

Zinc finger protein Loz1 is required for zinc-responsive regulation of gene expression in fission yeast

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In *Schizosaccharomyces pombe*, alcohol dehydrogenase 1 (Adh1) is an abundant zinc-requiring enzyme that catalyses the conversion of acetaldehyde to ethanol during fermentation. In a zinc-replete cell, *adh1* is highly expressed. However, in zinc-limited cells, *adh1* gene expression is repressed, and cells induce the expression of an alternative alcohol dehydrogenase encoded by the *adh4* gene. In our studies examining this zinc-dependent switch in alcohol dehydrogenase gene expression, we isolated an *adh1Δ* strain containing a partial loss of function mutation that resulted in higher levels of *adh4* transcripts in zinc-replete cells. This mutation also led to the aberrant expression of other genes that are typically regulated by zinc. Using linkage analysis, we have mapped the position of this mutation to a single gene called Loss Of Zinc sensing 1 (*loz1*). Loz1 is a 55-kDa protein that contains a double C₂H₂-type zinc finger domain. The mapped mutation that disrupts Loz1 function leads to an arginine to glycine substitution in the second zinc finger domain, suggesting that the double zinc finger domain is important for Loz1 function. We show that *loz1Δ* cells hyperaccumulate zinc and that Loz1 is required for gene repression in zinc-replete cells. We also have found that Loz1 negatively autoregulates its own expression. We propose that Loz1 is a unique metalloregulatory factor that plays a central role in zinc homeostasis in *S. pombe*.

metalloensor | metallothionein | ncRNA | noncoding RNA

Zinc is an important trace metal element that has diverse biological roles. At the cellular level, zinc is an essential cofactor in a range of enzymes, including alkaline phosphatases, Cu-Zn superoxide dismutases, and alcohol dehydrogenases (1). Many regulatory proteins also contain small domains that are stabilized by zinc; these domains include the widely used C₂H₂-type zinc finger domain, the LIM domain, and the RING finger domain (2, 3). In addition to being a protein cofactor, a growing amount of evidence suggests that zinc can act as an intracellular signaling molecule (4, 5). Thus, zinc is essential for all life and has many cellular roles.

Although zinc is an essential nutrient, in excess, it is toxic to growth. Cells therefore rely on mechanisms to maintain a relatively constant intracellular level of zinc despite fluctuations in the extracellular environment. In eukaryotes, zinc-responsive transcription factors play a central role in zinc homeostasis by regulating the expression of genes required for zinc transport or zinc storage (6). In *Saccharomyces cerevisiae*, the transcription factor Zap1 binds to zinc-responsive elements located in target gene promoters and activates gene expression when cytoplasmic zinc levels are low (7). Zap1 target genes include *ZRT1* and *ZRT3*, which are required for zinc uptake and the release of zinc from intracellular stores, respectively (7, 8). In mammals, fish, and flies, a different protein, metal responsive transcription factor 1 (MTF-1), prevents zinc from accumulating to toxic levels. In contrast to Zap1, MTF-1 activates target gene expression when zinc is in excess. MTF-1 regulates genes required for zinc efflux (*ZnT-1*) and zinc storage (*MT-1*) (9). Although studies with MTF-1 and Zap1 have

revealed much about zinc homeostasis, zinc-dependent changes in gene expression are observed in organisms that lack homologs of MTF-1 and Zap1 (10, 11). Moreover, in mammals, zinc-dependent changes in gene expression occur in a manner that is independent of MTF-1 (12, 13). These observations suggest that other unidentified zinc-regulated factors play a critical role in zinc homeostasis in eukaryotes.

In the fission yeast *Schizosaccharomyces pombe*, ~25 genes are induced in response to zinc deficiency (14, 15). These genes include, zinc regulated transporter 1 (*zrt1*), which encodes a high-affinity zinc uptake system, *adh4*, which encodes a putative mitochondrial iron-dependent alcohol dehydrogenase, and *adh1AS*, an antisense transcript generated at the *adh1* gene locus. Other genes that are highly expressed under zinc-limited conditions include *SPAC977.05c*, *SPBC1348.06c*, and *SPBPB2B2.15*, which encode three highly related fungal proteins of unknown function. Genes that are repressed in response to zinc limitation include alcohol dehydrogenase 1 (*adh1*) and zinc binding metallothionein (*zym1*) (15, 16). Despite tight regulation of gene expression in response to zinc availability, there are no homologs of Zap1 or MTF-1 in fission yeast, suggesting that *S. pombe* uses a different factor(s) to sense and regulate gene expression in response to zinc. In our studies examining the regulation of *adh1* gene expression by zinc, we isolated a second site mutation that led to increased expression of *adh4*, *zrt1*, *SPBC1348.06c*, and the *adh1AS* transcript in zinc-replete cells. This mutation also led to lower levels of *adh1* and *zym1* gene expression. In this report, we have mapped the position of this mutation to a single gene and demonstrate that this gene is essential for zinc-dependent regulation of gene expression. We have named this gene Loss Of Zinc sensing 1 (*loz1*).

