch, 2005; Gunisova and Valasek, 2014). Such a mode of regulation is consistent with the presence of uORFs in the extended *BOI1* 5′-UTR, and our finding that *ume6* cells grown in YPD, which de-repress the meiotic isoform, contain very little Boi1.

We noticed that the protein migrates slower in the absence of Ume6 than in wild-type cells. This might be a consequence of protein modification rendering it unstable or somehow unable to fold correctly, which would make it a target for the unfolded protein response pathway (Schröder *et al.*, 2000). An alternative – or rather additional – explanation to isoform-mediated translational control is that Boi1 becomes unstable in cells growing in the presence of acetate and gets rapidly degraded by a target-specific protease in cells as they initiate meiosis; this would also explain why the cells do not appear to completely switch from the short to the long isoform during meiosis. Such a two-step mechanism was found to downregulate Ume6 levels during respiration and early/middle meiosis (Mallory *et al.*, 2007; Law *et al.*, 2014). Interestingly, while Ume6 in *S. cerevisiae* appears to be regulated via protein stability during growth and development (Mallory *et al.*, 2007; 2012; Law *et al.*, 2014), the orthologous protein in the human pathogen *C. albicans* is negatively controlled via its 5′-UTR when cells switch to filamentous growth (Chiders *et al.*, 2014).

**A complex mechanism involving the conserved HDAC complex Rpd3/Sin3/Ume6 regulates Boi1 during growth and development**

A large-scale in vivo binding assay revealed that Ume6 binds to the *BOI1* upstream region but this interaction did not have the same effect as in the cases of early meiotic genes that are, contrary to *BOI1*, repressed during mitotic growth (Harbison *et al.*, 2004). An obvious explanation for this result – given that we identified a URS1 motif bound by Ume6 in vivo at the 5′-end of the isoform, and that growing *rdp3* and *ume6* mutant cells de-repress the isoform – is that the Rpd3/Sin3/Ume6 HDAC complex shuts down the transcription of *BOI1*’s meiotic isoform in mitosis. In the presence of acetate, cells partially degrade Ume6, which enables the long isoform to accumulate. Once cells have initiated meiosis, Ume6 is destroyed and the extended isoform is fully induced during early and middle meiosis, while the mitotic isoform continues to be detectable. A similar regulatory mechanism controls meiotic isoforms encoded by *CFT2* and *RTT10*; however, in these cases proteins levels do not decline, but rather increase when cells progress from respiration to sporulation (Fig. 5); (Lardenois *et al.*, 2014). This underlines how important it is to experimentally validate predicted 5′-UTR functions at the molecular level in follow-up studies. As
far as BOI1 is concerned, it is unclear what happens during late stages of sporulation formation but from tile array data, RNA-Seq, Northern blotting, and 5′-RACE assays we conclude that after exiting meiotic M-phase cells progressively switch back to expressing only the short mitotic isofrom. We speculate that the short isoform persists until late stages of sporulation because Boi1 – perhaps together with Boi2 – is important for the first round of mitosis immediately after germination (Joseph-Strauss et al., 2007; Geijer et al., 2012).

Our results provide initial insight into how BOI1 expression is altered by a conserved HDAC complex when cells respond to nutritional cues. This is also the first case of an early mitotic isoform that negatively correlates with protein levels during initial stages of meiosis (Lardenois et al., 2014). The HDAC Rpd3 is conserved during evolution, and a growing body of evidence shows that eukaryotic genes encode multiple isoforms (Nagalakshmi et al., 2008; Yang and Seto, 2008; Wara and Snyder, 2013; Andersson et al., 2014; Brown et al., 2014; Haberle et al., 2014). Therefore, our results likely have broad implications for flexible 5′-UTRs that influence protein levels during cell growth and cell differentiation in eukaryotes.

Experimental procedures

Yeast strains and media

The tiling array data were generated with sporulation competent SK1 MATα/α and sporulation deficient MATα/α strains. RT-PCR validation experiments were carried out in SK1 MATα/α and JHY222 MATα/α strains as previously reported (Lardenois et al., 2011). The induction of extended 5′-mUTR expression was analyzed in SK1 MATα/α ume6 and rpd3, and JHY222 MATα/α ume6 deletion strains (Table 1). Sporulation experiments were carried out using standard rich medium with glucose (YPD) or acetate (YPA) and sporulation medium (SPII).

Tiling array data

The molecular methods and bioinformatics approaches used for raw data processing and normalization and transcript identification were published in reference (Lardenois et al., 2011).

RT-PCR

RT-PCR oligonucleotide primers were designed with Primer3 (simgine.com/Primer3). To take strain-specific mutations into account, we downloaded the BOI1 open reading frame’s DNA sequence from the Saccharomyces Genome Database (SGD; yeastgenome.org), and aligned it with the SK1 genome using the Saccharomyces Genome Resequencing Project (SGRP) browser (sanger.ac.uk/research/projects/sgrodgrp). RT-PCR reactions were carried out using 2 μg of RNA reverse transcribed with Reverse Transcriptase (High Capacity cDNA Reverse Transcription kit; Life Technologies, USA) and amplified using Taq Polymerase (Qiagen, France) at 60°C for 26 cycles. DNA samples were separated on 2% agarose gels and photographed using an ImageQuant 350 digital Imaging System at the default settings (General Electric, USA). Primer sequences are given in Table 2.

Table 1. Yeast strains.

