

Repression of *ADH1* and *ADH3* during zinc deficiency by Zap1-induced intergenic RNA transcripts

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The transcriptional activator Zap1 induces target gene expression in response to zinc deficiency. We demonstrate that during zinc starvation, Zap1 is required for the repression of *ADH1* expression. *ADH1* encodes the major zinc-dependent alcohol dehydrogenase that is utilized during fermentation. During zinc starvation, Zap1 binds upstream of the activator Rap1 and induces an intergenic RNA transcript, *ZRR1*. *ZRR1* expression leads to the transient displacement of Rap1 from the *ADH1* promoter resulting in *ADH1* repression. Using a microarray-based approach, we screened for additional genes repressed by Zap1 intergenic transcripts. We found that *ADH3*, the major mitochondrial alcohol dehydrogenase, is regulated in a manner similar to *ADH1*. Thus, during zinc deficiency, Zap1 mediates the repression of two of the most abundant zinc-requiring enzymes.

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Introduction

Metals such as zinc, copper and iron are essential for cell growth and yet are toxic when found in excess. Consequently, all cells employ a variety of mechanisms to survive periods of metal deficiency and sufficiency. In *Saccharomyces cerevisiae*, the transcriptional activator, Zap1, is essential for growth during periods of zinc starvation (Zhao and Eide, 1997). Zap1 protects cells from zinc limitation by inducing the expression of genes that encode proteins involved in zinc uptake (*ZRT1* and *FET4*) (Zhao and Eide, 1996a; Waters and Eide, 2002) and the compartmentalization and release of zinc from the vacuolar stores (*ZRC1* and *ZRT3*) (MacDiarmid *et al.*, 2000, 2003). Zap1 mediates this response by binding in a site-specific manner to zinc-responsive elements (ZRE) located

in the promoter regions of Zap1 target genes (Zhao *et al.*, 1998). As zinc levels increase, Zap1 is inactivated by zinc post-translationally (Bird *et al.*, 2000, 2003; Herbig *et al.*, 2005).

A second mechanism of cell survival during metal ion starvation is the downregulation of the expression of proteins that utilize the required metal cofactor and upregulation of the expression of an alternative enzyme or metabolic pathway. For example, under iron starvation conditions in *S. cerevisiae*, three genes involved in biotin synthesis (*BIO3*, *BIO4* and *BIO2*) are transcriptionally repressed (Shakoury-Elizeh *et al.*, 2004). *BIO2* encodes the iron-sulfur-containing enzyme biotin synthetase, which catalyses the rate-limiting step of biotin synthesis. Iron deficiency simultaneously leads to the upregulation of the biotin uptake system (Shakoury-Elizeh *et al.*, 2004). Thus, cells utilize iron-independent pathways (biotin uptake) during iron starvation and iron-dependent pathways (biotin synthesis) when iron is plentiful.

Microarray analysis suggests that a number of genes are repressed during zinc deficiency in a Zap1-dependent manner (Lyons *et al.*, 2000). One of the most highly repressed genes in response to zinc limitation is *ADH1*. *ADH1* encodes the major zinc-dependent alcohol dehydrogenase utilized by yeast during fermentation. Proteomic studies have estimated that the abundance of Adh1 protein in fermenting yeast cells is anywhere between 2 and 7.5×10^5 molecules per cell (Norbeck and Blomberg, 1997; Gygi *et al.*, 1999). Given that yeast cells contain 1.5×10^7 atoms of zinc under these growth conditions (MacDiarmid *et al.*, 2000) and each monomer of Adh1 binds two zinc ions, as much as 5% of the cellular zinc quota is potentially bound to Adh1. One of the major target genes of Zap1 is *Adh4*, an alcohol dehydrogenase that resembles the iron-dependent ADHII of *Zymomonas mobilis* (Drewke and Ciriacy, 1988). Thus, transcriptional remodeling of *ADH1* and *ADH4* expression could be an important homeostatic mechanism used to conserve zinc during zinc deficiency.

Although Zap1 is primarily involved in gene activation, during severe zinc deficiency it represses the expression of *ZRT2*. *ZRT2* encodes a low-affinity zinc uptake system. Zap1 mediates repression by binding to a low-affinity ZRE that overlaps with the *ZRT2* transcriptional start site (Bird *et al.*, 2004). Binding at this site inhibits transcription. This regulatory mechanism limits the production of Zrt2 under conditions where it is unable to transport zinc. It is likely that the mechanism of *ADH1* repression differs from that of *ZRT2*, as there is no ZRE located between the *ADH1* TATA element and translational start site. In this study, we have characterized the mechanism by which Zap1 represses *ADH1* expression and have investigated whether this mechanism is used at other promoters to limit gene expression during zinc deficiency.

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Results

ADH1 is repressed in zinc-limited cells in a Zap1-dependent manner

Microarray studies revealed that during zinc deficiency, *ADH1* gene expression is repressed in a Zap1-dependent manner (Lyons *et al*, 2000). Zap1-mediated repression of *ADH1* was confirmed by S1 nuclease analysis. Zap1 target genes, such as *ADH4*, are induced in zinc-deficient cells (Figure 1A), whereas *ADH1* expression is repressed. *CMD1* (calmodulin), a gene whose expression is not regulated by zinc status, was used as a loading control. As a *zap1Δ* mutant is unable to grow in the absence of zinc, we examined *ADH1* expression in the *zap1Δ* mutant strain containing a plasmid expressing the *ZRT1* high-affinity uptake permease from the constitutive *PGK1* promoter (pZRT1). The increase in cellular zinc from Zrt1 overproduction partially alleviates the growth