Significance

Zinc is an essential nutrient that is a cofactor in a wide range of enzymes and regulatory proteins. However, in excess, zinc is toxic to growth. As a consequence, all cells required mechanisms to maintain an optimal level of zinc. Despite a wealth of knowledge of proteins that use a structural or catalytic zinc cofactor, relatively little is known about the factors that sense and regulate intracellular zinc levels in eukaryotes. This report demonstrates that Loz1, a transcriptional repressor that contains a double C₂H₂-type zinc finger domain, plays a central role in zinc homeostasis in *Schizosaccharomyces pombe*. These studies provide important insight into the types of proteins and possible domains that can be used to sense zinc ions.

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Results

Spontaneous Mutation in *adh1*Δ Cells Leads to Aberrant Zinc-Dependent Regulation of Gene Expression. In our previous work, we found that *adh1* and *adh4* gene expression was regulated by zinc (15). During these studies, an *adh1*Δ colony was isolated that contained a spontaneous mutation resulting in aberrant zinc-dependent regulation of gene expression. The effects of this mutation (named *loz1-1*; Loss of Zinc Sensing 1 Allele 1) on gene expression are shown by RNA blot analysis in Fig. 1A and B. In wild-type cells grown in the zinc-rich yeast extract + supplements (YES) medium, *adh1* and *zym1* transcripts accumulate to high levels, and *zrt1*, *adh4*, *adh1AS*, and *SPBC1348.06c* transcripts are not detected (Fig. 1A, lane 1). In cells lacking *adh1* and/or *adh4* grown under similar conditions, *zrt1*, *adh1AS*, and *SPBC1348.06c* transcripts were not detected (Fig. 1A, lanes 2, 5, and 6). Unexpectedly, *zym1* transcripts levels were reduced relative to the wild-type control in the *adh1*Δ and *adh4*Δ cells. However, *zym1* expression is tightly controlled in response to a number of environmental and stress pathways (16), suggesting that the changes of *zym1* mRNA levels in these mutants could result from zinc-independent effects on gene expression. In contrast, in *adh1*Δ *loz1-1* cells, *zrt1*, *adh4*, *adh1AS*, and *SPBC1348.06c* transcripts accumulated to high levels (Fig. 1A, lane 4). *Zym1* transcripts levels were also lower than those observed in *adh1*Δ and wild-type cells. Thus, the gene expression profile of *adh1*Δ *loz1-1* cells grown in YES medium was atypical and resembled that of a cell grown under zinc-limiting conditions.

The *loz1-1* mutation spontaneously occurred in a strain lacking the *adh1* gene. To determine whether the aberrant gene expression observed in *adh1*Δ *loz1-1* cells depended on the loss of *adh1* gene function, *adh1*Δ *loz1-1* cells were crossed to a wild-type strain of the opposite mating type. When the resulting diploid was sporulated and individual asci isolated by tetrad dissection analysis, half of the ascospores accumulated high levels of *adh4* transcripts in YES medium. This phenotype segregated independently from *adh1*Δ, and ascospores were isolated that had aberrant *adh4* expression and the endogenous *adh1* gene (Fig. 1A, lane 3 strain *loz1-1*). These results are consistent with the aberrant gene expression observed in *adh1*Δ *loz1-1* being independent of *adh1*Δ.

To further examine the effects of the *loz1-1* mutation on gene expression, total RNA was purified from wild-type and *loz1-1* cells grown in a zinc-limited minimal medium (ZL-EMM), or in this medium with supplemented with 50, 200, or 500 μM zinc. In wild-type cells, high levels of *adh4*, *zrt1*, *SPBC1348.06c*, and *adh1AS* transcripts levels accumulated when cells were grown in ZL-EMM with no zinc supplement (Fig. 1B, lane 1). As expected, growth of wild-type cells in the presence of a zinc supplement led to a significant reduction in the levels of these transcripts, and an increase in *zym1* and *adh1* mRNA transcripts (Fig. 1B, lanes 2–4). In *loz1-1* cells, high levels of *adh4*, *zrt1*, *SPBC1348.06c*, and *adh1AS* transcripts were detected in cells that had been grown in ZL-EMM with and without a 50 μM zinc supplement (Fig. 1B, lanes 5 and 6). Although there was a clear misregulation of zinc-dependent gene expression in *loz1-1* cells grown in ZL-EMM + 50 μM zinc, at higher zinc levels (ZL-EMM + 200 or 500 μM zinc) there was a significant decrease in *adh4*, *SPBC1348.06c*, and *adh1AS* transcripts levels, and a modest reduction in *zrt1* transcript levels. *zym1* and *adh1* mRNAs were also detected at these higher zinc levels in *loz1-1* cells. Thus, the *loz1-1* allele leads to impaired zinc-dependent gene expression that can be partially restored by the addition of zinc in excess.