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<th>Genotype</th>
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<td>(Lardenois et al., 2011)</td>
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<td>(Lardenois et al., 2011)</td>
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</table>
Table 2. RT-PCR primers.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOI1</td>
<td>5'-CAAGGGGCCAAATCTTTTC-3'</td>
<td>5'-AAATTGCGCCATAATACCA-3'</td>
<td>150</td>
</tr>
<tr>
<td>mBOI1</td>
<td>5'-AGCCGCATGAAGATGAAAGT-3'</td>
<td>5'-CCGGAGAACACTCAATTCC-3'</td>
<td>236 (SK1 237)</td>
</tr>
<tr>
<td>lmBOI1</td>
<td>5'-AGCCGCATGAAGATGAAAGT-3'</td>
<td>5'-AAATTGCGCCATAATACCA-3'</td>
<td>1042 (SK1 1048)</td>
</tr>
</tbody>
</table>

Table 3. 5'-RACE primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOI1 GSP1</td>
<td>5'-TGGTCTTTGCAATTCTGTGG-3'</td>
</tr>
<tr>
<td>BOI1 GSP2</td>
<td>5'-TCGTGTTTTCCTCATTTCTGG-3'</td>
</tr>
<tr>
<td>BOI1 sequencing</td>
<td>5'-AAATTGCGCCATAATACCA-3'</td>
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Table 4. Northern blot primers.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOI1</td>
<td>5'-CGCATCAACAGGAGAACAGA-3'</td>
<td>5'-TCCGGAGACTTGATGCTCTT-3'</td>
<td>434</td>
</tr>
<tr>
<td>ACT1</td>
<td>5'-CTCGTGCTGTCTTCCCATCT-3'</td>
<td>5'-AGATGGACCACTTTCTGCGT-3'</td>
<td>1025</td>
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</table>

Table 5. ChIP primers.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOI1</td>
<td>5'-GACCTCTGAATGGTGCTAATTAAG-3'</td>
<td>5'-TTGCGAACATGATGACAGTTA-3'</td>
<td>434</td>
</tr>
</tbody>
</table>

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(Thermo Scientific). First strand cDNA was synthesized using a gene-specific primer (GSP1) and SuperScript II reverse transcriptase, and the mRNA template was removed by treatment with RNase H and RNase T1. Unincorporated dNTPs, GSP1, and proteins were separated from cDNA using a S.N.A.P. Column. A homopolymeric tail was added to the 3'-end of the cDNA using Terminal deoxynucleotidyl Transferase and dCTP. DNA amplification was carried out with GSP2 and UAP (Universal Amplification Primer). The PCR products of the expected size were purified from the agarose gel and sequenced. The primer sequences are shown in Table 3.

Northern blotting

Total RNA samples were prepared from fermenting (YPD), respiring (YPA) and sporulating (SPII) diploid cells and further processed as published (Lardenois et al., 2014). Oligonucleotide sequences are shown in Table 4.

URS1 motif prediction

We searched for predicted URS1 sites in a 2 kb region ranging from 61876 to 63876 immediately upstream of BOI1. We used several PWMs (M01503, M01898 and M02531) provided by the TRANSFAC Professional database (Matys et al., 2006) and Jaspar (Mathelier et al., 2014). The Match tool was employed with minFP cut-offs scores. Among 10 sites predicted, we selected the one that was detected with the high quality matrix M02531. The motif logo was generated with R seqLogo package (Bembom).

Chromatin immunoprecipitation (ChIP) assay

In vivo Ume6 binding to the URS1 motif present in the BOI1 up-stream region was assayed as published (Lardenois et al., 2014). Oligonucleotides used are shown in Table 5. Oligonucleotides used for the NUP85 control are published in (Lardenois et al., 2014).

Analysis of the BOI1 5'-UTR

We extracted the DNA sequence corresponding to the 5'-UTR according to the boundaries of the segmentation algorithm in S288C on chromosome2 (chr02 + : 62921–63876). The Expasy tools (Artimo et al., 2012) and ORF Finder (Rombel et al., 2002) were used to identify ORFs with the classical genetic code, start and stop codons being required and the DNA length set at > 100 bp. An ORF at 62926–63063 was identified corresponding to a 45 amino acid peptide in frame with the annotated BOI1 ORF.

Protein tagging

A one-step tagging method based on PCR was employed to construct a strain expressing Boi1 with a C-terminal myc tag using cassette plasmids and oligonucleotides as published (Wach et al., 1994; Janke et al., 2004). Colonies were first examined by diagnostic PCR for correct integration and then validated by Western blotting. Oligonucleotides used are shown in Table 6.
Western blotting

Samples were prepared from fermenting (YPD), respiring (YPA) and sporulating (SPII) cells as previously described. 25 μg of total protein extract was run on a 4–20% gradient gel (BioRad, USA) for one hour. Proteins were transferred onto ImmobilonPSQ membranes (Millipore, France) using an electro-blotter (TE77X; Hoefer, USA) in modified Towbin buffer (48 mM Tris base, 40 mM glycine and 0.1% SDS) and methanol (20% vol/vol anode; 5% vol/vol cathode) for two hours. Tagged Boi1 was detected with a monoclonal anti-myc-HRP antibody (Life Technologies, USA) at 1:1000. The antibody was incubated in hybridization buffer overnight at 4°C. The signals were revealed using the ECL-Plus Chemiluminescence kit (GE Healthcare, USA) and the ImageQuant 350 system (GE Healthcare, USA). Band intensities determined using the ImageQuant TL 7.0 software set at default parameters. A rabbit polyclonal anti-Pgk1 antibody (Invitrogen, USA) was used as a loading control.

Acknowledgements

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References


Table 6. Primers for C-terminal myc tagging.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmid</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BOI1</em></td>
<td>pYM18</td>
<td>5′-GATACCTGGAAGTTCGACAT</td>
<td>5′-AGGTGTTAAGTGTGTCAGAAG</td>
<td>(Wach et al., 1994; Janke et al., 2004)</td>
</tr>
</tbody>
</table>

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.