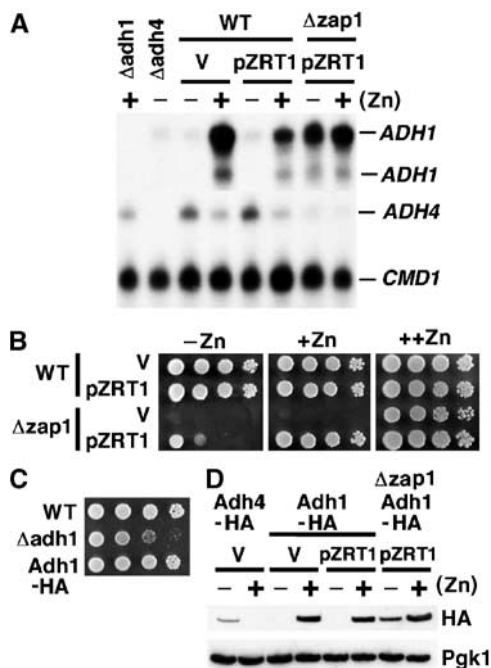


Figure 1 Differential regulation of *ADH1* and *ADH4* expression in response to zinc. The wild-type strain BY4741 and *zap1Δ* mutant strain were transformed with pZRT1 or the empty vector (V). Total RNA was extracted following growth to exponential phase in LZM media supplemented with either 5 μM zinc [−Zn] or 3000 μM zinc [+Zn] and the levels of *ADH4* and *ADH1* analyzed using S1 nuclease analysis (A). RNA from *adh4Δ* and *adh1Δ* mutant strains was used to confirm the specificity of the probes, whereas calmodulin (*CMD1*) was used as a loading control. The strains BY4741 and *zap1Δ* containing the indicated plasmids were grown overnight in synthetic deplete medium lacking uracil (SD −URA). Five microliters of a cell suspension (OD₆₀₀ 1.0) and three 10-fold serial dilutions were plated onto SD (−URA) medium [+ +Zn] or SD medium (−uracil) containing 1 mM EDTA supplemented with 50 μM zinc [+Zn] or 3 μM zinc [−Zn]. Plates were grown for 3 days at 30°C (B). The strains BY4741 (WT), *adh1Δ* and ABY69 (Adh1-HA) were grown in synthetic complete (SC) medium overnight before serial dilutions were made onto SC medium, as described in panel B (C). The strains BY4741 (WT), ABY64 (Adh4-HA), ABY69 (Adh1-HA) and ABY77 (*Δzap1*, Adh1-HA) containing the indicated plasmids were grown as described in panel A. Crude protein extracts were made and analyzed by immunoblotting. Phosphoglycerate kinase 1 (Pgk1) was used as a loading control (D).

defect of a *zap1Δ* mutant (Figure 1B). Under these conditions, *ADH1* expression is constitutively derepressed (Figure 1A).

To examine the effects of zinc on Adh1 protein level, Adh1 was genomically tagged at the C-terminus with three HA epitope tags (Adh1-HA). The Adh1-HA strain grew at a rate similar to the wild-type strain and did not show the slow growth phenotype of an *adh1Δ* mutant (Figure 1C). Immunoblotting revealed that Adh1 accumulates under zinc-replete conditions, a profile opposite to Adh4, and that the absence of Adh1 in low zinc is dependent on Zap1 (Figure 1D). Thus, the Zap1 transcriptional activator is required for the repression of *ADH1* expression in zinc-deficient cells.

Zap1 induces an RNA transcript 5' of *ADH1* that is required for repression

Rap1 is the major activator of *ADH1* expression in glucose-grown cells (Lieb *et al*, 2001). The Rap1 UAS (AAACCCATACATC) is located 664 bp upstream of the *ADH1* ATG translational start codon. A consensus Zap1-binding site (ACCTTGAAGGT) was identified upstream of the Rap1 UAS at position −1006 bp relative to the *ADH1* translational start site. The role of the ZRE in repression was investigated using nested *ADH1* promoter fusions to the integrated *lacZ* reporter gene (Figure 2A). Introduction of the promoter region containing the ZRE resulted in attenuated *lacZ* expression in zinc-deficient cells (compare constructs 3 and 4). This low zinc attenuation was abrogated when the ZRE was mutated (construct 5) or in *zap1Δ* cells (data not shown). Mutation of a putative TATA box sequence, located downstream of the *ADH1* ZRE at position −936 bp, also resulted in impaired zinc-responsive regulation (construct 6).

As Zap1 is a transcriptional activator and an upstream TATA element is essential for *ADH1* repression, Northern analysis was used to investigate whether Zap1 induced a transcript across the *ADH1* promoter. A diffuse small RNA transcript (≤0.5 kb), designated *ZRR1* (for Zinc Regulated RNA 1), was identified in zinc-deficient cells (Figure 2B). A longer ~2.2-kb transcript was also observed, which most likely runs through the entire *ADH1* ORF. Both transcripts were lost when the *ADH1* ZRE was deleted from the chromosome, leading to constitutive *ADH1* expression (Figure 2B, ABY79). The constitutive expression of *ADH1* in low-zinc cells with the mutated ZRE was not reversed by the presence of the fully regulated *ADH1-lacZ* reporter fusion that yields a functional *ZRR1* transcript (data not shown). Thus, *ZRR1* acts in *cis* and not in *trans*.

ZRR1 mediates repression of *ADH1* gene expression via transcriptional interference

Transcription of an intergenic mRNA transcript can inhibit the binding of transcriptional regulators to promoter regions. For example, during growth in rich medium, the promoter region of *SER3* involved in serine biosynthesis is highly transcribed, yielding an intergenic RNA (*SRG1*) that attenuates the binding of activators to the *SER3* promoter leading to *SER3* repression (Martens *et al*, 2004). If a similar transcriptional interference mechanism occurs within the *ADH1* promoter, repression should occur with an *ADH1* activator other than Rap1 and with a *ZRR1* activator other than Zap1. To determine whether Rap1 and Zap1 are essential for the

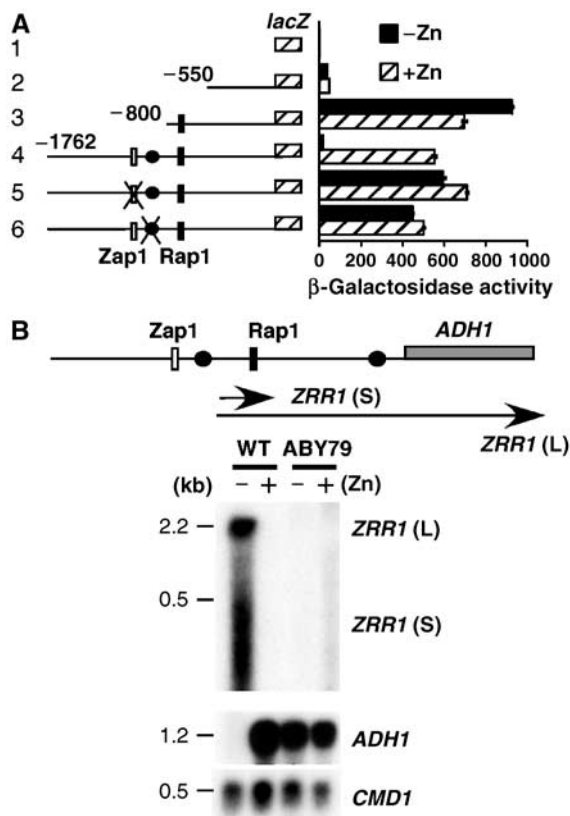


Figure 2 *ADH1* repression requires the Zap1 dependent RNA transcript *ZRR1*. The indicated reporter constructs were transformed into in the wild-type strain BY4741. Transformants were grown to exponential phase in LZM supplemented with either 5 μ M zinc [-Zn] or 3000 μ M zinc [+Zn] and β -galactosidase activity measured in triplicate by standard procedures (A). The ZRE (white rectangle), Rap1 UAS (black rectangle) and upstream TATA box (black oval) are shown. Mutation of the ZRE element (ACCTTGAAGGT to TGCTTGAAGGT) or TATA box sequence (TATAAA to CCTAGG) is indicated by a cross. All numbers are relative to the first base of the initiation codon of *lacZ*, which is designated as +1. Northern analysis of *ADH1*, *ZRR1* and loading control *CMD1*. The wild-type strain BY4741 and strain ABY79 in which the *ADH1*-ZRE (-1006 to -995 bp) is replaced by the *K. lactis URA3* ORF, were grown as described in panel A (B). The northern probe for *ADH1* was a 54 bp oligonucleotide that hybridized to -27 to +27 bp relative to the ATG translational codon.