The aberrant expression of zinc-regulated genes observed in *loz1-1* cells could result from a mutation that impaired zinc uptake or a mutation that interfered with zinc-dependent regulation of gene expression. To distinguish between these possibilities,

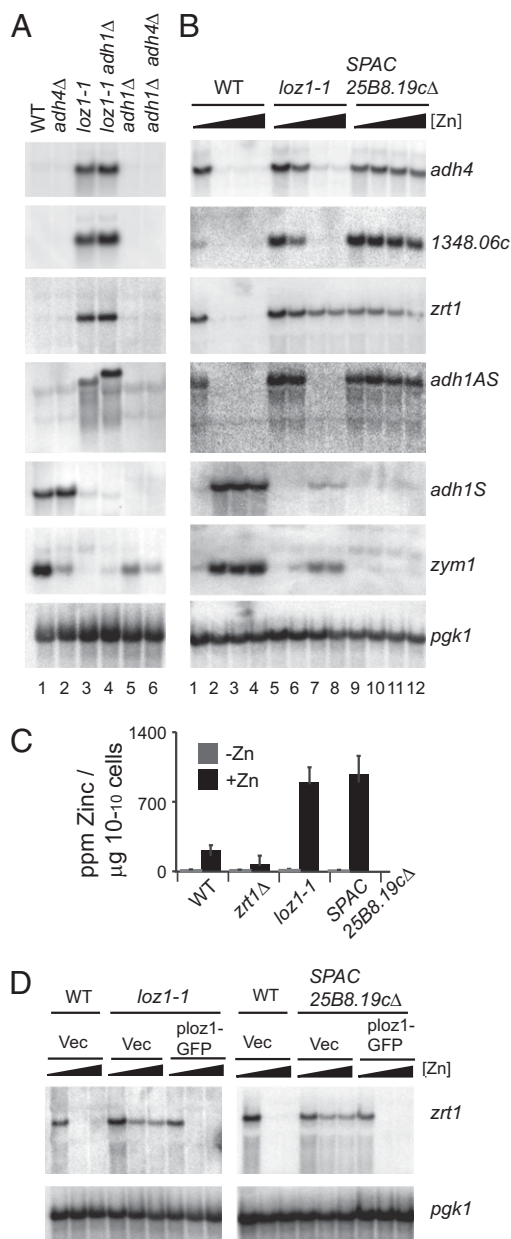


Fig. 1. A mutation in *loz1* disrupts zinc-dependent regulation of gene expression. (A) Wild-type, *adh4*Δ, *loz1-1*, *adh1*Δ *loz1-1*, *adh1*Δ, and *adh1*Δ *adh4*Δ cells were grown to exponential phase in YES medium and total RNA purified for RNA blot analysis. RNA blots were incubated with strand-specific probes that hybridized to the *adh4*, *zrt1*, *SPBC1348.06c*, *adh1AS*, *adh1*, and *zym1* transcripts. The *adh1AS* probe hybridizes to the extreme 5' end of this transcript, a region that does not overlap with the *adh1* ORF and is present in *adh1*Δ cells. Because of the high level of sequence conservation at the DNA level (>99% identity), the *SPBC1348.06c* probe can cross-hybridize to *SPAC977.05c* and *SPBPB2B2.15* transcripts. For a loading control, blots were also probed for *pgk1*. (B) Wild-type, *loz1-1*, and *loz1*Δ cells were grown in ZL-EMM supplemented with 0, 50, 200, or 500 μM zinc. Total RNA was purified for RNA blot analysis. RNA blots were probed as described in A. (C) Wild-type, *zrt1*Δ, *loz1-1*, and *loz1*Δ cells were grown in ZL-EMM supplemented with 0 μM (-Zn) or 200 μM zinc (+Zn). Cells were harvested, and total cellular zinc levels measured by inductively coupled plasma mass spectrometry. (D) Wild-type, *loz1-1*, and *loz1*Δ cells containing the vector JK148 or plasmid pLoz1-GFP were grown in ZL-EMM supplemented with 0, 100, or 200 μM zinc, and total RNA was purified for RNA blot analysis.

total cellular zinc levels were measured in wild-type and *loz1-1* cells that had been grown in ZL-EMM or in this medium

supplemented with 200 μ M zinc. As a control, zinc levels were also examined in *zrt1 Δ* cells that lack the high-affinity zinc uptake system. In zinc-replete medium, *loz1-1* cells accumulated higher levels of zinc compared with the wild-type and *zrt1 Δ* cells (Fig. 1C). Thus, under zinc-replete conditions, *loz1-1* cells hyperaccumulate zinc, but “sense” that they are zinc limited. These results are consistent with a mutation in *loz1* disrupting zinc-dependent regulation of gene expression.

Loz1 Is Required for Zinc-Dependent Regulation of Gene Expression.

The *loz1-1* allele provided a tool to understand how *S. pombe* cells sense intracellular zinc levels. To determine how the *loz1-1* mutation disrupted zinc sensing, the position of the mutation in *loz1-1* cells was mapped by linkage analysis (Table S1). These experiments mapped the mutation to an ~44-kb region on the right arm of chromosome 1. DNA sequencing through this region revealed a single C-G mutation at nucleotide position 1528 in the gene *SPAC25B8.19c*. To determine whether the mutation in *SPAC25B8.19c* was the cause of the impaired zinc-dependent regulation of gene expression, a plasmid that expressed a GFP-tagged *SPAC25B8.19c* gene from its native promoter (pLoz1-GFP) was integrated into *loz1-1* cells. When *loz1-1* cells containing the empty vector or pLoz1-GFP were grown in ZL-EMM with or without zinc supplements, pLoz1-GFP was able to restore zinc-dependent gene expression (Fig. 1D). To further examine the function of *SPAC25B8.19c*, a strain was constructed in which the entire *SPAC25B8.19c* ORF was deleted from the genome. In this cell type, *adh4*, *SPBC1348.06c*, *zrt1*, and *adh1AS* transcripts accumulated under all conditions, and no *zym1* and *adh1* transcripts were detected by RNA blot analysis (Fig. 1B, lanes 9–12). This aberrant gene expression could be fully restored by the addition of pLoz1-GFP (Fig. 1D) and partially rescued by the addition of a plasmid expressing *loz1-1* (Fig. S1). Taken together, these results indicate that *SPAC25B8.19c* plays a central role in zinc homeostasis and strongly suggest that the single base-pair mutation in *SPAC25B8.19c* is the cause of impaired zinc-dependent regulation of gene expression in *loz1-1* cells. *SPAC25B8.19c* was therefore named Loz1.

