Metalloregulation of yeast membrane steroid receptor homologs

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Zinc is an essential micronutrient that can also be toxic. An intricate mechanism exists in yeast that maintains cellular zinc within an optimal range. The centerpiece of this mechanism is the Zap1p protein, a transcription factor that senses zinc deficiency and responds by up-regulating genes involved in zinc metabolism. A microarray screen for novel Zap1p target genes suggested a role in zinc homeostasis for four homologous yeast genes. The expression of two of these genes, *YDR492w* and *YOL002c*, suggested direct regulation by Zap1p, whereas the expression of *YOL002c* and a third homologous gene, *YOL101c*, was induced by high zinc. *YDR492w* and *YOL002c* are confirmed to be direct Zap1p target genes. The induction of *YOL002c* and *YOL101c* by toxic metal ion exposure is shown to be mediated by the Mga2p hypoxia sensor. Furthermore, *YOL101c* is induced by deletion of the Aft1p iron-responsive transcription factor. These three genes, along with a fourth yeast homolog, *YLR023c*, have phenotypic effects on zinc tolerance and Zap1p activity. Because of their metalloregulation, zinc-related phenotypes, and highly conserved motifs containing potential metal-binding residues, this family has been renamed the *IZH* gene family (Implicated in Zinc Homeostasis). Furthermore, these genes are regulated by exogenous fatty acids, suggesting a dual role in lipid metabolism. The *IZH* genes encode membrane proteins that belong to a ubiquitous protein family that includes hemolysin III and vertebrate membrane steroid receptors. We propose that the *IZH* genes affect zinc homeostasis either directly or indirectly by altering sterol metabolism.

Zinc is an essential micronutrient and many of the proteins involved in maintaining zinc homeostasis are highly conserved throughout evolution. For example, the ZIP and CDF families of zinc transporter proteins can be found in organisms ranging from bacteria to humans (1). The coordinate induction of *ZIP* (*ZRT1*, *ZRT2*, and *ZRT3*) and *CDF* (*ZRC1*) gene transcription in zinc-limited *Saccharomyces cerevisiae* is administered by the zinc-sensing transcription factor, Zap1p (2). Zap1p binds to an 11-bp site in its target promoters called a zinc responsive element (ZRE) with the consensus sequence ACCTTNAAGGT. In a previous study, DNA microarrays were used to define the Zap1p regulon as well as to identify genes induced by zinc excess (2). Herein, we describe the initial characterization of a previously unrecognized family of four yeast genes discovered in the course of that study. Two of these genes are induced by zinc-limitation in a Zap1p-dependent manner and two respond to excess metals via the hypoxia sensor Mga2p. All four genes encode membrane proteins with mutant phenotypic effects on zinc tolerance and homeostasis. Therefore, we have designated these genes *IZH1-4* (Implicated in Zinc Homeostasis).

Two observations suggested other roles for the Izh proteins unrelated to zinc metabolism. First, some *IZH* genes are transcriptionally regulated by fatty acids (3). Second, the proteins they encode are homologs of vertebrate membrane steroid receptors (mSRs) that mediate rapid, posttranslational (also referred to as nongenomic) effects of steroids (4).

This report demonstrates metalloregulation of three of the four *IZH* genes as well as zinc-dependent mutant phenotypes for all four genes. Increased *IZH* gene dosage is also shown to affect zinc homeostasis. Lipid- and oxygen-dependent expression of *IZH2* and *IZH4* is confirmed, and analysis of related genes link *IZH* gene function with sterol metabolism. We propose that the Izh proteins affect zinc metabolism either by altering membrane sterol content or by directly altering cellular zinc levels.

Materials and Methods

Yeast Strains. The strains used in this study are described in Table 2, which is published as supporting information on the PNAS web site. kanMX4::*IZH* deletion strains were either purchased from EUROSCARF or generated by PCR-based gene disruption using short flanking homology (5). Multiple mutants were generated from a heterozygous quadruple knockout strain engineered by successive rounds of mating and sporulation. The kanMX4 markers in the *izh2*, *izh3*, and *izh4* strains were replaced with the hphMX4 (hygromycin), natMX4 (nourseothricin), and ura3MX4 (URA^+) cassettes, respectively (6, 7). Nourseothricin was obtained from WERNER BioAgents (www.webioage.com).