regulation observed at the *ADH1* promoter, the Rap1 UAS and Zap1 ZRE were mutated to the binding site for the transcriptional activator Gal4 (Figure 3A). As carbon source can influence *ADH1* expression, each reporter construct was coexpressed with the pGEV transcriptional activator. pGEV is a hybrid transcription factor that utilizes the Gal4-DNA-binding domain, a β -estradiol-responsive element and the VP16 activation domain (Gao and Pinkham, 2000). pGEV can activate the expression of Gal4 target genes in glucose-grown cells in the presence of β -estradiol. As predicted, conversion of the Rap1 UAS to the Gal4 UAS (Figure 3A, construct 3) led to a marked reduction in the activation of *ADH1-lacZ* in the absence of β -estradiol. The addition of β -estradiol increased activity under zinc-replete but not zinc-limiting conditions. We predicted that the conversion of the Zap1 ZRE to a Gal4 UAS (construct 4) would lead to the constitutive production of *ZRR1* in the presence of β -estradiol and consequently diminished β -galactosidase activity. The observed greater

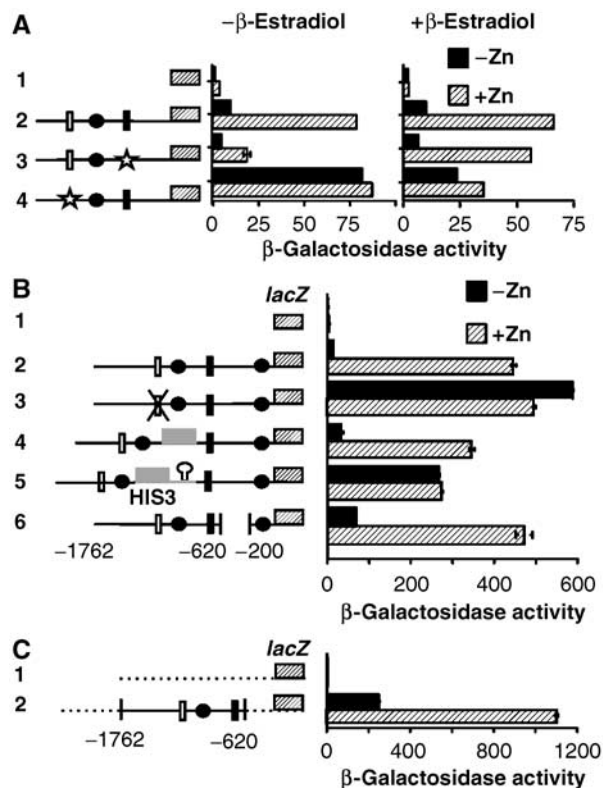


Figure 3 Characterization of the promoter elements required for *ADH1* repression. The strain YM4271 (*gal4* Δ) was transformed with the indicated constructs. Transformed cells were grown as described in Figure 2A in the presence or absence of 10⁻⁵M β -estradiol. β -Galactosidase activity measured in triplicate by standard procedures (A). The indicated reporter constructs were transformed into the wild-type strain BY4741 and cells grown as described in Figure 2A. β -Galactosidase activity measured in triplicate by standard procedures. The *HIS3* ORF (gray box), *HIS3* terminator (hairpin loop), and *CYC1* promoter region (dashed line) and Gal4 UAS (star) are shown. All other symbols are described in Figure 2A (B, C).

repression seen with the Zap1 ZRE relative to the Gal4 UAS may imply that full repression may require an additional factor recruited by Zap1. Alternatively, the weaker effect of Gal4 may arise from the inclusion of only a single Gal4 UAS. Highly expressed Gal4 target genes such as *GAL1* contain multiple Gal4-binding sites in their promoters.

To confirm that the intergenic transcript was interfering with *ADH1* expression, we replaced 150 bp of the *ZRR1* sequence (-700 to -850) with the *HIS3* ORF or the *HIS3* ORF inclusive of the *HIS3* transcriptional terminator (Figure 3B). Replacement of *ZRR1* with the *HIS3* ORF did not affect repression of *ADH1-lacZ* under zinc-limiting conditions (construct 4). Conversely, *ADH1-lacZ* expression was derepressed when the *HIS3* ORF and its terminator were introduced (construct 5). If repression is mediated through the transcriptional interference of Rap1 binding, then sequences downstream of the Rap1-binding site should not be necessary for repression. Deletion of the *ADH1* promoter region between -200 and -620 bp had little effect on repression (construct 6). Similarly, repression was observed in response to zinc limitation when the *ADH1* promoter region containing the Zap1 and Rap1 sites (-620 to -1762 bp) was introduced into a plasmid containing a UAS-less *CYC1* promoter (Figure 3C).

To test whether the repression was a direct result of impaired Rap1 binding to the *ADH1* promoter, chromatin immunoprecipitation (ChIP) analysis was performed using strains containing genomic TAP-tagged Zap1 and Rap1. The TAP-tagged Zap1 strain was fully functional, showing no growth defect in zinc-limiting media (data not shown). Zap1 accumulates to high levels in zinc-limited growth medium owing to autoregulation (Figure 4A). Under these growth conditions, TAP-Zap1 could be detected binding to the *ADH1* promoter (Figure 4B). If transcriptional interference of *ADH1* was similar to that of *SER3*, we predicted that Rap1 binding to the *ADH1* promoter would be attenuated in zinc-deficient cells. However, no zinc-dependent change in occupancy of Rap1 binding was observed (Figure 4B, primer pair 2). Rap1 is a well-characterized DNA-binding protein that has a variety of cellular roles, including chromatin opening and silencing at the telomeres. The ability of Rap1 to affect chromatin suggests that it might behave differently

to ordinary transcription factors during a collision with an elongating RNA polymerase. For example, a collision of RNA polymerase with Rap1 might transiently dislodge Rap1 from DNA, allowing its rapid re-association. Alternatively, transcription through the Rap1 site may alter the transcriptional activation function of Rap1 without altering its DNA-binding ability. To address the dynamic regulation of Rap1 binding, we examined Rap1 promoter occupancy by *in vivo* dimethyl sulfate (DMS) footprinting analysis. Surprisingly, the modification of guanine (G) residues by DMS was severely impaired in our low-zinc media in the BY4741 strain background (data not shown). We therefore generated a constitutive allele of Zap1, by fusing the Zap1 DNA-binding domain to the VP16 activation domain (Zap1^c), to examine the effects of *ZRR1* expression on Rap1 occupancy in zinc-replete media. Zap1^c was able to complement a *zap1Δ* strain but could not confer zinc-responsive regulation onto a minimal ZRE-*lacZ* reporter construct (Supplementary Figure 1). Expression of Zap1^c in a wild-type strain resulted in increased levels of the *ZRR1* transcript and decreased *ADH1* expression and protein under zinc-replete conditions (Figure 4C and D). In addition, the presence of Zap1^c resulted in protection of G residues at positions -1004 and -1005 within the *ADH1* ZRE (Figure 4E), but significantly exposed G residues at positions -652, -659 and -660 within the Rap1-binding site and at positions -680 and -681 within an upstream Gcr1-binding site to methylation. Thus, Zap1-induced *ZRR1* expression leads to the transient displacement of Rap1 and Gcr1 from the *ADH1* promoter.