Loz1 is a 55-kDa protein that contains a double C₂H₂ type zinc finger domain at the extreme C terminus (Fig. 2A). The mutation that impairs Loz1 function results in an Arg510Gly substitution at position 1 of the alpha helix of the second zinc finger. In other fungal Loz1 homologs, the double zinc finger domain is highly conserved, consistent with it being important for Loz1 function (Fig. 2B). Outside of the double zinc finger domain, Loz1 shares little homology with other fungal proteins with the exception of a few smaller domains that are conserved in *Schizosaccharomyces japonicus*, a close relative of *S. pombe* (Fig. 2A).

Because deletion of *loz1* resulted in increased expression of *adh4* and *zrt1*, we hypothesized that Loz1 played a central role in zinc homeostasis by acting as a transcriptional repressor in zinc-replete cells. To test whether Loz1 acted as a zinc-regulated transcriptional repressor, constructs were generated in which the *adh4* and *SPBC1348.06c* promoters were fused to a *lacZ* reporter gene. When these constructs were integrated into wild-type or *loz1 Δ* cells, β -galactosidase activity was robustly regulated by zinc in a manner that depended on *loz1* (Fig. 3A). No zinc-dependent changes in β -galactosidase activity were observed in cells expressing a control *sod1-lacZ* reporter. When the *adh4* and *SPBC1348.06c* reporters were introduced into *loz1-1* cells, high levels of β -galactosidase activity were observed at intermediate zinc levels, consistent with the partial loss of gene expression in this strain (Fig. S2). Thus, Loz1 is necessary for the zinc-dependent regulation of the *adh4-lacZ* and *SPBC1348.06c-lacZ* reporter genes.

The *adh4-lacZ* reporter contained promoter and 5' UTR sequences. A 5' RACE revealed that the *adh4* 5' UTR was 620 nt in length and contained a previously unannotated intron

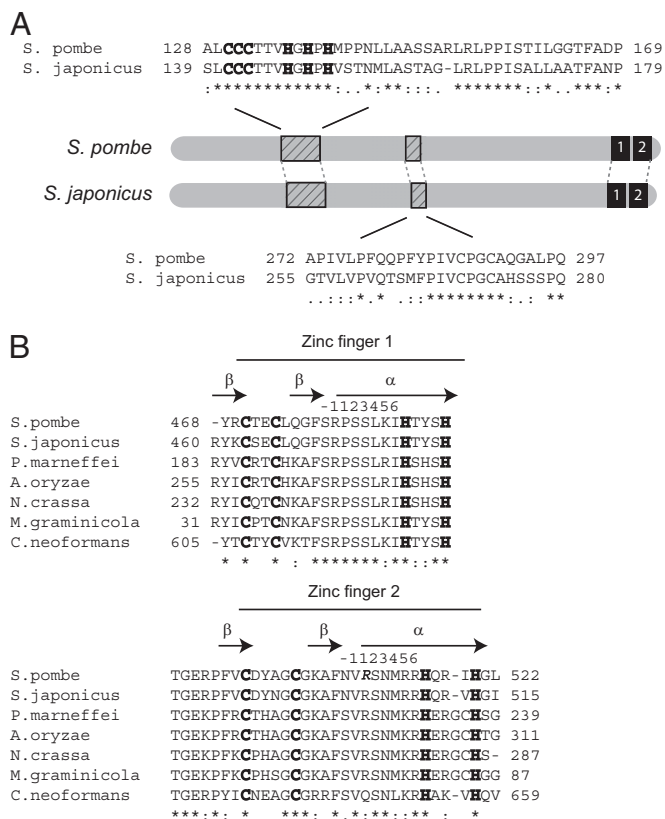


Fig. 2. Conserved Loz1 domains. (A) A schematic diagram of the Loz1 protein from *S. pombe* and *S. japonicus*. To identify conserved domains, the amino acid sequence of Loz1 was aligned with the Loz1 ortholog from *S. japonicus* by using CLUSTAL W. Black numbered boxes indicate the positions of the double zinc finger domains. The sequence alignment of other conserved domains has been shown and their positions indicated by striped rectangles on the schematic diagram. (B) An alignment of the Loz1 zinc finger domain with the corresponding region of Loz1 orthologs from *S. japonicus*, *Penicillium marneffeii*, *Aspergillus oryzae*, *Neurospora crassa*, *Mycosphaerella graminicola*, and *Cryptococcus neoformans*. The cysteine and histidine residues that are predicted to coordinate zinc are shown in bold. The arginine altered to a glycine in *loz1-1* is shown in bold and italics.