DNA Manipulations. A complete list of primers is given in Data Set 1, which is published as supporting information on the PNAS web site. All PCR products were cloned into their respective plasmids by gap repair (8). *IZH-lacZ* fusions were generated as described (2). Only the fusion of the second in-frame ATG in the *IZH2* ORF to *lacZ* resulted in a functional promoter construct (2, 3). $IZH1_{mutZRE}$-*lacZ* and $IZH2_{mutZRE}$-*lacZ* constructs in which each position in the ZREs was altered by transversion mutation (mutZRE) were generated by overlap extension PCR (9). *HIS4-lacZ* (10), *CYC1-lacZ* (11), *FET3-lacZ* (gift of A. Dancis, University of Pennsylvania, Philadelphia), and *OLE1-lacZ* (p62::934) (12) reporters were used as controls. pNB404 contains *lacZ* driven by a minimal *CYC1* promoter lacking upstream activating sequences (*CYC1*Δ*UAS-lacZ*) (13). Insertion of putative regulatory elements between the *Xba*I and *Xho*I sites of this plasmid was used to test element function. An *IZH1* ZRE insert was generated by overlap extension PCR. LORE-*lacZ* (pAM6) and ZRE-*lacZ* (pDg2 *URA3*/pDg2 *LEU2*) were made previously (12, 14). The inserts for these constructs contain the *OLE1* low-oxygen response element (LORE, ACTCAACAA) and the *ZRT1* ZRE (ACCCTCAAGGT), respectively.

Analysis of the promoter regions of zinc-inducible genes was performed by using RSA-TOOLS (http://rsat.ulb.ac.be/rsat) (15). The *OLE1* LORE and the *FET3* FeRE (iron response element, TGCACCCA) were used as seed sequences for the program.

Single- and multicopy plasmids were generated by the insertion of PCR-amplified genomic fragments (from ≈1,000 bp

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Abbreviations: FeRE, iron response element; LORE, low-oxygen response element; mSR, membrane steroid receptor; TM, transmembrane domain.

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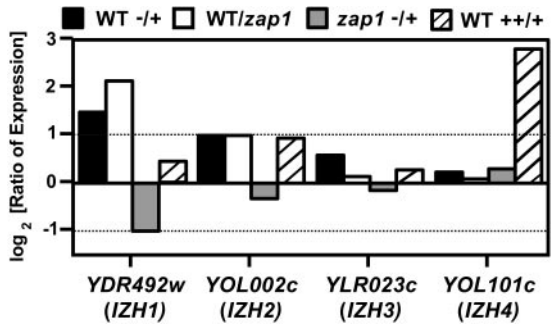

Fig. 1. *IZH* gene expression response to Zn^{2+}. Comparative expression of *IZH* genes across four experimental regimes ($n = 2$ for each). In this and all subsequent figures, −, +, and ++ indicate 50 nM, 10 μM, and 3 mM Zn^{2+} in the growth medium, respectively. Data are presented as $\log_2$ of the ratio of expression. Dotted lines demarcate a 2-fold difference in expression.

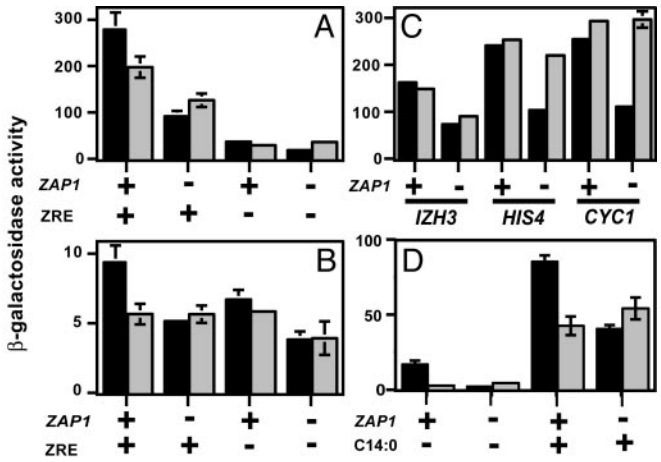

Fig. 2. Zap1p-dependent regulation of *IZH1* and *IZH2*. IZH1-*lacZ* and $IZH1_{mutZRE}$-*lacZ* (*A*) and IZH2-*lacZ* and $IZH2_{mutZRE}$-*lacZ* (*B*) reporter fusion constructs show Zap1p-dependent induction during Zn^{2+}-limitation. (*C*) A similar analysis of the *IZH3-lacZ*, *HIS4-lacZ*, and *CYC1-lacZ* control constructs. (*D*) The *IZH2-lacZ* reporter responds to both Zn^{2+} and exogenous myristate (C14:0). Black bars show reporter activity in − Zn^{2+}, and gray bars show reporter activity in + Zn^{2+}. In this and all other figures showing *lacZ* data, a representative experiment performed in triplicate is shown and the error bars represent ±1 SD.

