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The Double Zinc Finger Domain and Adjacent Accessory Domain from the Transcription Factor Loss of Zinc Sensing 1 (Loz1) Are Necessary for DNA Binding and Zinc Sensing

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Background: Loz1 represses gene expression when zinc is in excess.
Results: A double zinc finger domain and accessory domain preceding zinc finger 1 are required for zinc-dependent repression.
Conclusion: The zinc-sensing domain in Loz1 coincides with the DNA binding domain.
Significance: Data presented here provide new insights into mechanisms of zinc sensing.

The Loz1 transcription factor from *Schizosaccharomyces pombe* plays an essential role in zinc homeostasis by repressing target gene expression in zinc-replete cells. To determine how Loz1 function is regulated by zinc, we employed a genetic screen to isolate mutants with impaired zinc-dependent gene expression and analyzed Loz1 protein truncations to map a minimal zinc-responsive domain. In the screen, we isolated 36 new *loz1* alleles. 27 of these alleles contained mutations resulting in the truncation of the Loz1 protein. The remaining nine alleles contained point mutations leading to an amino acid substitution within a C-terminal double zinc finger domain. Further analysis of two of these substitutions revealed that they disrupted Loz1 DNA activity *in vitro*. By analyzing Loz1 protein truncations, we found that the last 96 amino acids of Loz1 was the smallest region that was able to confer partial zinc-dependent repression *in vivo*. This 96-amino acid region contains the double zinc finger domain and an accessory domain that enhances DNA binding. These results were further supported by the findings that MtfA, a transcription factor from *Aspergillus nidulans* that contains a related double zinc finger, is unable to complement *loz1*Δ, whereas a chimera of MtfA containing the Loz1 accessory domain is able to complement *loz1*Δ. Together, our studies indicate that the double zinc finger domain and adjacent accessory domain preceding zinc finger 1 are necessary for DNA binding and zinc-dependent repression.

Zinc is a metal nutrient that is essential for all life. At the cellular level, zinc is a cofactor in over 300 enzymes, including alcohol dehydrogenases, RNA polymerases, and alkaline phosphatases (1). Several structural motifs, including the zinc finger and RING finger, are also stabilized by zinc ions (2). These types of domains are commonly found in regulatory factors and often play a role in mediating interaction with DNA, RNA, or proteins. In addition to its role in protein structure/function, zinc is an important signaling molecule in some cells (3).

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Although zinc has many important biological roles, in excess, zinc can be toxic to cell growth. As a consequence, all organisms rely on mechanisms to maintain optimal intracellular levels of zinc. In eukaryotes, zinc-responsive transcription factors play a primary role in maintaining zinc homeostasis by controlling the expression of genes necessary for zinc transport and/or zinc storage (4). Transcription factors that regulate gene expression in response to zinc levels include Zap1 from *Saccharomyces cerevisiae*, MTF-1 in mammals and fish, Loz1 in *Schizosaccharomyces pombe*, and bZip19 and bZip23 in *Arabidopsis thaliana* (5–8).

The majority of what is known about how eukaryotic cells sense zinc comes from studies of the two transcriptional activators, Zap1 and MTF-1 (4). Zap1 is active in zinc-limited cells, whereas MTF-1 is active in zinc-replete cells. Both factors contain multiple zinc-responsive domains. Zap1 contains two transactivation domains that are independently regulated by zinc (9, 10). Zinc also inhibits Zap1 DNA binding activity (11). In mammals, zinc regulates MTF-1 DNA binding activity, cellular localization, and transactivation domain function (8). The presence of these multiple zinc-responsive domains ensures that the activity of each factor can be precisely controlled by changes in cellular zinc status.

In *S. pombe*, Loz1 represses gene expression under conditions of zinc excess. Loz1 target genes include *zrt1*, *adh4*, and the *adh1AS* transcript, which encode a zinc uptake transporter, a mitochondrial alcohol dehydrogenase, and an antisense transcript that inhibits the expression of *adh1* (alcohol dehydrogenase 1), respectively (6, 12). Loz1 also negatively regulates the expression of its own gene (6). Although most studies to date have focused on the mechanisms by which zinc-responsive transcriptional activators sense zinc, in this study, we investigated how Loz1 is regulated by zinc. We found that a minimal domain containing two C_2H_2-type zinc finger domains and a neighboring accessory domain is sufficient for DNA binding and zinc-dependent repression.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—All strains created in this study are derivatives of the wild-type strain JW81 (*h*−

ade6-M210 leu1-32 ura4-D18) (13), *adh1*Δ strain ABY83 (*h+ ade6-M210 leu1-32 ura4-D18 adh1*Δ::*kanMX6*), or *loz1*Δ strain ABY540 (*h− ade6-M210 leu1-32 ura4-D18 loz1*Δ::*kanMX6*) (6). Strains were created by integrating linearized plasmid constructs using standard transformation procedures. *S. pombe* strains were grown in YES medium or in zinc-limited Edinburgh minimal medium (ZL-EMM)[2] with or without the indicated zinc supplement (12). For all experiments with ZL-EMM, cells were pregrown to exponential phase in YES medium. Cells were washed twice in ZL-EMM, diluted to a final A_{600} of 0.5 and grown for a further 14–16 h in ZL-EMM with or without zinc.

Plasmid Construction—All plasmids used in this study were linearized with NruI, BsiWI, or NcoI before integration at the *leu1* or *ade6* locus in the yeast strains, JW81, ABY83, or ABY540 (6). The fusion of the *zhf1* promoter to the *lacZ* reporter gene was generated by PCR-amplifying an ~1-kb fragment of the *zhf1* promoter. PCR primers contained EagI and BamHI restriction sites to facilitate cloning into similar sites in the vector JK-*lacZ* (12). The construction of the *adh4-lacZ* and *loz1-lacZ* reporters has been described previously (6, 12). The reporter pTN-*zrt1-lacZ* is a derivative of the plasmid pTN215 (NBRP ID FYP484), which was obtained from the National BioResource Project, Japan. pTN-*zrt1-lacZ* was generated by introducing the *lacZ* gene into the PstI/ApaI sites of pTN215 to generate pTN-*lacZ*. The *zrt1* promoter was then introduced as a SacII/PstI fragment to generate pTN-*zrt1-lacZ. pZ-loz1GFP* is a derivative of *pL-loz1GFP* (6) in which a KpnI/EcoRI *loz1* promoter fragment was replaced with a PCR-amplified 1-kb *zhf1* promoter fragment. The S489F and M513I mutations were introduced into pTH-Loz1 plasmid using QuikChange mutagenesis (Agilent Technologies). The pTH-Loz1 ZF fusion was generated by amplifying the C terminus of Loz1 with primers containing BamHI and XhoI sites. The resulting PCR product was digested with BamHI/XhoI and was cloned into similar sites in the vector Pet32a (EMD Millipore). Constructs expressing pL-Loz1GFP truncations were generated by PCR-amplifying the respective region of the *loz1* ORF. PCR primers contained EcoRI and BamHI restriction sites to facilitate cloning into EcoRI/BamHI-digested *pL-loz1GFP*. Constructs expressing Loz1-GFP truncations from the *zhf1* promoter were generated by replacing the *loz1* promoter with a KpnI/EcoRI *zhf1* promoter fragment released from *pZ-loz1GFP*. *pZ-MtfAGFP* was generated by using overlapping PCR to amplify the *mtfA* ORF from an *Aspergillus nidulans* genomic DNA template. Primers contained EcoRI/BamHI sites to allow replacement of the *loz1* ORF with MtfA in the vector *pZ-loz1GFP*. Chimeras of Loz1 and MtfA were generated by a similar strategy using overlapping PCR and *pZ-MtfAGFP* and *pL-Loz1GFP* plasmid templates. All plasmid constructs were confirmed by sequence analysis.