***ZRR1* expression lowers but does not prevent *ADH1* transcriptional initiation**

Our data are consistent with a model where the displacement of Rap1 and Gcr1 from the *ADH1* promoter during zinc

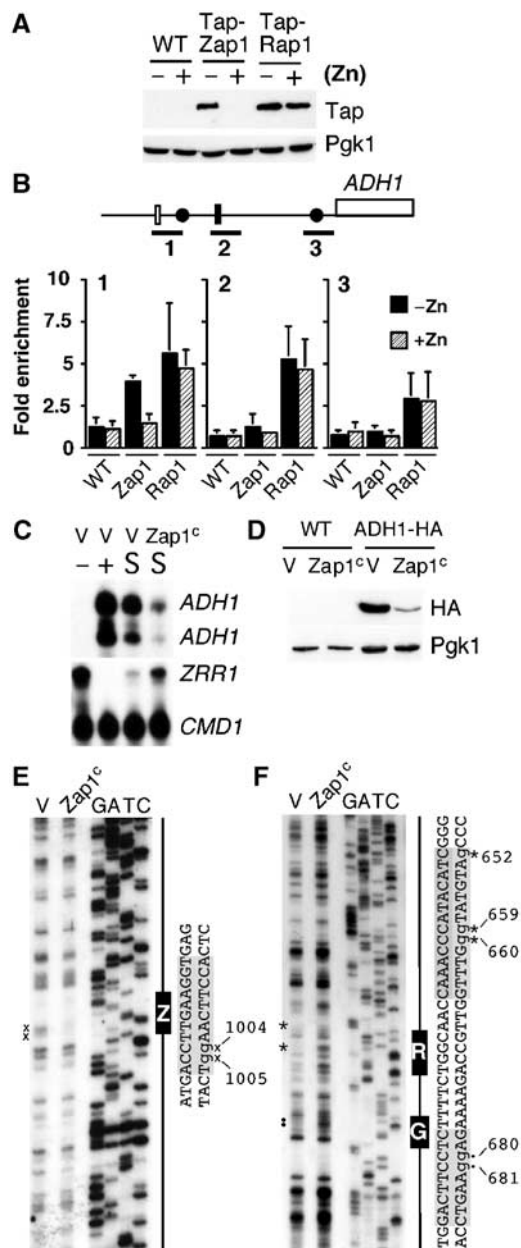


Figure 4 Expression of *ZRR1* leads to the transient dissociation of Rap1 from the *ADH1* promoter. The strains BY4741, ABY61 (TAP-Zap1) and TAP-Rap1 were grown to exponential phase in LZM supplemented with either 3 μ M zinc [-Zn] or 3000 μ M zinc [+Zn]. (A) Crude protein extracts were generated and analyzed by immunoblotting. Blots were incubated with peroxidase-anti peroxidase (for TAP) or Pgk1 for the loading control. (B) Cells grown under the conditions in panel A were crosslinked for ChIP analysis. PCR was performed with primer pairs that amplified the following sequences relative to the first base of the initiation codon of *ADH1*, which is designated as +1 (bar 1 -1163 to -778 bp, bar 2 -837 to -486 bp and bar 3 -290 to +58 bp). Amplified sequences were normalized against input DNA and binding at the *HIS3* ORF (negative control). Data represents an average of three independent experiments. The extension of Rap1 binding in bar 1 and bar 3 sequences likely arises from incomplete sonication. (C) The strain BY4741 containing the empty vector pRS-VP16 (V) or VP16-ZAP1 (Zap1^c) was grown to exponential phase in LZM supplemented with 5 μ M zinc (-), 3000 μ M zinc (+) or in Zn-replete SD (-URA) (S). Total RNA was extracted and the levels of *ADH1*, *ZRR1* and *CMD1* mRNAs were analyzed by S1 nuclease analysis. (D) The strains BY4741 and ABY69 (ADH1-HA) containing pRS-VP16 (V) or Zap1^c were grown in SD (-URA). Crude protein extracts were made and analyzed by immunoblotting. (E, F) Exponential cultures of BY4741 containing the above plasmids were grown to exponential phase in SD (-URA) media and analyzed by *in vivo* DMS footprinting. The positions of the ZRE (Z), Rap1 (R) and Gcr1 (G) binding sites have been indicated schematically and by gray shading in the DNA sequence. Protected G residues have been indicated by \times (Zap1), * (Rap1), \bullet (Gcr1) and by lowercase letters in the DNA sequence. Data is a representative of 3 independent experiments.

deficiency prevents transcriptional initiation of *ADH1* mRNAs. To test this model, we measured the extent of histone H3K4 trimethylation (H3K4me3) across the *ADH1* promoter using ChIP analysis (Figure 5A). Histone H3K4me3 generally occurs at the beginning of actively transcribed genes (Pokholok et al, 2005). We were therefore surprised by an increase in H3K4me3 during zinc deficiency over the region of the *ADH1* promoter amplified with the primer pair A3 (and not A2). More notable was H3K4me3 at the *ADH1* transcriptional start site under both zinc-limiting and zinc-replete conditions (primer pair A4). Although no *ADH1* transcripts were detected during zinc limitation by Northern analysis, we re-investigated the abundance of *ADH1* and *ADH1-lacZ* mRNAs using S1 nuclease analysis. To differentiate between the long *ZRR1* intergenic transcripts and the *ADH1* or *lacZ* transcripts, S1 nuclease primers were designed to overlap with the two major *ADH1* transcriptional initiation start sites (Figure 5B). The two sites correspond to -27 and -37 bp upstream of the ATG codon. Accordingly, the long *ZRR1-lacZ* and *ZRR1-ADH1* transcripts will be digested to final lengths of 65 and 64 bp, whereas *lacZ* and *ADH1* transcripts that initiate at the *ADH1* transcriptional start