upstream of ATG to ≈500 bp downstream of STOP) into the centromeric pRS315 and episomal YEp353 vectors. Fragments were inserted into YEp353 between the *Eco*RI and *Bam*HI sites and into pRS315 between the *Sac*I and *Sal*I sites. For *GAL1*-driven overexpression, each ORF was amplified by PCR and inserted between the *Sac*I and *Sal*I sites of the pRS316-*GAL1* plasmid (16). For *IZH2* the *GAL1* promoter was fused to both the first and second in-frame ATG codons to make *GAL1-IZH2.1* and *GAL1-IZH2.2*. *GAL1*-hZip1 and *GAL1-ZRT1*Δ2 express the human hZip1 and mutant yeast Zrt1p zinc transporter proteins, respectively. Overexpression was achieved by using an estradiol-inducible system (17) or by 2% galactose.

Yeast Growth and Assays. Yeast and bacterial transformations were performed by standard methods. Microarrays were previously published (2). It was necessary to culture yeast for 4 days in selectable minimal media before re-inoculation in high-zinc media to ensure consistent results. Protocol for the preparation of chelexed synthetic media (CSD) (2) and low-zinc media (14) are published. Zinc, as $ZnCl_2$, was added back to CSD to a final concentration of 50 nM (deficiency), 10 μM (repletion), and 3 mM (excess) for microarrays and *lacZ* assays. To induce zinc toxicity, 6 mM zinc was added to liquid media and 12 mM zinc was added to agar plates. A 37.5% wt/vol stock of sodium myristate was first dissolved in 50% EtOH/25% Tween-40 and then added to CSD to a final concentration of 0.375% myristate. Doubling time was determined by dividing ln 2 by the slope of a line generated by plotting growth time (log phase only) versus ln OD_{600}. β-galactosidase activity was measured by using published procedures (2) and is expressed in Miller units.

Results

Microarray Analysis. Previously published microarray data showed higher mRNA levels for two genes, *YDR492w* and *YOL002c*, in zinc-deficient vs. zinc-replete wild-type cells and greater expression in a zinc-deficient wild-type strain vs. a zinc-limited *zap1* strain. These genes also showed decreased expression in zinc-deficient vs. zinc-replete *zap1* cells (Fig. 1). *YDR492w* and *YOL002c* are highly similar in sequence and analysis of the *S. cerevisiae* genome revealed two other related genes, *YLR023c* and *YOL101c*. *YOL002c* and *YOL101c* showed higher expression in cells exposed to excess zinc. *YLR023c* did not show significantly altered expression in response to zinc status. These genes are herein named *IZH1* (*YDR492w*), *IZH2* (*YOL002c*), *IZH3* (*YLR023c*), and *IZH4* (*YOL101c*).

***IZH1* and *IZH2* Are Zap1p Target Genes.** *IZH1* and *IZH2* possess putative ZREs in their promoter regions, located at −416 (ACCTTTAGGGT) and −225 (TCCTCTAGGGT), respectively. Confirmation of the functionality of the *IZH2* ZRE was reported previously (2). The *IZH1* ZRE-*lacZ* construct yielded 30-fold more activity under zinc deficiency than under zinc repletion (455.8 ± 12.2 vs. 15.3 ± 0.7). In a *zap1* strain, this effect disappeared (9.3 ± 1.4 vs. 17.6 ± 6.6).

IZH1- and *IZH2-lacZ* reporters were inducible by zinc deficiency in a wild-type strain, but not in a *zap1* strain (Fig. 2 *A* and *B*). *IZH3-lacZ*, *CYC1-lacZ*, and *HIS4-lacZ* controls were not induced by zinc deficiency, demonstrating that general changes in the levels of transcription or translation are not responsible for the observed induction of *IZH1* and *IZH2* (Fig. 2*C*). Moreover, $IZH1_{mutZRE}$-*lacZ* and $IZH2_{mutZRE}$-*lacZ* reporters, in which the ZREs were mutated, were not inducible by zinc limitation (Fig. 2 *A* and *B*).

***IZH2* Is Regulated by Fatty Acids.** A previous report showed that *IZH1*, *IZH2*, and *IZH4* expression is induced by fatty acids (3). We have confirmed that *IZH2-lacZ* responds independently to both zinc and the addition of exogenous myristate (Fig. 2*D*). Fatty acids can induce gene expression via the Oaf1p/Pip2p complex that binds to oleate response elements (OREs). Putative OREs are present in the *IZH2* (−159 to −167 bp), *IZH1* (−302 to −328 bp), and *IZH4* (−240 to −263 bp) promoters (3).