RNA Blot, Immunoblot Analysis, and β-Galactosidase Assays—For RNA blot analysis, total RNA was purified using hot acidic phenol, and total RNA was separated on formaldehyde gels by standard procedures. All single-stranded ^{32}P-labeled RNA probes were generated from a PCR template using a MAXISCRIPT T7 kit (Ambion) according to the manufacturer's instructions. PCR primers used for probe generation have been described previously (6, 12). Total protein extracts were prepared from yeast as described previously (6). Immunoblots were incubated with the primary antibodies anti-GFP (G1544, Sigma) and anti-Act1 (ab3280-500, Abcam) and secondary antibodies IRDye800CW-conjugated anti-mouse IgG (LI-COR) and IRDye680-conjugated anti-rabbit IgG (LI-COR). Signal intensities were analyzed using the Odyssey infrared image system (LI-COR). β-Galactosidase assays were performed as described previously (12).

Recombinant Protein Purification and EMSAs—TH-Loz1 truncations and mutated derivatives were expressed in *Escherichia coli* and were purified using Ni^{2+}-nitrilotriacetic acid Superflow (Qiagen) columns as described previously (6). Double-stranded DNA probes for the EMSAs were created by end labeling with [γ-^{32}P]dATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase. Binding reactions were performed in buffer containing a final concentration of 50 μg/ml BSA (New England Biolabs), 1 μg of poly(dI-dC) (Sigma), 200 ng of the indicated protein, and 40 μmol of labeled probe. Competition studies were performed by incubating protein with the indicated unlabeled probe for 20 min at room temperature before the addition of the labeled probe. Reactions were incubated for an additional 10 min before protein·DNA complexes were resolved on 6% (w/v) acrylamide Tris borate-EDTA gels. Gels were dried and subjected to phosphorimaging analysis.

RESULTS

Loz1 Function Is Regulated by Zinc at a Post-translational Level—In previous studies, we found that Loz1 represses its own expression in zinc-replete cells (6). Because the levels of Loz1 within a cell could potentially influence target gene regulation, we initially investigated how autoregulation of *loz1* levels contributed to the overall zinc-dependent regulation of Loz1 target genes. To determine the extent to which autoregulation affected gene expression, constructs were generated in which a Loz1-GFP fusion protein was expressed from its own promoter (*pL-loz1GFP*) or the *zhf1* promoter (*pZ-loz1GFP*). The *zhf1* promoter was used because previous studies suggested that *zhf1* expression is not regulated by zinc (12, 14, 15). These results were also confirmed by measuring β-galactosidase activity in wild-type cells expressing a *zhf1-lacZ* reporter gene and control zinc-regulated *adh4-lacZ* and *loz1-lacZ* reporters (Fig. 1*A*). Following growth in ZL-EMM with or without a zinc supplement, no major changes in β-galactosidase activity were observed in wild-type cells expressing the *zhf1-lacZ* reporter gene, consistent with zinc not affecting expression from the *zhf1* promoter. To determine the extent to which *loz1* expression level influences target gene expression, *pL-loz1GFP*, *pZ-loz1GFP*, or the empty vector was introduced into *loz1*Δ cells, and zinc-dependent changes in gene expression were examined by RNA blot analysis (Fig. 1*B*). Expression of *pL-loz1GFP* or *pZ-loz1GFP* led to the strong repression of *adh4* and *zrt1* expression in zinc-replete cells. Interestingly, the introduction of *pL-loz1GFP* or *pZ-loz1GFP* resulted in slightly lower levels of *adh4* expression under zinc-limiting conditions relative to the *adh4* transcript levels observed in *loz1*Δ. How-

[2] The abbreviations used are: ZL-EMM, zinc-limited Edinburgh minimal medium; nt, nucleotide(s).

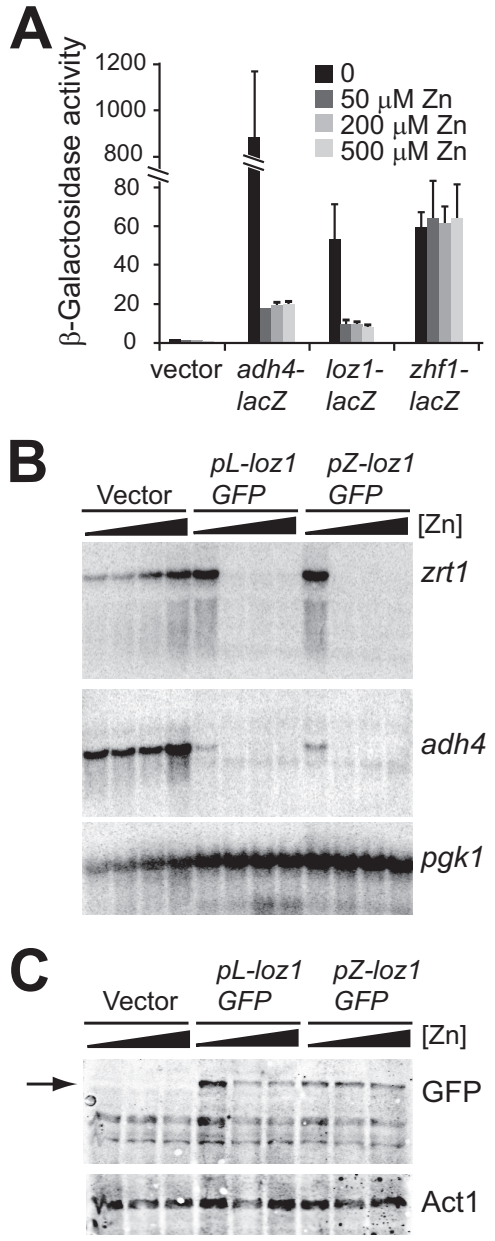

FIGURE 1. **Loz1 is regulated at a post-translational level by zinc.** *A*, wild-type cells containing the empty vector or the integrated *adh4-lacZ*, *loz1-lacZ*, or *zhf1-lacZ* reporter constructs were grown in ZL-EMM supplemented with the indicated amount of zinc before β-galactosidase activity was measured. *B*, *loz1*Δ cells containing the empty vector, *pL-loz1GFP*, or *pZ-loz1GFP* were grown in ZL-EMM supplemented with 0, 50, 200, or 500 μM zinc. Total RNA was extracted and subjected to RNA blot analysis. Blots were probed for *zrt1*, *adh4*, and the loading control *pgk1* (phosphoglycerate kinase 1). *C*, immunoblot analysis of crude protein extracts prepared from *loz1*Δ cells containing the indicated plasmids. Cells were grown in ZL-EMM supplemented with 0, 50, or 200 μM zinc. Immunoblots were probed with antibodies raised against GFP or the loading control actin (*Act1*). An *arrow* indicates the band that is specific to Loz1-GFP. Shown are the means from three independent experiments, and the *error bars* indicate ± S.D.