(Fig. S3). To test whether the *adh4* 5' UTR was necessary for the Loz1-dependent regulation of *adh4-lacZ* reporter activity, a construct was generated in which the *adh4* 5' UTR was exchanged for the *nmt1* 5' UTR (Fig. S3). *nmt1* (no message in thiamine 1) is a gene that is not regulated by zinc (15). When this hybrid reporter was expressed in wild-type cells, β -galactosidase activity was regulated by zinc (Fig. 3B, construct 2). In contrast, zinc responsiveness was severely reduced in *loz1 Δ* cells expressing this reporter. Together these results indicate that the *adh4* 5' UTR is not necessary for zinc-dependent regulation. To further map the region of the *adh4* promoter required for Loz1-dependent regulation, additional truncated *adh4-nmt1* hybrid reporters were introduced into wild-type and *loz1 Δ* cells (Fig. 3B, constructs 3–6). These truncations resulted in a number of reductions in the total level of β -galactosidase activity, consistent with the loss of upstream activating sequences. When the zinc-dependent regulation was examined, the hybrid reporter containing *adh4* nucleotides –1050 to –621 (construct 3) was not regulated by zinc in *loz1 Δ* cells but retained full zinc responsiveness when expressed in a wild-type strain. In contrast, a shorter reporter containing *adh4* nucleotides –713 to –621 (construct 4) was only weakly regulated by zinc in a wild-type strain. Together these results suggest that an element(s) critical

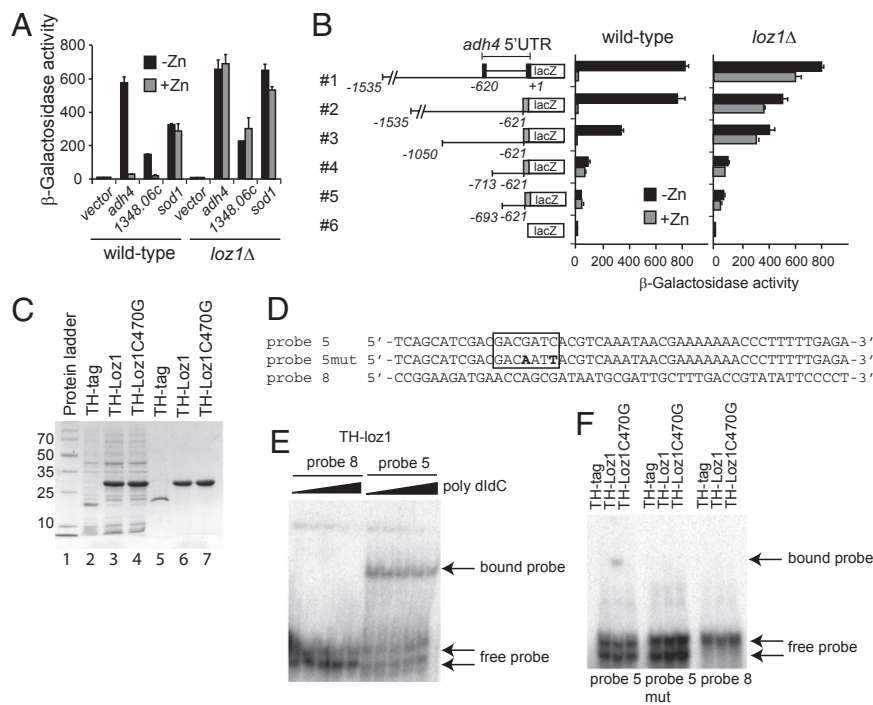


Fig. 3. Loz1 is required for zinc-dependent transcriptional regulation of gene expression. (A) Wild-type and *loz1* Δ cells containing the empty vector, *adh4-lacZ*, or *SPBC1348.06c-lacZ* reporters were grown in ZL-EMM supplemented with 0 μ M (-Zn) or 200 μ M zinc (+Zn) before cells were harvested for β -galactosidase assays. (B) Wild-type and *loz1* Δ cells containing the indicated reporter constructs were grown as described in A. Cells were harvested and β -galactosidase activity was measured by standard procedures. (C) SDS/PAGE analysis of crude lysates from isopropyl β -D-1-thiogalactopyranoside-induced *E. coli* cells containing the Pet32a empty vector (TH tag), Pet32a-Loz1 (TH-Loz1), or Pet32a-Loz1Cys470Gly (TH-Loz1Cys470Gly) (lanes 2–4), or the Ni-NTA affinity-purified TH tag, TH-Loz1, or TH-Loz1Cys470Gly (lanes 5–7). Proteins were visualized by using Coomassie blue. The sizes of a molecular mass ladder (lane 1) are shown in kilodaltons on the left. (D) Sequence of the probes used for EMSA. All probes used were double-stranded; the sequence of the 5' strand for each probe is shown. The GN(A/C)GATC element is boxed. Mutations in probe 5mut are highlighted in bold. (E) A representative EMSA using 0.1 ng of P32-labeled double-stranded probes T8 and T5 in the presence of increasing levels (0, 0.5, 1, 2.5, and 5 μ g) of poly dIdC. (F) A representative EMSA using 0.1 ng of P32-labeled double-stranded probes T5, T5mut, and T8, and affinity-purified TH tag, TH-tagged Loz1, and TH-Loz1Cys470Gly in the presence of 1 μ g of poly dIdC. Free probe and bound probe are indicated.

to the *loz1*-dependent regulation of *adh4* was located between nucleotides -1050 to -713 .