***IZH4* Is Induced by Excess Zinc.** The *IZH4-lacZ* reporter showed elevated activity (2-fold) when grown in elevated zinc, whereas a general inhibitory effect of high zinc on *lacZ* activity is demonstrated by a significant decrease in *IZH1-lacZ*, *IZH3-lacZ*, *HIS4-lacZ*, and *CYC1-lacZ* activities in cells exposed to 3 mM zinc (Fig. 3*A*). Indeed, high levels of zinc have been shown to inhibit both protein and RNA synthesis by up to 70% in certain yeast species (18). Unlike the control constructs, the activity of *IZH2-lacZ* did not decrease in high zinc, suggesting the existence of some factor that maintains elevated expression.

***IZH2* and *IZH4* Are Part of the Hypoxic Response.** Table 1 lists genes with an average induction of >2-fold in cells exposed to 3 mM zinc ($n = 2$) and includes known targets of the Mga2p hypoxia

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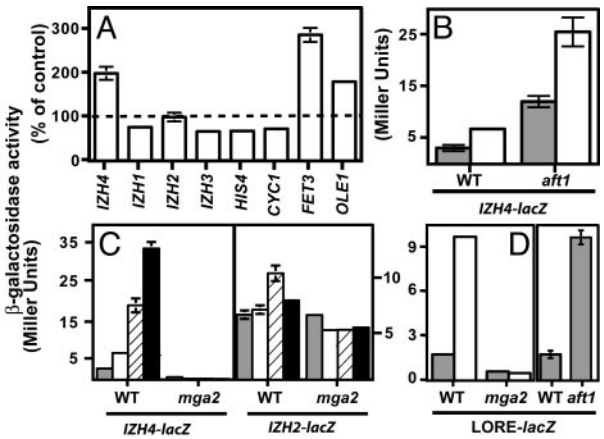

Fig. 3. Metal induction of *IZH2* and *IZH4*. (*A*) Relative activities of promoter-*lacZ* reporters shown as % change in activity in cells exposed to ++ zinc relative to activity in + zinc. (*B*) Effect of *aft1* mutation on *IZH4-lacZ* activity. (*C*) Mga2p dependence of *IZH4-lacZ* and *IZH2-lacZ* activity in cells exposed to 3 mM Zn^{2+} (white bars), 400 μM Co^{2+} (hatched bars), or 400 μM Ni^{2+} (black bars). Control treatment has no metals added (gray bars). (*D*) Effect of Zn^{2+}, Mga2p, and Aft1p on LORE-*lacZ* activity. In *B* and *D*, gray bars show activity in + zinc, and white bars show activity in ++ zinc.

sensor, *OLE1* (12) and Ty1 elements (19). The remaining genes are known to be induced by either low pO_2, by deletion of the Ssn6p O_2-activated repressor complex (20, 21) or by iron deficiency via the Aft1p iron-responsive transcription factor (22). Indeed, Fig. 3*A* confirms zinc induction for the *OLE1-lacZ* and *FET3-lacZ* reporters. A screen for regulatory elements in the promoters of the O_2-regulated genes by using RSA-TOOLS generated a probability-based consensus matrix that matched the LORE (low-oxygen response element). A similar screen of the promoters of the Aft1p-target genes generated a consensus matrix that matched the FeRE. With these matrices, we scanned 750 bp of the promoters of all genes in Table 1 and found that most of the O_2-regulated promoters contained putative LOREs and that all of the Aft1p-target promoters contained putative FeREs (Data Set 2, which is published as supporting information on the PNAS web site).

The *IZH4* promoter contains a potential LORE between −189 and −197 bp but does not contain an FeRE, suggesting that it is a target of Mga2p instead of Aft1p. To address this, we tested the effects of *mga2* and *aft1* mutations for their effects on *IZH4-lacZ* activity. Fig. 3*B* shows that although the induction of *IZH4-lacZ* in response to zinc was still 2-fold in an *aft1* mutant strain, the basal level of activity of the reporter construct was increased 5-fold. Fig. 3*C* shows that basal and zinc-inducible expression of *IZH4-lacZ* depends on Mga2p. In addition, other stimuli that are known to induce the hypoxic response in yeast, such as high Co^{2+} and Ni^{2+} (12), also induce *IZH4-lacZ*. These responses are not seen in an *mga2* strain. Fig. 3*D* confirms that the LORE-*lacZ* hypoxia reporter is also induced by high zinc in an Mga2p-dependent fashion, as well as by *aft1* deletion. The *IZH2* promoter also contains a putative LORE sequence between −137 and −145 bp. *IZH2-lacZ* is weakly induced by Co^{2+} and Ni^{2+} in an Mga2p-dependent manner. Like the control plasmids in wild-type yeast exposed to high zinc, elevated *IZH2-lacZ* activity is not maintained in an *mga2* mutant, suggesting that Mga2p is responsible for maintaining elevated expression in high zinc (Fig. 3*C*).