ever, when related constructs lacking the GFP tag were expressed in *loz1*Δ cells, *adh4* expression was fully induced under zinc-limiting conditions (data not shown). Thus, the slight increase in repression observed at the *adh4* locus in the presence of *pL-loz1GFP* and *pZ-loz1GFP* is probably a result of the introduction of the C-terminal GFP tag. Despite this slight overcompensation, zinc-dependent changes in gene expression were similar whether *loz1GFP* was expressed from its own promoter or at a constant level from the *zhf1* promoter. These results indicate that although Loz1 regulates its own expression, other post-transcriptional mechanisms must play a major role in the regulation of Loz1 function by zinc.

Zinc could directly or indirectly alter Loz1 function by affecting mRNA stability, protein translation, protein stability, cellular localization, DNA binding activity, and/or a repressor function. To determine whether zinc affected Loz1 protein levels, *loz1*Δ cells expressing *pZ-loz1GFP* were grown in ZL-EMM supplemented with 0–200 μM zinc, and crude protein extracts were prepared for immunoblot analysis (Fig. 1*C*). As controls, crude protein extracts were also prepared from *loz1*Δ cells expressing the empty vector or *pL-loz1GFP*. When immunoblots were probed for GFP, a single band was detected that was specific to cells expressing either of the Loz1GFP fusions. Consistent with *loz1* autoregulating its own expression, an ~2-fold reduction in Loz1GFP levels was observed in zinc-replete cells expressing *pL-loz1GFP*. However, when *loz1GFP* was expressed from the *zhf1* promoter, Loz1GFP accumulated at a constant level. The *zhf1*-driven Loz1GFP was also localized to the nucleus under both zinc-limiting and zinc-replete conditions (data not shown). Thus, when expressed at a constant level, Loz1 protein accumulates in the nucleus under both zinc-limiting and zinc-replete conditions; however, Loz1 only represses target gene expression when zinc levels are high. Together, these results suggest that zinc directly or indirectly regulates Loz1 function at a post-translational level.

Isolation of Mutations That Disrupt Loz1 Function—To understand how Loz1 is regulated by zinc, we used a genetic approach to isolate amino acid residues that were critical for Loz1 function. In our previous study, we isolated an *adh1*Δ strain containing a spontaneous partial loss of function mutation in *loz1* (designated *loz1-1*) (6). Under nutrient-rich conditions, the *loz1-1* allele conferred a growth advantage to *adh1*Δ cells. It also resulted in *adh1*Δ cells surviving in the presence of the respiration inhibitor antimycin A. Based on these observations, we screened for further antimycin A-resistant *adh1*Δ colonies. We hypothesized that these colonies might also contain spontaneous mutations that directly or indirectly affected Loz1 function.

To identify antimycin A-resistant *adh1*Δ strains, 1×10^7 *adh1*Δ cells were plated onto YES medium. Cells were grown for 3 days at 31 °C and then were transferred to YES plates containing antimycin A. After 2–5 days of growth, ~5–8 colonies were detected per 1×10^7 cells. Similar results were obtained when *adh1*Δ cells were transferred onto plates supplemented with antimycin A and 500 μM zinc or with antimycin A and a 50 μM concentration of the zinc ion chelator EDTA (Fig. 2*A*) (data not shown). During these studies, we also noted that when *adh1*Δ cells are plated onto YES medium, a few colonies grew faster than others. When one of these faster growing colonies was isolated and transferred to antimycin A plates, it was also able to survive.

To determine if zinc homeostasis was impaired in any of the antimycin A-resistant *adh1*Δ cells, *adh4* transcript levels were examined by RNA blot analysis in 36 independently isolated colonies. In *adh1*Δ cells grown in nutrient-rich YES medium,