sites will be digested to a final length of 40 or 50 and 39 or 49 bp, respectively. To examine the effects of *ZRR1* on *lacZ* expression, cells were grown under zinc-limiting conditions before exposure to zinc. In zinc-starved cells, two transcript sizes were detected the long *ZRR1-lacZ* transcript that accumulated to high levels (Figure 5C, 0 min *lacZ*⁶⁵) and a *lacZ* transcript that initiated from the -37 transcriptional initiation start site (Figure 5C, 0 min *lacZ*⁵⁰). No *lacZ* transcript initiating from the -27 transcriptional initiation start site (*lacZ*⁴⁰) was observed. It is unlikely the *lacZ*⁵⁰ transcript arises from an overlap with the low-abundance *ZRR1-ADH1* transcript (that would give an S1 nuclease product of 55 bp), as the *lacZ*⁵⁰ mRNA was present in low-zinc conditions in a strain that lacked the chromosomal *ADH1* ZRE (Figure 5E, 0 min *lacZ*⁵⁰). Both the long *ZRR1-lacZ* and the full-length *lacZ* transcript were also observed by Northern analysis during zinc-limiting conditions (data not shown). The addition of zinc resulted in the rapid loss of the *lacZ*⁵⁰ transcript (Figure 5C, 3 min), the slower loss of the *ZRR1-lacZ* mRNA and then the re-appearance of *lacZ* transcripts that initiated from both the -37 and -27 transcriptional initiation start sites (Figure 5C, 90 min *lacZ*⁵⁰ and *lacZ*⁴⁰). Importantly, the

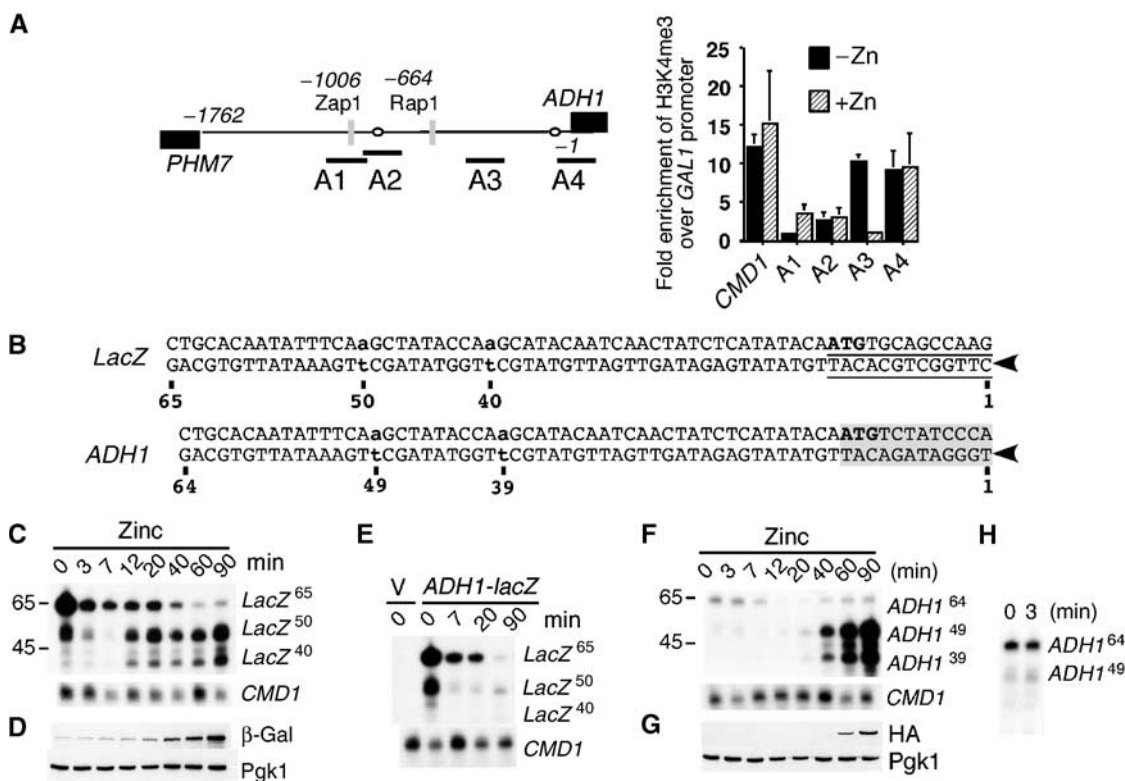


Figure 5 Effects of *ZRR1* on *ADH1* transcriptional initiation. The wild-type strain BY4741 was grown under conditions described in Figure 4A. ChIP was performed with anti-histone H3K4me3 antibodies and real time quantitative PCR performed with PCR primers that amplified the following sequences relative to the *ADH1* ATG codon; A1 -1136 to -949 bp, A2 -974 to -820 bp, A3 -479 to -345 bp, A4 -94 to +84 bp. The *CMD1* control primers amplified sequences -227 to +61 bp relative to the *CMD1* ATG codon. Values are an average of three quantifications (A). DNA sequence of the S1 oligonucleotides used in panels C-F. Arrowheads indicate the 5' ends of the oligonucleotides. Shown are the complementary DNA strands (the positions of the previously mapped -27 and -37 *ADH1* transcriptional start site (bold lowercase letters) (Bennetzen and Hall, 1982), the translational ATG (bold uppercase letters), the *ADH1* ORF (gray shading), the TATA boxes (open ovals) and *lacZ* ORF (underlined sequence). Numbers indicate the base-pair position in the oligonucleotide (B). Strain BY4741 containing the integrated *ADH1-lacZ* fusion (Figure 2A construct 4) was grown to exponential phase in LZM media supplemented with 5 μ M Zn²⁺. Cells were harvested, washed 3 times in PBS and then resuspended in SD (-URA) supplemented with 100 μ M Zn²⁺. Aliquots were removed at the indicated time points for S1 nuclease analysis (C) or immunoblot analysis (D). The strain ABY79 (Δ *ADH1*ZRE) containing pYep353 or pYep*ADH1* (multi-copy version of Figure 2 construct 4) was grown as described in panel C. Aliquots were removed at the indicated times for S1 nuclease analysis (E). Strain ABY69 (*ADH1*-HA) was grown as described in panel C. Cells were removed for S1 nuclease analysis (F) or immunoblot analysis (G). A longer exposure of lanes 1 and 2 of panel F was performed (H).

'wild-type' *lacZ* transcript accumulated to similar levels in both zinc-limited and zinc-replete cells (Figure 5C, compare 0 and 90 min *lacZ*⁵⁰), but was translated into protein only under zinc-replete conditions (Figure 5D, β -Gal). Analysis of *ADH1* transcripts under the same conditions revealed similar trends, although the long *ZRR1-ADH1* transcript was less stable than its *ZRR1-lacZ* counterpart (Figure 5F). An *ADH1* transcript that was initiated from the -37 transcriptional start site was detected during zinc limitation (*ADH1*⁴⁹). However, this transcript was very unstable and could only be detected on long exposures (Figure 5H).