Motif-based sequence analysis tools (17) revealed that three GN(A/C)GATC elements were located within nucleotides -1050 to -713 of the *adh4* promoter. This element was also present in multiple copies in the promoters of other Loz1-regulated genes. To test whether Loz1 directly interacted with this candidate motif, in vitro DNA binding studies were performed by using a recombinant 96 aa C-terminal truncation of Loz1 and a 50-bp *adh4* promoter fragment containing this motif. The C-terminal of Loz1 was chosen for these studies because this region contained the double zinc finger domain, a motif known to mediate site-specific interactions with DNA in other proteins (18). For the purification, the C-terminal of Loz1 was expressed in *Escherichia coli* as a thioredoxin-His tag fusion protein and was purified by using Ni-NTA affinity chromatography (Fig. 3C, TH-Loz1). The tag only (TH-tag) and a mutated form of the tagged Loz1 fusion protein containing a Cys470Gly substitution (TH-Loz1C470G) were also expressed and purified as controls. The Cys470Gly substitution disrupts the first cysteine in zinc finger 1. Results showed that the radio-labeled *adh4* promoter probe containing an GN(A/C)GATC element (Fig. 4D, probe 5) formed a complex in the presence of the TH-Loz1 and the nonspecific competitor poly dIdC (Fig. 4E). No complex was detected with this probe, and the thioredoxin-His tag only or the Cys470Gly mutant Loz1 protein (Fig. 4F, probe 5). In addition, no complex was detected when the GN(A/C)GATC element was mutated to GN(A/C)AATT (Fig. 4F, probe 5mut), or when TH-Loz1 was incubated with an adjacent *adh4* promoter fragments lacking this motif (Fig. 4F, probe 8). These results suggest that the C-terminal 96 amino acids of Loz1 associate with a 50-bp region of the *adh4* promoter that contains a GN(A/C)GATC element, and that the GATC core of this sequence is critical for this interaction.

Loz1 Negatively Autoregulates Its Own Expression. Loz1 is required for gene repression in zinc-replete cells. To determine how Loz1 was regulated by zinc in *S. pombe*, we initially tested whether *loz1* transcript levels depended on zinc levels. When wild-type were

grown in ZL-EMM, an ~ 3.1 -kb *loz1* transcript was detected by RNA blot analysis (Fig. 4A, see arrow). This band was absent from *loz1* Δ cells and was present at reduced levels in wild-type cells that had been grown in the presence of 50–500 μ M zinc. A smaller band of unknown origin was also detected in *loz1* Δ cells. In *loz1-1* cells, *loz1* transcript levels were regulated by zinc, but to a lesser extent than the regulation observed in wild-type cells. These results suggested that Loz1 might negatively autoregulate its own expression. To test this hypothesis, the activity of a *loz1-lacZ* reporter was examined in wild-type, *loz1-1*, and *loz1* Δ cells that had been grown over a range of zinc levels (Fig. 4B). Consistent with Loz1 controlling its own expression, β -galactosidase activity was regulated by zinc in a manner that depended on Loz1.

The above results revealed that *loz1* mRNA transcripts accumulate at lower levels in zinc-replete cells compared with zinc-limited cells. To determine whether a similar reduction was observed at the protein level, immunoblot analysis was used to examine Loz1 levels. To facilitate these studies, a yeast strain was generated in which a 13 \times myc epitope tag was fused to the C terminus of the endogenous Loz1 (Loz1-myc). In cells expressing the Loz1-myc fusion protein, *adh4* transcript levels were regulated by zinc indicating that the Loz1-myc fusion is functional (Fig. 4C). When crude protein extracts were purified from Loz1-myc cells grown over a range of zinc levels, a major band of ~ 75 kDa was detected by immunoblot analysis (Fig. 4D). This band was not detected in wild-type cells, suggesting that it was specific to the Loz1-myc strain. In addition, higher levels of Loz1-myc accumulated in zinc-limited cells, consistent with the higher expression of *loz1* under this condition. Because Loz1 accumulated under all conditions, but plays a role in gene repression in zinc-replete cells, we hypothesized that the zinc-dependent changes in Loz1 function might result from zinc modulating the cellular distribution of Loz1. To test this hypothesis, *loz1* Δ cells expressing pLoz1-GFP were grown under zinc-limiting and zinc-replete conditions and the localization of Loz1-GFP visualized by immunofluorescence microscopy. Under all conditions, Loz1-GFP colocalized with a Hoechst DNA stain (Fig. 4E). Thus, Loz1 accumulates in the nucleus in zinc-limited and zinc-replete