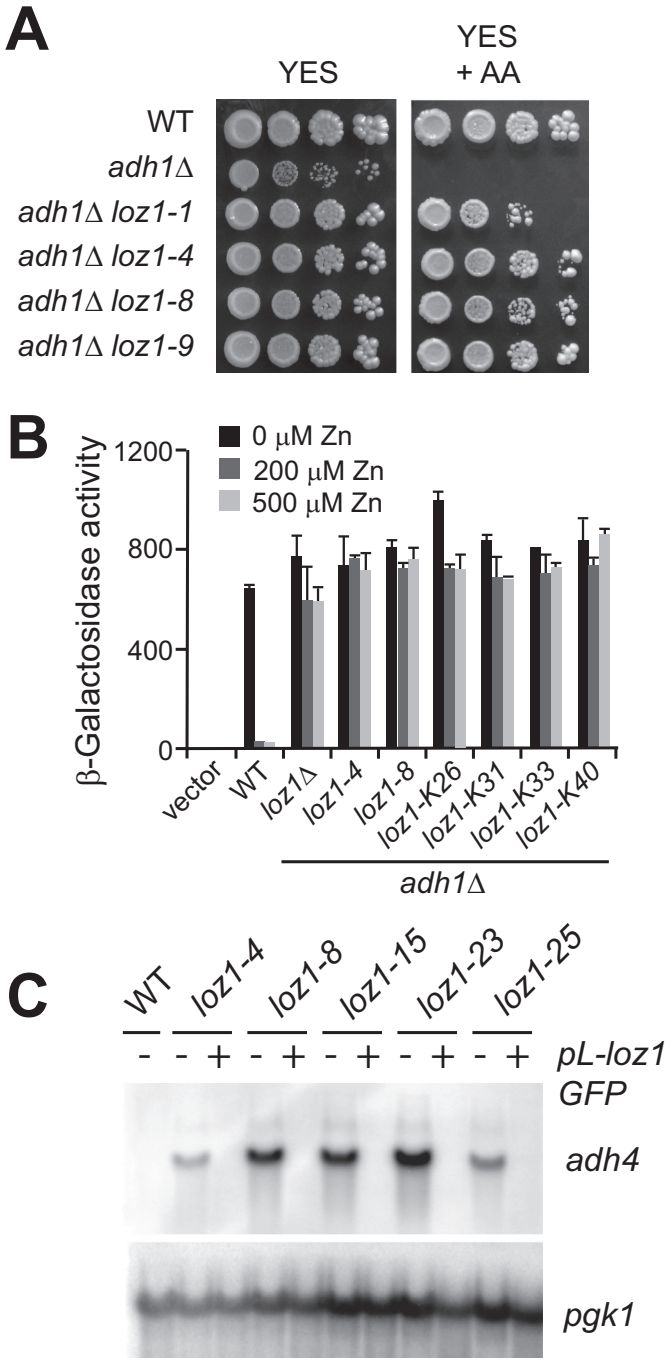

FIGURE 2. **Abnormal zinc homeostasis in antimycin A-resistant *adh1Δ* cells.** *A*, wild-type, *adh1Δ*, *adh1Δ loz1-1*, *adh1Δ loz1-4*, *adh1Δ loz1-8*, and *adh1Δ loz1-9* cells were grown overnight in YES medium before cells were spotted in 10-fold serial dilutions onto YES medium without or with (+*AA*) a 10 μg/μl antimycin A supplement. Plates were incubated for 3 days at 31 °C before photography. *B*, wild-type cells expressing the empty vector (*Vector*) or *adh4-lacZ* reporter (*WT*) or *adh1Δ* cells containing the indicated *loz1* allele and the *adh4-lacZ* reporter were grown in ZL-EMM with or without a 200 or 500 μM zinc supplement. Cells were harvested, and β-galactosidase activity was measured using standard procedures. *C*, wild-type cells or haploid strains containing the indicated *loz1* alleles or these strains with the plasmid *pL-loz1GFP* were grown overnight in YES medium. Total RNA was extracted and subjected to RNA blot analysis. *Error bars*, S.E.

adh4 gene expression is repressed. However, when the 36 antimycin A-resistant strains were grown under similar conditions, *adh4* transcripts accumulated to high levels, suggesting that each of these isolates contained a mutation(s) that resulted in *adh4* expression under nutrient-rich conditions (data not shown). Because Loz1 is required for the transcriptional repression of *adh4* in zinc-replete cells, we also tested whether the increased *adh4* expression observed in the antimycin A resistant strains was typically dependent upon the *adh4* promoter. For this, an *adh4-lacZ* reporter gene was introduced into six of the antimycin A-resistant colonies, and β-galactosidase activity was measured following growth in ZL-EMM with or without a 200 or 500 μM zinc supplement (Fig. 2*C*). As controls, *adh4-lacZ* reporter activity was also examined in wild-type and *adh1Δ loz1Δ* cells grown under similar conditions. We were unable to measure *adh4-lacZ* activity in *adh1Δ* cells because this strain has a severe growth defect in ZL-EMM with or without zinc (data not shown). Growth in ZL-EMM with a 200 or 500 μM zinc supplement led to an ~30-fold decrease in β-galactosidase in wild-type cells. Although a small zinc-dependent change in β-galactosidase was observed in the presence of the *loz1-K26* allele, for the most part, there was no major change in β-galactosidase activity in any of the antimycin A-resistant colonies. These results are consistent with the mutation(s) in these strains impairing gene repression. Further genetic backcrosses to wild type cells revealed that the impaired zinc-responsive gene expression observed in the antimycin A-resistant colonies was a result of a single site mutation in the genome (data not shown). In addition, introduction of *pL-loz1GFP* into five backcrossed isolates fully restored zinc-dependent repression of *adh4* (Fig. 2*C*). Because these results were consistent with at least five of the strains containing a mutation within the *loz1* ORF, DNA was isolated from each antimycin A-resistant *adh1Δ* colony, and the *loz1* ORF was sequenced. All 36 colonies contained a mutation in the *loz1* ORF (Tables 1 and 2). Of these, 18 were nucleotide insertions, deletions, or duplications resulting in a frameshift; 9 contained point mutations leading to the introduction of a premature stop codon; and 9 contained point mutations that resulted in a change to an amino acid residue within the double zinc finger domain (Fig. 3).

TABLE 1
***loz1* alleles containing a point mutation**

Allele	DNA mutation	Amino acid substitution	Isolation condition
loz1-1	C1528G	R510G	Spontaneous mutation identified via transformation[a]
loz1-4	T1430C	F477S	10 μg/ml antimycin A
loz1-5	G865T	G289Stop	10 μg/ml antimycin A
loz1-8	G1418A	C473Y	10 μg/ml antimycin A
loz1-15	T1404G	Y468Stop	10 μg/ml antimycin A
loz1-K5	T1408C	C470R	Spontaneous mutation in YES medium
loz1-23	T1417G	C473G	5 μg/ml antimycin A
loz1-25	C1466T	S489F	5 μg/ml antimycin A
loz1-K25	G298T	E100Stop	10 μg/ml antimycin A
loz1-K26	C1558G	H520D	10 μg/ml antimycin A
loz1-K27	C931T	Q311Stop	10 μg/ml antimycin A
loz1-K29	C952T	Q318Stop	10 μg/ml antimycin A + 500 μM zinc
loz1-K30	C874T	Q292Stop	10 μg/ml antimycin A + 500 μM zinc
loz1-K31	G1436T	R479M	10 μg/ml antimycin A
loz1-K33	G1539A	M513I	10 μg/ml antimycin A + 500 μM zinc
loz1-K34	C889T	Q297Stop	10 μg/ml antimycin A + 500 μM zinc
loz1-K35	C1327T	R443Stop	10 μg/ml antimycin A + 500 μM zinc
loz1-K36	T666G	Y222Stop	10 μg/ml antimycin A + 500 μM zinc
loz1-K40	C1456T	H486Y	10 μg/ml antimycin A

[a] The original *loz1-1* allele reported in Ref. 6.

TABLE 2
Other *loz1* alleles

Type of mutation	Allele	Mutation	Summary	Isolation condition
Insertion	*loz1-9*	nt 428TC	2-bp insertion	10 μg/ml antimycin A
	loz1-12	nt 486T	1-bp insertion	10 μg/ml antimycin A
	loz1-K18	nt 1404A	1-bp insertion	10 μg/ml antimycin A
	loz1-K20	nt 529A	1-bp insertion	10 μg/ml antimycin A
	loz1-K22	nt 796A	1-bp insertion	10 μg/ml antimycin A
	loz1-K23	nt 1283T	1-bp insertion	10 μg/ml antimycin A
	loz1-K24	nt 636T	1-bp insertion	10 μg/ml antimycin A
	loz1-K32	nt 349C	1-bp insertion	10 μg/ml antimycin A + 500 μM zinc
	loz1-K37	nt 486T	1-bp insertion	10 μg/ml antimycin A + 500 μM zinc
	loz1-K38	nt 486T	1-bp insertion	10 μg/ml antimycin A
Deletion	*loz1-K13*	nt 1484	1-bp deletion	10 μg/ml antimycin A
	loz1-K17	nt 515	1-bp deletion	10 μg/ml antimycin A
	loz1-K19	nt 1193	1-bp deletion	10 μg/ml antimycin A
	loz1-K28	nt 609	1-bp deletion	10 μg/ml antimycin A + 50 μM EDTA
	loz1-K39	nt 1341–1342	2-bp deletion	10 μg/ml antimycin A
Duplication	*loz1-K14*	Duplication of nt 681–709	29-bp duplication	10 μg/ml antimycin A
	loz1-K16	Duplication of nt 681–709	29-bp duplication	10 μg/ml antimycin A
	loz1-K21	Duplication of nt 870–880	11-bp duplication	10 μg/ml antimycin A