***ADH3* gene expression is repressed during zinc deficiency**

To investigate whether Zap1 mediated repression of other genes via transcriptional interference, we used a microarray-based approach with Agilent intragenic and intergenic oligonucleotide arrays. These arrays contain oligonucleotide probes that are spaced ~ 266 bp apart, allowing changes in the expression of both ORF and intergenic regions to be examined. We screened for genes exhibiting a Zap1-induced intergenic transcript in zinc-limited cells and repression of the downstream ORF.

The LZM medium used to make yeast cells zinc deficient contains two metal chelators, EDTA and sodium citrate. As a consequence, our medium is slightly limited for other metal ions, such as iron. We therefore performed our microarrays under zinc-replete conditions using the constitutive Zap1^c. Microarray analysis of a *zap1 Δ* strain containing Zap1^c versus the empty vector revealed that the major Zap1 target genes were induced (Supplementary Figure 2). Figure 6A shows the expression profile around the *ZRT1* locus of chromosome VII. For Zap1 targets (such as *ZRT1*, *ADH4* and *VEL1*), we found higher levels of expression of probes that fell within their open reading frames (ORF) and low expression in their promoter regions (arrows indicate the positions of ZRE motifs). This expression profile was reversed at the *ADH1* locus where we observed high expression in the 5' promoter region, whereas the ORF was repressed (Figure 6B).

Microarray analysis revealed that another highly induced intergenic region was the *ADH3* promoter (Figure 7A). *ADH3* encodes the major zinc-dependent mitochondrial alcohol dehydrogenase (Pilgrim and Young, 1987). An *ADH3-lacZ* reporter fusion was used to confirm that *ADH3* expression was repressed under zinc-limiting conditions (Figure 7B, construct 2). Mutations that disrupted the ZRE led to marked derepression under zinc-limiting conditions and a slight loss of activity under zinc-replete conditions (Figure 7B, construct 3). To further investigate whether Zap1 was involved in the repression of *ADH3* expression during zinc limitation, we analyzed *ADH3* transcript levels by Northern analysis (Figure 7C-E). The major 1.2-kb *ADH3* transcript seen in zinc-replete cells was attenuated in zinc-deficient cells and two additional transcripts were seen. The production of both of these transcripts was dependent upon the presence of the *ADH3* ZRE and Zap1 (Figure 7D and E). To determine the effect of the ZRE-mediated transcripts on Adh3 protein levels, a strain containing Adh3 tagged at the C-terminus with three HA epitope tags was generated (Figure 7F). Little Adh3 protein accumulated in zinc-limited cells. Adh3 protein accumulated to some extent in *zap1 Δ* cells, but not to the level seen in zinc-replete cells. As the *ADH3* transcript size is normal in

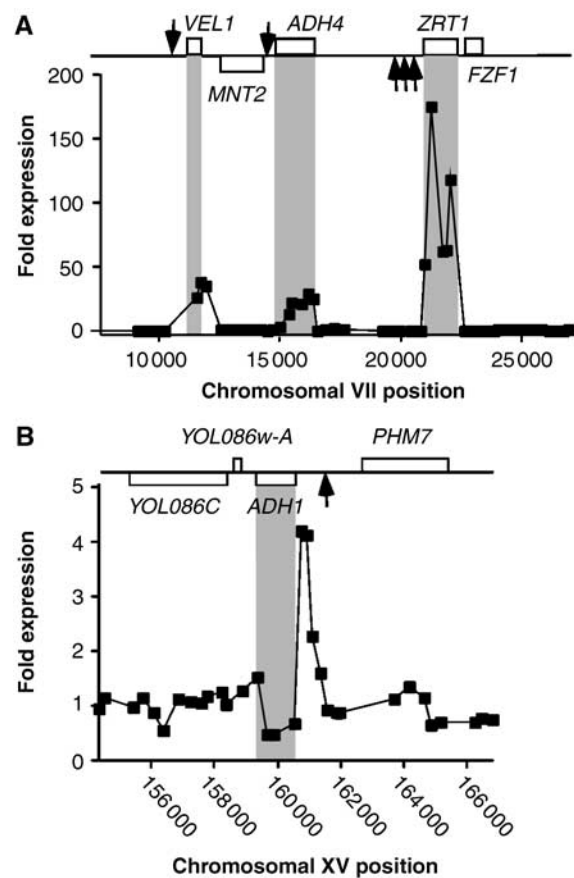


Figure 6 Identification of additional Zap1-dependent intergenic RNA transcripts. Shown are data for the *ZRT1* (A) and *ADH1* (B) chromosomal regions. The strain *zap1 Δ* containing either pRS-VP16 (vector) or pZap1^c was grown to exponential phase in SD ($-$ uracil) media before cells were harvested. RNA was extracted and hybridized to yeast intergenic microarrays as described in Supplementary Figure 2. The differential expression between the vector and pZap1^c labeled samples was plotted against chromosomal map position. The positions of ORFs have been indicated where upper boxes represent the Watson strand ORFs and lower boxes represent the Crick strand. Arrows indicate the positions of ZREs. Gray panels highlight the positions of Zap1 regulated ORFs.

zap1 Δ cells, the reduction in the protein level in zinc-deficient cells is likely due to Adh3 protein instability.

A number of additional genes were reproducibly repressed in Zap1^c-containing cells and exhibited an apparent elevated transcript within the 5' promoter region. To test whether Zap1-dependent intergenic transcripts repressed these genes, 1100 bp of the *OM45*, *MTH1* or *HXT17* promoter regions was fused to the *lacZ* reporter gene. We found that none of these reporter constructs led to attenuated *lacZ* expression in zinc-limiting medium (data not shown). Furthermore, Northern analysis did not substantiate a change in the transcript size in zinc deficient cells (data not shown). Thus, zinc repression of gene expression by intergenic non-coding RNAs appears limited to *ADH1* and *ADH3*.