***IZH* Genes Encode Homologs of Vertebrate Membrane Steroid Receptors.** The *IZH* genes encode related proteins that belong to a large family of membrane proteins. An alignment of the four

Table 1. Genes induced >2-fold by zinc excess

Group	Gene name	Fold induction
Induced by *SSN6* deletion or by low oxygen	***IZH4****	6.6
	OLE1*	3.4
	HSP26*	3.4
	YGL039w*	2.9
	ERG3*	2.8
	PIR3	2.6
	YOR338w*	2.3
	HSP30*	2.3
	COS10*	2.2
	IZH2*	2.1
	NCE103*	2.0
	AHP1*	2.0
	YGR161c*	2.0
	HSP104	2.0
	YOL106w†	2.8
Miscellaneous	***PDR3****	2.6
	MGA2*	2.3
	UBS1	2.1
	HSP150	2.0
Iron metabolism	***FIT3***†	7.6
	FIT2†	7.2
	TAF1†	6.2
	TIS11†	3.6
	ENB1†	3.4
	ARN1†	3.0
	FTR1*†	2.4
	FRE1†	2.3
	SIT1†	2.2
	FET3†	2.0
	HMX1†	2.0
Ty retrotransposons	*YBL005w-A* (YBLWTy1-1)	2.1
	YER138c (YERCTy1-1)	2.2
	YER160c (YERCTy1-2)	2.3
	YHR214c-B (YHRCTy1-1)	2.2
	YML045w (YMLWTy1-2)	2.6
	YBR012w-A/-B (YBRWTy1-2)	2.2/2.2
	YCL019w/20w (YCLWTy2-1)	2.1/2.4
	YJR026w/27w* (YJRWTy1-1)	2.2/2.5
	YJR028w/29w (YJRWTy1-2)	2.2/2.2
	YML039w/40w (YMLWTy1-1)	2.2/2.6
	YMR045c/46c* (YMRCTy1-3)	2.0/2.6
	YMR050c/51c (YMRCTy1-4)	2.3/2.6

Genes in bold have promoters containing putative regulatory elements scoring >7.0 when using the LORE or FeRE matrices generated by RSA-TOOLS.
*LORE-containing.
†FeRE-containing.

yeast proteins, a highly similar human protein (HsAdipoR1, NP_057083), three human membrane progestin receptors (HsmPRβ, NP_588608; HsmPRγ, NP_060175; and HsmPRα, NM_178422), and hemolysin III (Hly3, AAM90670) from *Bacillus cereus* is shown in Fig. 4. Each polypeptide has at least seven putative transmembrane domains (TMs) and a similar predicted topology with cytoplasmic N termini and extracytoplasmic C termini, although known vertebrate membrane progestin receptors possess an eighth predicted TM at the C terminus. This predicted topology has been confirmed for Izh4p (23). Five motifs that cluster on the cytoplasmic side of the membrane are conserved in the Izh-like proteins. These conserved regions, which contain potential metal-binding residues, can be summarized as follows: (*i*) a long motif N-terminal to TM1 that generally resembles $Px_nGYRx_nNEx_2Nx_2T/SH$; (*ii*) an Sx_2Hx_5S motif at the C terminus of TM2; (*iii*) a Dx_9GS motif at the