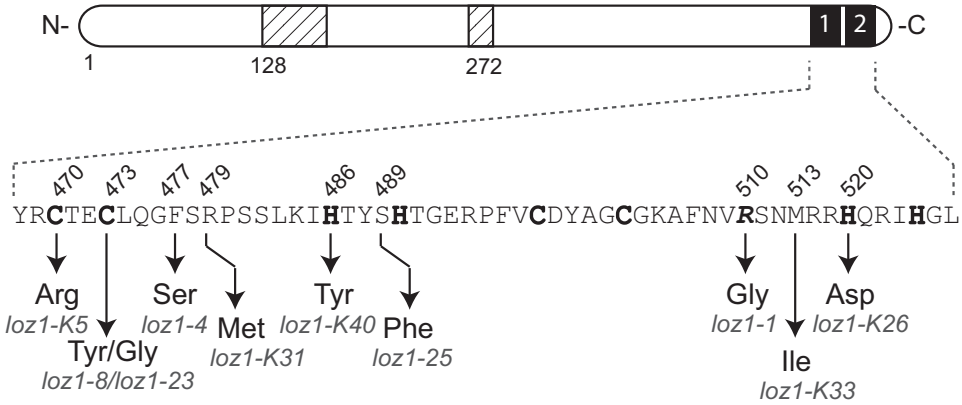

FIGURE 3. **A schematic diagram illustrating amino acid substitutions present in different Loz1 alleles.** Cysteine and histidine residues predicted to coordinate zinc are shown in *boldface type*, and the original *loz1-1* mutation is shown in *boldface* and *italic type*.

Mutations in the Loz1 Zinc Finger Domain Affect DNA Binding Function—Previously, we had found that a C470G substitution in zinc finger 1 prevented Loz1 from binding to a GNNGATC cis-acting element *in vitro*, suggesting that the Loz1 double zinc finger domain was necessary for DNA binding (6). In further support of the Loz1 zinc finger domains having a direct role in DNA binding, all of the newly isolated alleles primarily targeted amino acid residues predicted to be involved in zinc ion coordination (C470R, C473Y, C473G, H486Y, and H520D), the formation of the zinc finger hydrophobic core (F477S), or making site-specific interactions with DNA (R479M). Two exceptions were the *loz1-25* allele (S489F) and the *loz1-K33* allele (M513I), each containing amino acid substitutions that do not target amino acids known to be critical for zinc finger formation or interactions with DNA. To determine whether these amino acid substitutions affected DNA binding function, recombinant proteins containing amino acids 427–522 of Loz1 fused to a Trx-His tag (TH-Loz1) with or without S489F or M513I substitutions were purified from *E. coli* using nickel-nitrilotriacetic acid affinity chromatography (Fig. 4*B*). The tag alone and TH-loz1 C470G were also purified as controls. The ability of each protein to bind to a radiolabeled double-stranded oligonucleotide containing the predicted Loz1 binding site (GNNGATC) was then examined using electrophoretic mobility shift assays (EMSAs) (Fig. 4, *A* and *C*). As expected, a DNA·protein complex was detected when the TH-Loz1 fusion was incubated with the wild-type oligonucleotide. Formation of this complex was inhibited by incubation with excess levels of a non-radiolabeled wild-type oligonucleotide but not a non-radiolabeled mutant oligonucleotide. When similar levels of TH-Loz1 recombinant proteins containing the C470G, S489F, or M513I substitutions were incubated with the wild-type oligonucleotide, no complex was detected in the presence of the C470G and S489F substitutions. A weak DNA·protein complex was observed in the presence of the M513I substitution, suggesting that this mutation significantly impaired but did not entirely disrupt DNA binding activity. Thus, consistent with the zinc finger domain being critical for Loz1 function, all of the amino acid substitutions identified in the screen impaired or disrupted DNA binding or targeted amino acids predicted to be critical for zinc finger formation.

A Minimal Region Required for Zinc Responsiveness Maps to the DNA Binding Domain—Because the genetic screen did not identify any amino acid substitutions outside of the DNA binding domain and Loz1 contains a number of other conserved regions, a series of Loz1-GFP truncations were generated to determine which region(s) of Loz1 was required for zinc-responsive regulation (Fig. 5*A*). Each truncation was expressed from its native promoter in *loz1*Δ cells, and immunoblot analysis and fluorescence microscopy were performed to confirm that the fusion protein was produced and was localized to the nucleus (Fig. 5*B*) (data not shown). For unknown reasons, the

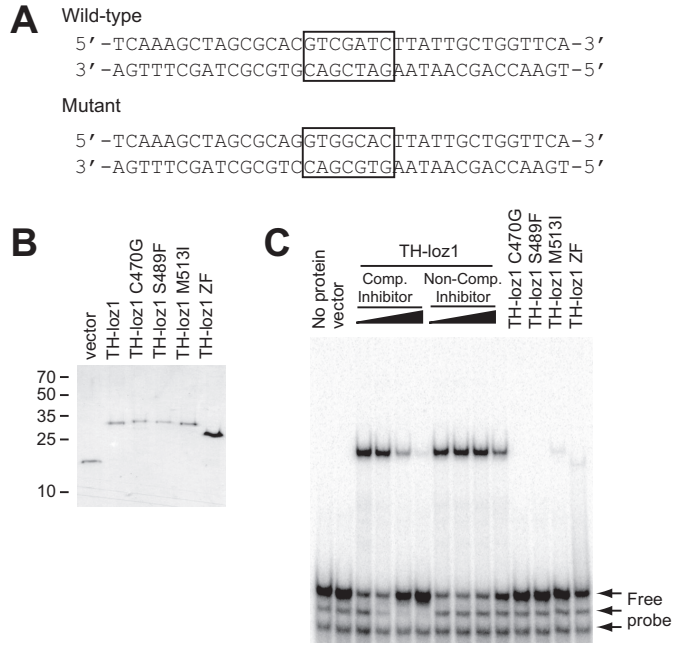

FIGURE 4. **S489F and M513I substitutions affect Loz1 DNA binding *in vitro*.** *A*, DNA sequence of the wild-type and mutated oligonucleotide probes. The *loz1* binding site is *boxed*. *B*, SDS-PAGE analysis of the purified Trx-histidine tag (*vector*) and recombinant proteins TH-loz1, TH-loz1 C470G, TH-loz1 S489F, TH-loz1 M513I, or TH-loz1 ZF. Proteins were visualized by staining with Coomassie Blue. The sizes of the molecular mass markers are shown in kDa on the *left*. *C*, representative EMSA using 40 μmol of ^{32}P-labeled double-stranded oligonucleotide and 200 ng of the indicated purified recombinant protein. For competition studies, 0, 50×, 200×, or 500× of the WT unlabeled oligonucleotide (*Comp Inhibitor*), or mutant oligonucleotide (*Non-Comp Inhibitor*) was added to the reactions.

smaller truncations (Fig. 5*B*, *constructs #3–5*) accumulated to higher levels than those of the full-length Loz1GFP under zinc-limited conditions (*construct #1*). With the exception of construct 2, all fusion proteins were found within the nucleus. A heterologous nuclear localization signal from the SV40 large T antigen was therefore added to construct 2 to ensure its nuclear localization. Although the nuclear localization signal containing construct was able to partially rescue *loz1*Δ phenotypes (see below), we were not able to detect it by immunoblot analysis (Fig. 5*B*, *construct #2*), suggesting that it may accumulate to lower levels or be less stable than the other truncations.