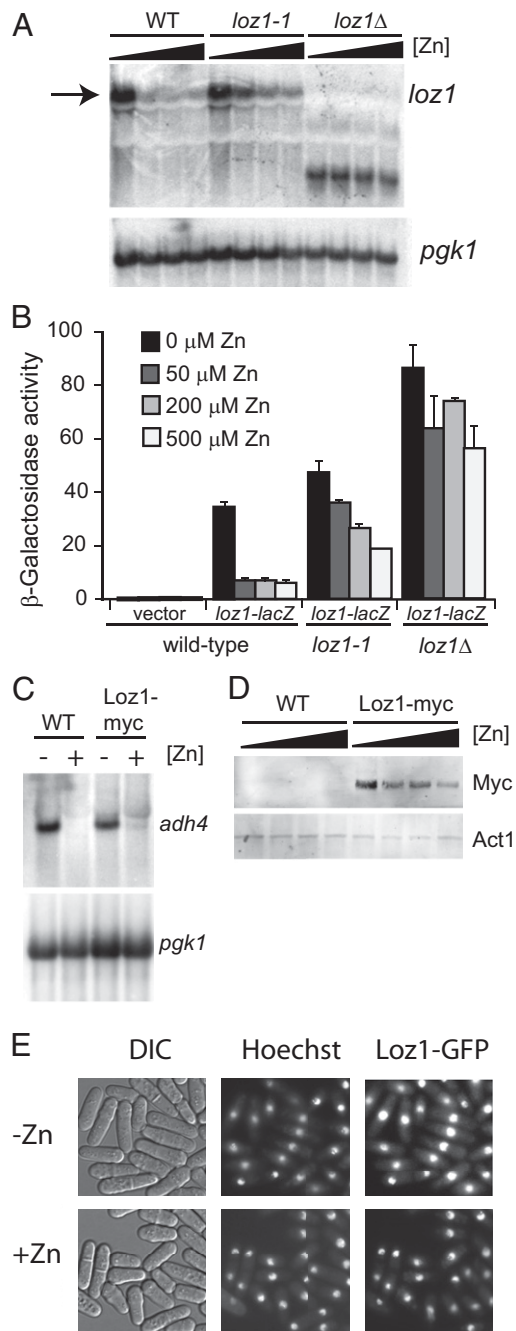


Fig. 4. (A) Wild-type, *loz1-1*, and *loz1Δ* cells were grown in ZL-EMM supplemented with 0, 50, 200, or 500 μ M zinc, cells were harvested, and total RNA was purified for RNA blot analysis. (B) Wild-type, *loz1-1*, and *loz1Δ* cells containing the *loz1-lacZ* reporter were grown in ZL-EMM supplemented with 0, 50, 200, or 500 μ M zinc, and cells were harvested for β -galactosidase assays. (C) Wild-type and Loz1-myc cells were grown in ZL-EMM supplemented with 0 or 200 μ M zinc, and total RNA was purified for RNA blot analysis. RNA blots were probed for *adh4* and *pgk1* mRNAs. (D) Wild-type and Loz1-myc cells were grown in ZL-EMM supplemented with 0, 50, 200, or 500 μ M zinc, and crude protein extracts were isolated for immunoblot analysis. (E) *loz1Δ* cells expressing pLoz1-GFP were grown in ZL-EMM supplemented with 0 (-Zn) or 200 μ M (+Zn) zinc. The cells were visualized for GFP by fluorescence microscopy. Hoechst stain was used to stain the nucleus, and differential interference contrast microscopy was used to confirm cell integrity.

cells but plays a role in gene repression under zinc-replete conditions.

Increased *adh4* Expression Confers a Growth Advantage to *adh1Δ* Cells. Why would a spontaneous mutation that impaired zinc-dependent regulation of gene expression have occurred in *adh1Δ* cells? In *S. cerevisiae*, gene duplications leading to increased *ADH4* copy number confer a significant growth advantage to *adh1Δ* cells (19). Because *adh4* expression is tightly regulated by zinc in *S. pombe*, a straightforward explanation for the occurrence of the *loz1-1* mutation is that it leads to increased *adh4* expression that, in turn, compensates for the absence of *adh1*. To test this hypothesis, we investigated whether the *loz1-1* mutation or increased *adh4* expression would confer a growth advantage to *adh1Δ* cells. *adh1Δ* cells have a severe growth defect on YES medium and are unable to grow in the presence of the respiration inhibitor Antimycin A (Fig. S4). In contrast, *adh1Δ loz1-1* cells had no growth defect in YES medium and were able to grow in the presence of antimycin A (Fig. S4). To determine whether increased *adh4* expression would enhance growth of *adh1Δ*, a plasmid expressing *adh4* from the constitutive *pgk1* promoter (*pgk1-adh4*) was introduced in *adh1Δ* cells, and control wild-type and *adh4Δ* cells. The addition of *pgk1-adh4* conferred a significant growth advantage to *adh1Δ* in both the presence and absence of antimycin A (Fig. S4). Thus, one potential explanation for the occurrence of the *loz1-1* mutation is that it results in increased *adh4* gene expression that, in turn, confers a growth advantage to *adh1Δ*.

Discussion

In *S. pombe*, *zrt1*, *adh4*, *SPBC1348.06c*, and *adh1AS* transcripts are specifically expressed in zinc-limited cells. In this study, we show that *loz1-1* and *loz1Δ* mutations result in the expression of *zrt1*, *adh4*, *SPBC1348.06c*, and *adh1AS* genes in zinc-replete cells and lead to the hyperaccumulation of zinc. We have also found that Loz1 is a nuclear protein that associates with a GN(A/C) GATC element located in the *adh4* promoter. Based on the above results, we propose that Loz1 plays a central role in zinc homeostasis in *S. pombe*.