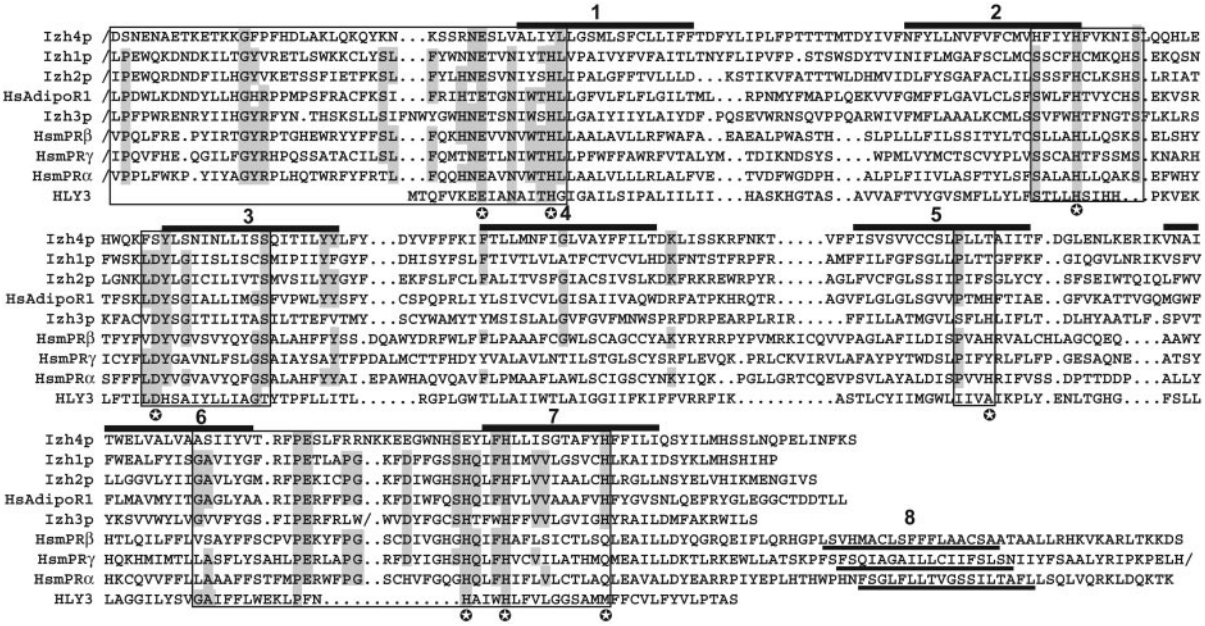

Fig. 4. Multiple sequence alignment. Izh1p-4p and similar proteins are aligned by using CLUSTALX. Predicted transmembrane segments are indicated by solid bars. Highly conserved regions are boxed. Stars indicate putative metal-binding residues that are highly conserved in the PAQR superfamily. Backslashes show locations where sequence was deleted to aid alignment.

beginning of TM3; (*iv*) a Px_2H motif in TM5 where the H residue is generally only conserved in higher eukaryotes; and (*v*) the loop between TM6 and TM7 containing PER/Kx_nPG and Hx_2F/WH motifs with a conserved histidine in the middle of TM7 being most common. Hly3-like members of this family share most of these motifs with the striking exceptions of truncated motifs 1 and 5 and a complete lack of motif 4.

***IZH* Genes Affect Zinc Tolerance.** A collection of strains in which all combinations of the *IZH* genes have been deleted was generated. All of these strains were viable, indicating that no single gene or combination of genes was essential for viability. When stationary-phase cells were inoculated into either zinc-replete or -limiting media, all strains grew with wild-type characteristics in terms of lag-phase duration, exponential growth rate, and final yield of cell number (data not shown).

When these strains were inoculated into a high-zinc medium (6 mM), mutant phenotypes were observed. First, the *izh3* strain showed slight but reproducible improvement in growth in high zinc when compared with the wild-type strain (Fig. 5*A*). This phenotype could be complemented by reintroduction of the *IZH3* gene on a high-copy plasmid and was found to be attributed to a decrease in lag-phase duration in the mutant rather than a change in exponential-phase growth rate because the doubling time (DT) of zinc-treated *izh3* mutant cells (3.25 ± 0.06 h) was identical to that of wild-type cells (3.22 ± 0.06 h). Mutation of *izh2* also altered zinc sensitivity. In contrast to *izh3*, *izh2* did not alter lag-phase duration but rather reduced the growth rate of mutant cells in exponential phase (DT = 4.28 ± 0.24 h).