To determine whether any of the truncations were functional, the ability of each fusion protein to repress target gene expression was examined by RNA blot analysis (Fig. 5*C*). Although the smallest truncation (*construct #5*) was not able to confer repression, introduction of constructs 2–4 resulted in the strong repression of *adh4* expression in zinc-replete cells and a more modest repression of *zrt1* expression in zinc-replete cells. To determine the extent to which the smaller truncation affected *zrt1* expression, a *zrt1-lacZ* reporter was co-expressed with the full-length Loz1GFP and the Loz1GFP truncation 3–5. When these cells were grown in ZL-EMM with or without a zinc supplement, only the full-length Loz1GFP resulted in the strong repression of β-galactosidase activity in zinc-replete cells (Fig. 5*D*). However, minor zinc-dependent changes in β-galactosidase activity were observed in cells expressing constructs 3 and 4, consistent with the mild zinc-dependent regulation observed in the RNA blot analysis.

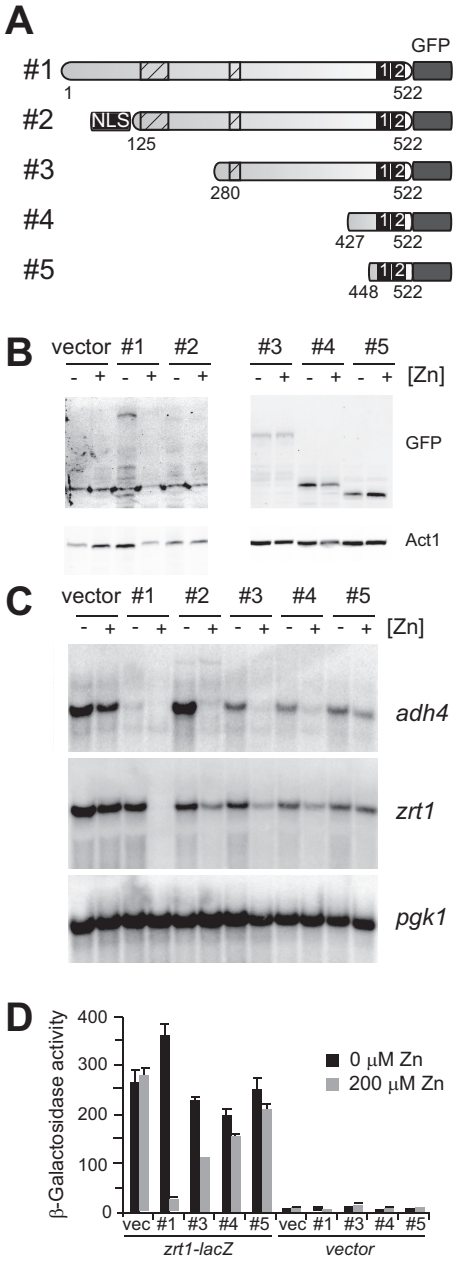

FIGURE 5. **Mapping a minimal zinc-responsive domain.** *A*, schematic diagram of *pL-Loz1GFP* truncations. Shown are a C-terminal GFP tag, the double zinc finger domain (*numbered black boxes*), regions conserved in Loz1 homologs from other *Schizosaccharomyces* sp. (*striped boxes*), and introduced nuclear localization signal. The amino acids included in each truncation are also indicated. *B* and *C*, *loz1*Δ cells expressing the constructs shown in *A* were grown in ZL-EMM with or without a 200 μM zinc supplement. Cells were harvested, and crude protein extracts were prepared for immunoblot analysis (*B*), or total RNA was extracted for RNA blot analysis (*C*). *D*, *loz1*Δ cells co-expressing the constructs shown in *A* and reporter gene pTN-*zrt1-lacZ* (*zrt1-lacZ*) or empty vector pTN-*lacZ* (*vector*) were grown as described in *B* before β-galactosidase activity was measured by standard procedures. *Error bars*, S.E.

Because the above truncations were expressed from the *loz1* promoter and were therefore subject to negative autoregulation, depending on their activity, similar experiments were performed using *zhf1*-driven truncations (Fig. 6, *A–C*). This analysis indicated that when expressed at a constant level, construct 4 was able to mediate partial zinc-responsive regulation of *adh4* and *zrt1* gene expression. Thus, when expressed at a constant

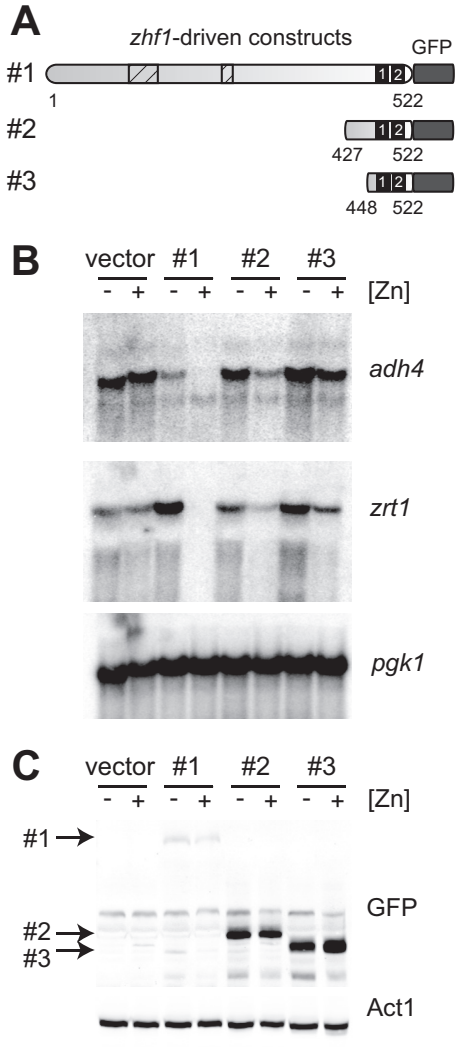

FIGURE 6. **A minimal domain containing the last 96 amino acids of Loz1 is sufficient to confer zinc-dependent repression of *adh4*.** *A*, schematic diagram of *pZ-loz1GFP* truncations. See the legend to Fig. 5*A* for details. *B* and *C*, *loz1*Δ cells expressing the constructs shown in *A* were grown in ZL-EMM with or without a 200 μM zinc supplement. Cells were harvested, and total RNA was extracted and subjected to RNA blot analysis (*B*), or crude protein extracts prepared for immunoblot analysis (*C*).

level, the minimal region of Loz1 containing the last 96 amino acids is sufficient to confer at least some zinc-dependent regulation. However, full repression of target gene expression requires additional N-terminal sequences.