Discussion

Zap1 induces the expression of over 40 genes in zinc-deficient cells. We show here that Zap1 mediates the repression of the *ADH1* and *ADH3* genes through generation of Zap1-dependent, intergenic, non-coding transcripts. The Zap1-dependent

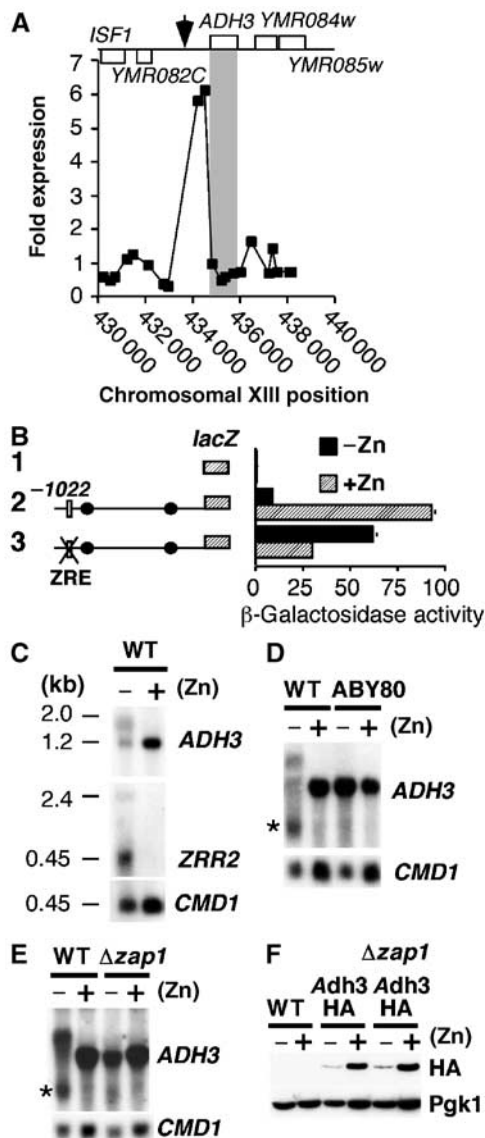


Figure 7 *ADH3* gene expression is regulated by the Zap1-dependent *ZRR2* transcript. (A) Expression differences in the strain *zap1Δ* containing either pRS-VP16 (vector) or pZap1^c. See legend of Figure 4C and D for details. (B) The indicated reporter constructs were transformed into the wild-type strain BY4741 and cells grown to exponential phase in LZM supplemented with either 3 μM zinc [-Zn] or 3000 μM zinc [+Zn] and β-galactosidase activity measured in triplicate by standard procedures. (C–E) Northern analysis of *ADH3*, *ZRR2* and loading control *CMD1*. Wild-type strain BY4741, strain ABY80 in which the *ADH3*-ZRE is replaced by the *K. lactis* *URA3* ORF (-755 to -745 bp relative to the ATG) or the *zap1Δ* mutant strain containing pZRT1 (*Δzap1*) was grown under the conditions described in panel B. The northern probe for *ADH3* was a 60 bp oligonucleotide that hybridized to +6 to +65 bp relative to the ATG translational codon. (F) The strains BY4741 (WT), ABY71 (Adh3-HA) and ABY74 (*Δzap1* Adh3-HA) were grown in LZM media supplemented with either 3 μM zinc [-Zn] or 3000 μM zinc [+Zn]. Crude protein extracts were made and analyzed by immunoblotting. Phosphoglycerate kinase 1 (Pgk1) was used as a loading control.

ZRR1 intergenic transcript mediates repression of *ADH1* through, in part, transcription interference. The *ZRR1* transcript results in a transient dislodging of Rap1 and Gcr1 from the *ADH1* promoter. Rap1 appears stably bound at the *ADH1* promoter by ChIP analysis in both zinc-limiting and zinc-

replete cells; however, *in vivo* footprinting analysis reveals a change in the occupancy of both Rap1 and Gcr1. Maximal activation of *ADH1* requires both Rap1 and Gcr1. *ZRR1* repression of *ADH1* requires the upstream ZRE and TATA elements and is abrogated by the inclusion of a transcriptional termination site 100 bp upstream of the candidate Gcr1 site. The repression of *ADH1* by *ZRR1* occurs only *in cis* and is effective with a heterologous downstream TATA element and ORF. Repression of *ADH3* through expression of an intergenic transcript will likely occur by a mechanism similar to that of *ADH1*.

Rap1 displacement by *ZRR1* was only observed by *in vivo* footprinting and not ChIP analysis. This difference may arise from the ability of the two different techniques to distinguish transient displacement. As Rap1 is known to induce a pronounced bend upon binding to DNA (Konig *et al.*, 1996), it is also possible that *ZRR1* alters the Rap1-DNA complex conformation such that it is more accessible to DMS methylation without perturbing crosslinking.

Transcriptional interference by regulated intergenic transcription was first demonstrated in *S. cerevisiae* at the *SER3* locus (Martens *et al.*, 2004). Transcription of the non-coding *SRG1* transcript attenuates expression of the downstream *SER3* gene. *SRG1* expression occurs in cells propagated in a growth medium containing high serine levels. The serine-responsive activator Cha4 mediates the induction of *SRG1* (Martens *et al.*, 2005). The Cha4-mediated repression of *SER3* requires two coactivators SAGA and Swi/Snf, which are chromatin-remodeling complexes. The Zap1 induction of *ZRR1* may also require additional components, as replacement of the Zap1 ZRE by a Gal4 UAS did not result in efficient Gal4-mediated repression of *lacZ*.

Although transcriptional interference is likely a dominant mechanism for the repression of *ADH1*, repression may have other features. Under conditions in which *ZRR1* is induced, transcriptional initiation at one of the two start sites in *ADH1* occurs. *ADH1* transcripts arising from initiation at the -37 start site are not translated into protein. Replacement of the *ADH1* ORF by *lacZ* results in the accumulation of a full-length mature transcript from the -37 start site, but this transcript also fails to be translated into protein. The observation of abundant transcripts from the -37 start site of the *ADH1-lacZ* construct but not the endogenous *ADH1* may arise from RNA instability of the *ADH1* transcript, or an effect of the *lacZ* fusion on chromatin structure. If the failure to see the substantial endogenous *ADH1* transcript initiating at -37 is only due to transcript instability, then Zap1 induction of *ZRR1* may alter the chromatin structure in such a way that only the -37 and not the -27 start site is used, and that the resulting transcript is incompetent for translation. If the observation of a significant *ADH1-lacZ* transcript starting from the -37 start is only an effect of *lacZ* on the chromatin structure, then the entire effect of *ZRR1* may relate to transcriptional interference. Studies are underway to discern these two possibilities.

Translationally defective transcripts are known. Defects in phosphorylation of the RNA polymerase C-terminal domain are known to affect mRNA processing (Muratani *et al.*, 2005). Ubiquitylation of Gal4 was shown to be important in the regulation of the quality of RNA transcripts. Cells lacking the Dsg1 ubiquitin ligase accumulate a Gal4 variant that induces transcription but yields transcripts that are incompletely processed and are nonfunctional. The effect is mediated

through changes in the phosphorylation state of the RNA polymerase C-terminal domain. Zap1 induction of *ZRR1* may likewise mediate an effect on RNA polymerase II that results in non-processed, non-functional RNAs from the *ADH1* locus. Future studies will address whether the transcripts are polyadenylated and associated with polysomes.