Effects on zinc sensitivity were also apparent on agar plates containing high levels of zinc (12 mM). The *izh2* mutant strain was sensitive under these conditions (Fig. 5*B*), a phenotype that could be complemented by a CEN plasmid containing the *IZH2* ORF driven by its own promoter (Fig. 5*C*) or the GAL1 promoter (Fig. 5*D*). Both *GAL1-IZH2.1* and *GAL1-IZH2.2* (first and second in-frame ATG constructs) complement the *izh2* phenotype when induced by galactose. As in liquid medium, strains bearing the *izh3* mutation grew slightly better in high zinc than the corresponding isogenic *IZH3* controls. For example, compare growth of *izh1izh2izh4* with the quadruple mutant (Fig. 5*B*). A slight zinc sensitivity was also apparent in the *izh1* single mutant (Fig. 5*B*). The contribution of *IZH1* to zinc tolerance was

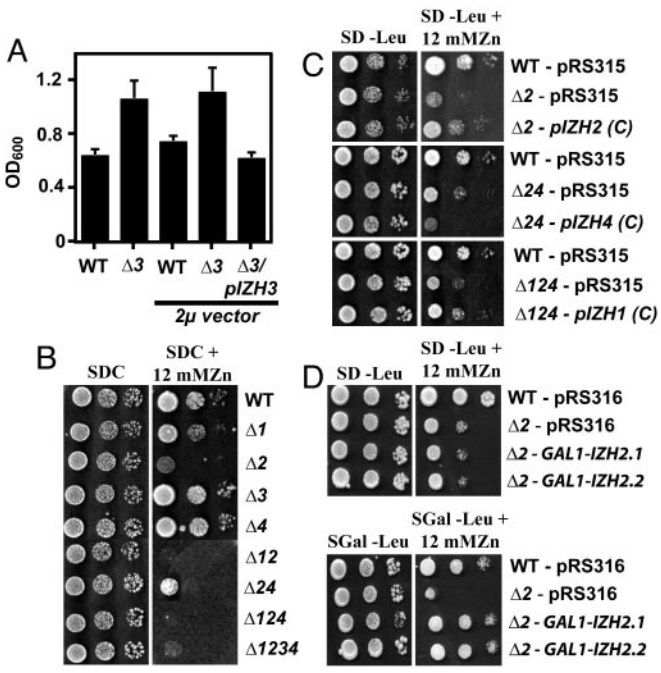

Fig. 5. Zn^{2+}-dependent phenotypes. (*A*) Growth of wild type and *izh3* mutants in liquid culture containing 6 mM zinc. *pIZH3* is a 2-μ plasmid bearing the *IZH3* gene under the control of its own promoter. (*B*) Growth of *izh* mutant strains on SD plates with or without 12 mM zinc added. (*C*) Complementation of zinc-dependent phenotypes by CEN (C) plasmids containing native-promoter-driven *IZH2* (*pIZH2*), *IZH4* (*pIZH4*), and *IZH1* (*pIZH1*) genes. (*D*) Complementation of the *izh2* mutant phenotype by CEN plasmids harboring the *GAL1-IZH2.1* and *GAL1-IZH2.2* constructs on plates containing 2% glucose (*Upper*) or 2% galactose (*Lower*) with or without 12 mM zinc. Error bars represent ±1 SD.

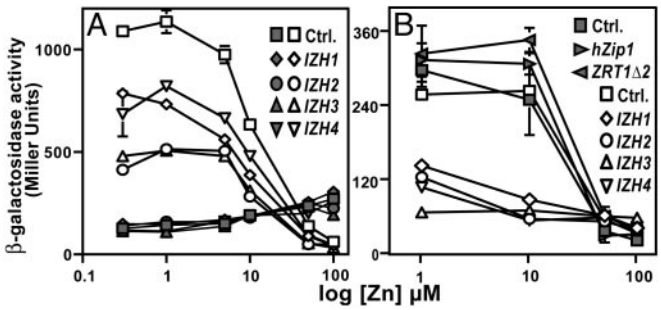

Fig. 6. *IZH* gene overexpression. (*A*) Overexpression of *IZH1-4* using 5 μM β-estradiol in cells grown in low-zinc media medium results in a significant decrease in Zap1p activity measured with a ZRE-*lacZ* reporter construct (open symbols). No effect was seen when the same experiment was performed by using the *HIS4-lacZ* control reporter construct (filled symbols). *IZH1-4* overexpression plasmids are *LEU2*-selectable and reporter plasmids are *URA3*-selectable. (*B*) Overexpression of *IZH1-4* by using 2% galactose gives similar results (open symbols), whereas overexpression of control membrane proteins hZip1 and *ZRT1*Δ2 does not inhibit ZRE-*lacZ* activity (filled symbols). For the filled symbols in *B* the overexpression plasmids are *URA3*-selectable and the reporter plasmids are *LEU2*-selectable. Ctrl., control.

clearly shown when comparing an *izh2izh4* mutant with an *izh1izh2izh4* strain. Moreover, the *izh2izh4* mutant grew better than the *izh2* strain indicating that mutation of *IZH4* makes cells zinc tolerant. These latter two phenotypes could be complemented by CEN plasmids harboring the *IZH1* or *IZH4* genes (Fig. 5*C*) driven by their native promoters.