An N-terminal Zinc Finger Accessory Domain Enhances DNA Binding—The 96-amino acid region that can confer partial zinc-dependent repression contains both C_2H_2 zinc fingers and an additional 40 amino acid residues adjacent to zinc finger 1. In other transcription factors that bind to DNA via zinc finger domains, an accessory domain that is adjacent to the first or last zinc finger can be critical for DNA recognition and/or high affinity binding (16–19). Because the minimal zinc-responsive domain included a 40-amino acid region that was not part of the double zinc finger domain, we tested whether additional N-terminal sequences that were adjacent to zinc finger 1 were required for DNA binding *in vitro*. For this, a recombinant protein containing amino acids 455–522 of Loz1 fused to a Trx-His tag (TH-Loz1 ZF) was purified from *E. coli*. The ability of this protein to bind to a GNNGATC element was then examined by EMSA (Fig. 4*C*, *TH-Loz1 ZF*). In EMSAs, a DNA·protein complex was detected in the presence of TH-Loz1 ZF and the double-stranded oligonucleotide containing the Loz1 binding site. However, the levels of this complex were significantly lower than those of the TH-Loz1·DNA complex. Similar results were also obtained using a recombinant protein that contained amino acids 448–552 of Loz1 (data not shown). Thus, Loz1 amino acids 427–552 are sufficient for high affinity DNA binding *in vitro* and can confer partial zinc-dependent repression *in vivo*. However, in the absence of the adjacent 40-amino acid "accessory domain," DNA binding affinity is severely reduced *in vitro*, and zinc-dependent regulation is lost *in vivo*. Together, these results suggest that the N-terminal accessory domain is necessary for high affinity DNA binding.

The partial zinc-dependent repression observed in cells expressing amino acids 427–522 of Loz1 (Fig. 5*A*, *construct #4*) could result from N-terminal sequences being necessary for gene repression (*e.g.* these sequences might be important for the recruitment of co-repressors or chromatin-remodeling proteins), or they might result from the N-terminal region of Loz1 being necessary for full zinc responsiveness. To determine whether the Loz1 C-terminal region was sufficient for zinc-dependent repression, we took advantage of the differences in sequence conservation between Loz1 and MtfA (master transcription factor A) from *A. nidulans*. MtfA is a transcription factor that plays a primary role in regulating secondary metabolism and morphogenesis (20). MtfA contains a double zinc finger domain that shares significant sequence similarities to the Loz1 zinc fingers (Fig. 7*A*). However, outside of this domain (including the 40-amino acid accessory domain), there is no sequence conservation. Because the sequence conservation between Loz1 and MtfA was limited to the zinc fingers, we tested whether expression of *mtfA-GFP* from the *zhf1* promoter would complement *loz1*Δ (Fig. 7, *B* and *C*). Despite the strong homology in the zinc finger domain, only a small zinc-dependent decrease in *adh4* or *zrt1* gene expression was observed in the *loz1*Δ cells expressing *mtfA-GFP*. However, when chimeras of MtfA containing the Loz1 zinc finger inclusive of the 40-amino acid accessory domain (construct 3) or in which the MtfA "accessory domain" was replaced with the Loz1 accessory domain (construct 4) were expressed in *loz1*Δ cells, both *zrt1* and *adh4* gene expression were robustly regulated by zinc. The derepression of *adh4* and *zrt1* expression in zinc-limited cells was not a result of altered protein stability or cellular localization, because all fusion proteins were stable (Fig. 7*D*) and nucleus-localized (data not shown). Together, these results are consistent with the Loz1 zinc finger domains and adjacent accessory domain having a dual role in DNA binding and zinc-dependent repression *in vivo*.

DISCUSSION

In our previous work, we isolated a partial loss of function mutation in *loz1*, which conferred a growth advantage to *adh1*Δ cells and enabled their survival on plates containing the respiration inhibitor antimycin A. In this study, we isolated 36 additional *adh1*Δ colonies that were able to grow in the presence of antimycin A. Further characterization of these colonies

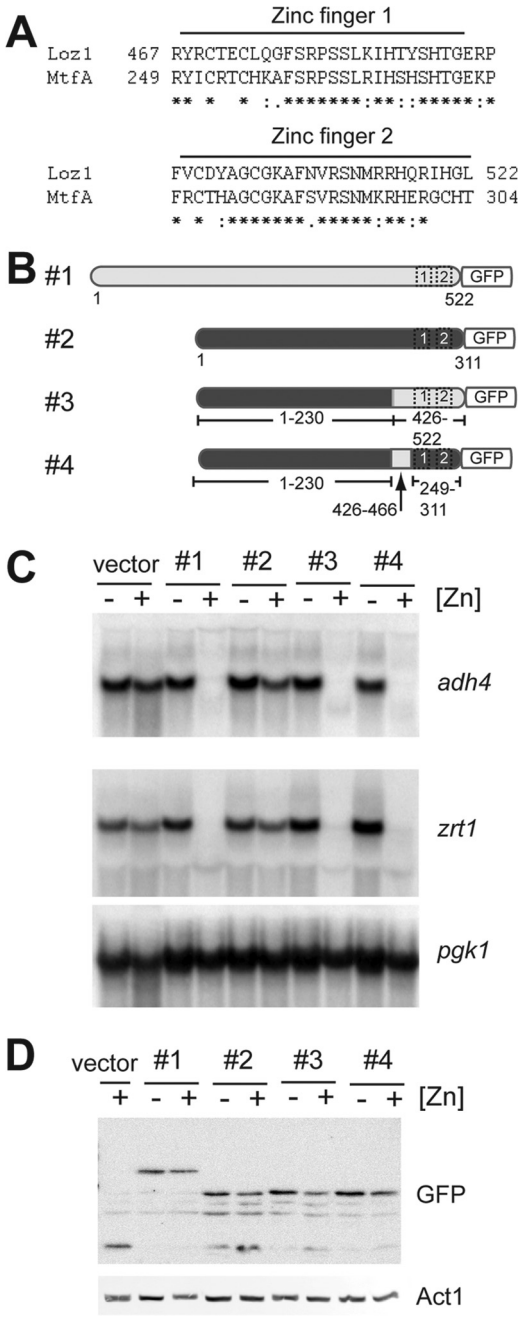

FIGURE 7. ***pZ-MtfAGFP* is regulated by zinc in *loz1*Δ cells.** *A*, an alignment of the double zinc finger domains from Loz1 and MtfA. *B*, schematic diagram of Loz1/MtfA chimeras. Loz1 sequences are shown in *light gray*, and MtfA sequences are shown in *dark gray*. *Numbers* represent amino acid number in the respective proteins. *C* and *D*, *loz1*Δ cells expressing the constructs shown in *B* were grown in ZL-EMM with or without a 200 μM zinc supplement. Cells were harvested, and total RNA was extracted for RNA blot analysis (*C*), or crude protein extracts were prepared for immunoblot analysis (*D*).

revealed that they all contained a mutation leading to the loss of Loz1 function. These results strongly support our previous observations that disruption of Loz1 function is advantageous to the growth of *adh1*Δ cells and highlight a novel approach to identify mutations that impair Loz1 function.