The defects observed in *loz1-1* cells are consistent with a loss-of-function mutation in a transcriptional repressor or a gain-of-function mutation in a transcriptional activator. We have found that the *loz1-1* mutation is recessive (Fig. S5) and that a wild-type copy of *loz1* can complement the aberrant gene expression observed in *loz1-1* cells (Fig. 1D). We also show that deletion of the *loz1* ORF results in the expression of *zrt1*, *adh4*, *SPBC1348.06c*, and the *adh1AS* transcript in high zinc (Fig. 1B). From these observations, we propose that *loz1-1* is a loss-of-function mutation in a gene required for transcriptional repression.

If Loz1 functions in transcriptional repression, then why are *adh1* and *zym1* mRNA levels reduced in *loz1Δ* and *loz1-1* cells? In *S. pombe*, *adh1* gene expression is inhibited by increased expression of the *adh1AS* transcript (15). In this study, we have found that the expression of the *adh1AS* transcript depends on Loz1 (Fig. 1B). Together, these results suggest that Loz1 indirectly controls *adh1* gene expression by regulating the levels of the *adh1AS* transcript. A potential clue to the regulation of *zym1* by zinc is that low levels of a larger *zym1* transcript are detected on RNA blots in *loz1Δ* cells and in wild-type cells grown under zinc-limiting conditions (Fig. 1B and Fig. S6). Intergenic transcripts that transverse through promoter regions can inhibit the recruitment of transcriptional activators, leading to decreased expression of the downstream gene (20, 21). Therefore, one explanation for the Loz1-dependent regulation of *zym1* is that Loz1 regulates the expression of an intergenic transcript that transcriptionally interferes with *zym1* expression. Supporting this hypothesis, probes that bind to the upstream region *zym1* hybridize to a larger Loz1-regulated transcript (Fig. S6). Thus, the

Loz1-dependent regulation of *adh1* and *zym1* genes is potentially indirectly mediated through the regulation of noncoding RNA transcripts at these loci.

A number of lines of evidence suggest that Loz1 is regulated at multiple levels by zinc. At a transcriptional level, Loz1 negatively regulates its own expression, leading to lower levels of *loz1* mRNA transcript in high zinc (Fig. 4A). This transcriptional regulation, in turn, leads to lower levels of Loz1 protein accumulating in zinc-replete cells compared with zinc-limiting cells (Fig. 4D). Despite modest changes in Loz1 protein levels by zinc, Loz1 is required for gene repression in zinc-replete cells, suggesting that Loz1 activity is also modulated by zinc. In the two characterized zinc-responsive transcription factors, Zap1 and MTF1, C₂H₂-type zinc fingers have been implicated in zinc sensing (22–24). In our study, we find that an Arg510Gly substitution in zinc finger 2 partially disrupts Loz1 function and that the zinc finger region is necessary for DNA-binding function. Thus, one possibility is that the occupancy of the Loz1 zinc finger domains with zinc depends on cellular zinc levels that, in turn, controls Loz1 DNA binding function. Alternately, the N-terminal region of Loz1 contains small domains rich in cysteine and histidine residues. Although these domains do not match any known zinc-binding motif, cysteine and histidine residues are commonly involved in the coordination of zinc ions. Further studies with Loz1 should provide insight into whether Loz1 activity is directly controlled by zinc ion binding to one or more of these domains.

A number of domains have been implicated in zinc sensing in eukaryotes, some of which closely resemble commonly found zinc structural motifs. In *Chlamydomonas reinhardtii*, deletion of a C-terminal metallothionein-like domain from the copper sensing transcription factor, *CRR1*, leads to increased expression of genes required for zinc uptake and zinc hyperaccumulation (10). In *Arabidopsis thaliana*, bZIP19 and bZIP23 are required for the adaptation to zinc (11). Although it is unclear how

bZIP19 and bZIP23 are regulated by zinc, both contain a small conserved Cys/His-motif that might be involved in zinc sensing. Zap1 contains multiple domains that are independently regulated by zinc (25). These domains include AD1, a transactivation domain that is rich in histidine and cysteine residues, and AD2, a transactivation domain that contains a unique regulatory zinc finger pair motif (26). In mammals, MTF-1 also contains multiple zinc-regulated domains including a zinc-responsive DNA binding domain containing six C₂H₂-type zinc fingers (9). The growing number of unique eukaryotic zinc-regulated factors that have been identified suggests that novel zinc-sensing domains may have evolved independently in different species. Intriguingly, studies with Zap1 have shown that AD1 plays a primary role in activating genes required to maintain zinc homeostasis, whereas AD2 plays a more major role in gene regulation when zinc deficiency is combined with other environmental stresses (26). Thus, one potential explanation for the diversity in zinc-regulated factors is that each unique factor is able to optimally sense changes in zinc levels under normal and specialized environmental conditions. Future studies with Loz1 and other zinc-regulated factors should therefore provide important insight into how unique domains have evolved to sense zinc and the role that different zinc sensors play in maintaining zinc homeostasis and adapting to changes in zinc levels.

Materials and Methods

Additional details are found as *SI Materials and Methods*. All of the yeast strains used in this study are shown in [Table S2](#). All primers used for plasmid generation are listed in [Table S3](#).

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