Zinc-dependent alcohol dehydrogenases are among the most abundant zinc metalloenzymes in yeast cells. The repression of Adh1 and Adh3 during zinc limitation may be a mechanism to conserve the zinc nutrient. This model might predict that Adh1 and Adh3 overproduction during zinc starvation would be detrimental to cell growth. However, we detected no growth defect in yeast strains that constitutively produced *ADH1* and *ADH3*. It is noteworthy that Adh3 protein fails to accumulate to high levels under zinc-limiting conditions in a *zap1Δ* strain. This result could explain why a strain constitutively producing Adh3 shows no zinc-dependent growth defect. Why can the Adh1 protein but not Adh3 accumulate under zinc-deficient conditions? Mechanisms that could explain this difference include that Adh3 stability may be regulated by zinc status. Alternatively, zinc in the mitochondria is less available to Adh3 during zinc deficiency leading to its turnover whereas more zinc is available to Adh1 in the cytoplasm. A third possibility is that protein turnover of unmetallated Adh3 is more efficient in the mitochondria compared to cytosolic apo-Adh1 turnover.

Zn-containing Adh1 and Adh3 are repressed in zinc-limited cells, whereas the iron-like alcohol dehydrogenase gene, *ADH4*, is maximally induced. Adh4 is the only alcohol dehydrogenase in yeast that resembles the iron-containing alcohol dehydrogenases, which are exemplified by ADHII from *Z. mobilis*. *Z. mobilis* ADHII forms a dimer that binds one atom of iron per subunit (Neale *et al*, 1986). Although the iron-binding ligands are conserved in Adh4, purification of Adh4 from *S. cerevisiae* revealed the presence of bound zinc and not iron. Furthermore, *in vitro* Adh4 reconstitution studies showed that only Zn(II) and not Fe(II) could activate the enzyme (Drewke and Ciriacy, 1988). If Adh4 binds zinc, why would this alcohol dehydrogenase be induced during zinc starvation? The simplest model is zinc conservation. In *S. cerevisiae*, Adh1 forms a tetrameric enzyme that binds two atoms of zinc per subunit. Adh4 is predicted to form a dimer with each subunit binding one zinc ion. Alternatively, Adh4 may be a more efficient alcohol dehydrogenase during zinc limitation. Kinetic analysis indicates that Adh4 closely resembles Adh1 in its affinities for ethanol, NADH and acetaldehyde (Drewke and Ciriacy, 1988). However, Adh4 has a higher affinity for NAD. This higher affinity for NAD, or some other property of Adh4, may be advantageous during zinc starvation. A final model is that Adh4 binds iron when zinc levels are limiting. Adh4 was purified as the zinc-containing enzyme from zinc-replete yeast cells, a condition where Adh4 is normally not synthesized. It is interesting to note that alcohol dehydrogenases that resemble Adh4 are found in many higher eukaryotes, including *Caenorhabditis elegans* and humans. It will be interesting to see if zinc reciprocally regulates the zinc-requiring and these alternative alcohol dehydrogenase isoforms in other organisms.

Zinc deficiency has broad effects on gene expression in yeast. In addition to the induction of many genes and the repression of *ADH1* and *ADH3*, multiple genes are reproducibly repressed in cells with a constitutively active Zap1. The

mechanism of repression of these genes is likely distinct from that of *ADH1* and *ADH3*, as no apparent intergenic transcripts are induced and they are not located adjacent to Zap1 target genes. Repression of these additional genes is also unlikely related to the Zap1-dependent repression of *ZRT2* through a proximal ZRE, as many of the other repressed genes lack a candidate ZRE in their proximal 5' sequences. Therefore, we have much more to learn about how Zap1 controls the expression of the many genes in the genome that respond to this factor. A full analysis of these other repressed genes will be presented in a subsequent report.

Materials and methods

Strains and growth conditions

Yeast strains used in this study are described in Supplementary Figure 3. Yeast cells were grown in synthetic defined (SD) medium supplemented with 2% glucose and the appropriate auxotrophic supplements. LZM was prepared as described previously (Zhao and Eide, 1996b). LZM contains the zinc chelator 1 mM EDTA.

Plasmid constructions

All clones were confirmed by sequencing. The *ADH1*-minimal *CYC1* promoter fusion (Figure 3A) was generated by PCR amplifying the indicated *ADH1* promoter region with primers that contained *XhoI*/*XbaI* sites and then subcloning the resulting PCR product into equivalent sites in the vector pNB404. All remaining *ADH1/ADH3 lacZ* fusions are integrated plasmids unless stated. These fusions were generated by PCR amplifying the indicated promoter fragment, inclusive of the translational start codon, and using homologous recombination to insert the PCR products into *BamHI/PstI*-digested pYep353 (Myers *et al*, 1986). Internal mutations and deletions were generated using two-step overlapping PCR. The *ADH1-lacZ* fusions containing the *HIS3* ORF were generated by initially replacing the *ADH1* promoter region between -700 and -850 bp with a *BamHI* site. The *HIS3* ORF (+1 to +663 with +1 at the *HIS3* ATG) or the *HIS3* ORF and terminator (+1 to +913) were subsequently introduced following digestion with *BamHI*, by homologous recombination (Kunes *et al*, 1987). Promoter inserts were subsequently released by a *BamHI/PstI* digest and subcloned into the corresponding sites in the vector Yp353 with the exceptions of promoter fusions containing the *HIS3* ORF that were subcloned into Yp353 using the *EcoRI/ClaI* restriction sites. The resulting plasmids were linearized with *NcoI* and the DNA was used to transform the indicated strains. The construct VP16-Zap1 has been reported previously (Bird *et al*, 2006).

DNA, RNA and protein analysis

RNA was extracted from cells grown to mid-log phase using the hot acidic phenol method. Intergenic microarrays were performed with Agilent yeast whole-genome oligonucleotide arrays as described in Supplementary data. For Northern analysis, 15 μg of yeast total RNA was separated on 2.2 M formaldehyde-agarose gel, before transfer to a nylon membrane at alkaline pH and Northern hybridization by standard conditions (Sambrook and Russell, 2001). S1 analysis was performed with total RNA as described by Dohrmann *et al* (1992). Crude protein extracts were generated by TCA precipitation, and Western analysis was performed as described previously (Bird *et al*, 2000). The primary antibodies used were anti-HA (Santa Cruz Biotechnology) and anti-Pgk1 (Molecular Probes). *In vivo* DMS footprinting was performed as described by Dumitru and McNeil (1994).

β-Galactosidase assays

β-Galactosidase activity was measured as described previously (Guarente, 1983). The reporter used was pGAL1-*lacZ*. pGAL1-*lacZ* contains 1000 bp of the *GAL1* promoter, inclusive of the translational ATG, fused to the *lacZ* gene in the vector pYep353.

Chromatin immunoprecipitation analysis

ChIP analysis with Tap-Rap1 and Tap-Zap1 was performed as described previously (Keller *et al*, 2005). Bands were imaged using a Bio-Rad phosphorimager. Data shown are averaged from three

independent experiments. ChIP analysis of histone H3K4me3 was performed as described previously (Roberts *et al.*) with the exception that immunoprecipitations were set up with histone H3 (trimethyl K4) antibodies (Abcam).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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