All of the newly identified *loz1* alleles resulted in the premature truncation of the Loz1 protein or an amino acid substitution within the Loz1 double zinc finger domain. Of the alleles leading to an amino acid substitution, most altered residues involved in zinc ion coordination or the formation of the hydrophobic core. One exception was the *loz1-K31* allele, which led to an R479M substitution at position −1 of the α helix in zinc finger 1. In C_2H_2-type zinc finger domains, side chains from amino acid residues at positions −1, 2, 3, and 6 of the α helix frequently make hydrogen bond contacts with DNA (2). Thus, the isolation of an allele encoding an R479M substitution is consistent with the Loz1 zinc finger domains being necessary for site-specific DNA binding. An unexpected mutation was S489F in zinc finger 1. This residue is not predicted to coordinate zinc or interact with DNA. However, it is adjacent to a histidine residue that is critical for zinc ion coordination. Thus, the introduction of a bulky, hydrophobic residue at this position could interfere with zinc ion binding. Another unanticipated mutation was an M513I substitution at position 4 of the α helix in zinc finger 2. This mutation disrupted Loz1 function *in vivo* (Fig. 2*B*) and impaired DNA binding function *in vitro* (Fig. 4*C*). Although the side chains of Met-513 are not predicted to make contacts with DNA, methionine residues have a higher helix-forming propensity than isoleucines (21). Thus, this substitution may have destabilized the α helix in zinc finger 2, preventing Loz1 from effectively interacting with DNA. Because this residue is highly conserved in other Loz1 homologs, it is also possible that this residue has another yet to be identified function. Thus, the *adh1*Δ screen revealed a number of novel amino acid substitutions that interfere with Loz1 activity and further emphasized that the double zinc finger domain is critical for Loz1 function.

To gain additional insight into how Loz1 senses zinc, N-terminal truncations and chimeric proteins were generated to map a minimal domain necessary for zinc responsiveness. These studies revealed that 1) an accessory domain that is adjacent to zinc finger 1 is necessary for high affinity DNA binding and zinc-dependent repression, 2) a minimal region containing the last 96 amino acids of Loz1 is sufficient to mediate partial zinc-dependent regulation of *adh4* and *zrt1*, and 3) a chimera of MtfA and the Loz1 accessory domain is able to complement *loz1*Δ. Because MtfA contains no regions of homology with Loz1 outside of the double zinc finger domain, this latter result is consistent with the double zinc finger domain and adjacent accessory domain being necessary for zinc-dependent repression. Based on the above observations, we propose that the last 96 amino acids of Loz1 play a dual role in DNA binding and in zinc sensing. Because truncations or mutations (*e.g.* M513I) that reduce DNA binding affinity *in vitro* result in the loss of zinc-dependent repression *in vivo*, our current working model is that changes in intracellular zinc levels affect Loz1 DNA binding activity and that high affinity DNA binding is critical for repression *in vivo*. In Zap1 and MTF-1, zinc finger domains can have regulatory functions and act as intracellular sensors of zinc (22). Because the two zinc finger domains in Loz1 are necessary for zinc-dependent repression, they may play a central role in zinc sensing. For example, if one or both of the zinc finger domains were only occupied by zinc when it is in excess, this would result in Loz1 binding to DNA and acting as a repressor in zinc-replete cells. Alternately, the accessory domain contains four histidine residues. Because these residues could potentially coordinate zinc, this domain might also bind

zinc in a regulatory capacity. For example, zinc binding to the accessory domain might result in a protein conformation that is able to bind to DNA with a high affinity. To test whether DNA binding is regulated by zinc *in vivo*, we have used ChIP to investigate whether Loz1 is bound to target gene promoters in zinc-replete cells. However, in these studies, we find that Loz1 is a relatively sticky protein in that it readily associates with chromatin purified from target and non-target gene promoters (data not shown). Our future studies will therefore use alternative strategies to address whether zinc affects DNA binding function *in vivo*.

Another aspect of Loz1 function our studies highlighted is that a minimal domain containing the last 96 amino acids of Loz1 is able to mediate strong repression of the *adh4* promoter but only weak repression of *zrt1* expression. In ongoing studies, we have found that the smaller truncations are also only able to modestly regulate the *loz1* promoter (data not shown), suggesting that that the N-terminal region of Loz1 is necessary for robust regulation of *zrt1* and *loz1*. Studies of the *adh4* promoter have found that it is complex, in that it contains multiple binding sites for other transcriptional activators and repressors (6, 15). In addition, multiple Loz1 DNA response elements are located downstream of the transcriptional start site. Differences in the affinity and positions of DNA response elements can affect the regulatory action of a transcription factor. For example, Zap1 binds to two high affinity zinc-responsive elements in the *ZRT2* promoter and activates gene expression in zinc-limited cells (23). However, when zinc is extremely limited, Zap1 binds to a third low affinity zinc-responsive element that is located downstream of the *ZRT2* transcriptional start site. Binding at this site restricts the progression of RNA polymerase II, inhibiting gene expression (23, 24). Because the *adh4* promoter contains multiple Loz1 binding sites that are located downstream of the transcriptional start site, binding of the smaller Loz1 proteins to these downstream sites may allow them to be effective repressors of *adh4* expression in zinc-replete cells.

So far, a number of different domains have been implicated in zinc sensing in eukaryotes. These include C_2H_2-type zinc fingers in Zap1, MTF-1, and now Loz1 and a cysteine/histidine-rich domain in AD1 from Zap1 (4, 22). Deletion of a cysteine-rich metallothionein-like domain from the copper-responsive regulator Crr1 in *Chlamydomonas* also leads to increased expression of genes required for zinc uptake and the hyperaccumulation of zinc, suggesting that this may be another type of zinc-sensing domain (25, 26). Despite a growing number of eukaryotic zinc-responsive factors, it is largely unknown whether these factors sense changes in zinc levels through zinc ion binding or if they are indirectly regulated by zinc (*e.g.* through a zinc-dependent post-translational modification). Thus, future studies with Loz1 will help to provide important knowledge concerning zinc homeostasis and zinc sensing.

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