Because the *IZH2* gene is adjacent to the *PHO80* gene, it is possible that the *izh2* phenotypes are due to *PHO80* inactivation. *pho80* mutants, like *izh2* mutants, have been shown to be zinc sensitive (24). However, complementation of *izh2* strains by plasmids bearing the *IZH2* gene demonstrates that the zinc-sensitivity phenotype is not due to *PHO80* inactivation.

Effects of *IZH* Overexpression on Zinc Homeostasis. By using the ZRE-*lacZ* reporter as a bioassay for Zap1p activity (indirect assay for labile zinc levels), the effect of *IZH* gene overexpression on cellular zinc homeostasis was measured. ZRE-*lacZ* activity was monitored over a range of zinc concentrations for wild-type strains in which the *IZH* genes were overexpressed from the *GAL1* promoter by using the GEV system (5 μM β-estradiol induction) (Fig. 6*A*). Although there was no effect of *IZH* gene overexpression on the control *HIS4-lacZ* reporter, overexpression of any *IZH* gene significantly decreased ZRE-*lacZ* activity. Similar results were seen when the *IZH* genes were induced with 2% galactose (Fig. 6*B*), indicating that the observed effects were not an artifact of β-estradiol treatment. No significant effect on zinc accumulation was seen in strains overproducing these proteins (data not shown). *GAL1*-driven overexpression of either hZip1, a human zinc uptake transporter that does not function in yeast (25), or *ZRT1*Δ2, a nonfunctional mutant of yeast *ZRT1* (26), has no significant effect on a ZRE-*lacZ* reporter, suggesting that the effect of *IZH*-gene dosage is not a generalized effect of aberrant protein folding or trafficking due to membrane protein overexpression (Fig. 6*B*).

Discussion

IZH1, *IZH2*, *IZH3*, and *IZH4* encode a family of paralogous proteins in yeast. The Izh proteins belong to a large and nearly ubiquitous family of proteins found in both prokaryotes and eukaryotes. This family has been named the PAQR (Progestin, AdipoQ-Receptor) family of proteins because several of its constituent members are steroid or adiponectin (AdipoQ) receptors. Structurally, PAQR proteins can be divided into two general subgroups: Hly3-like and Izh-like. Both subgroups are characterized by at least seven TMs and four highly conserved motifs rich in metal-binding amino acids. All of the conserved motifs are predicted to cluster on the cytoplasmic face of the membrane.

Little is known about the function of any member of this family. In the α-proteobacterium, *Azospirillum brasilense*, the Hly3-like gene encodes a chimeric protein with an Hly3-like N terminus fused to the CheA chemotaxis histidine kinase (27). Because *A. brasilense* is chemotactic toward hypoxic environments, Hly3-CheA may encode a sensor for hypoxia as well as the downstream kinase. Evidence that these proteins are receptors is more convincing for the vertebrate proteins. Expression of a sea trout mSR in cell culture resulted in progesterone-dependent inhibition of adenylate cyclase activity. This inhibition could be relieved by the addition of Pertussis toxin, suggesting that mSRs are inhibitory G protein-coupled receptors (4). Recombinant mSRs expressed in *E. coli* also bound progesterone with high affinity (28). Hly3 was originally discovered because of its hemolytic activity when overexpressed in *E. coli*, and evidence suggests that it is a pore-forming membrane protein with a pore diameter of $\approx$35 Å (29). These findings raise the possibility that PAQR proteins are receptors, channels, or both.

In yeast, gene regulation has provided the first functional clues. *IZH1* and *IZH2* are confirmed Zap1p-target genes, whereas *IZH4* is induced by excess zinc. We demonstrated that deletion of either *IZH1* or *IZH2* results in increased sensitivity to elevated zinc, whereas deletion of *IZH3* or *IZH4* has the opposite effect. Zinc sensitivity is variable and requires long preculture times before re-inoculation in zinc-containing medium. The *izh2* mutation increases the length of the cell cycle in zinc-treated cells, whereas *izh3* mutation decreases the lag phase under the same conditions. Overexpression of any of these four genes results in decreased activity of the Zap1p transcription factor when cells are grown in zinc-limiting medium. Based on their metalloregulation and their impact on zinc tolerance and homeostasis, we propose a role for these genes in zinc metabolism.

Other lines of evidence suggested that the function of the *IZH* genes goes beyond a role in zinc metabolism. *IZH4* is also induced by exposure to Zn^{2+}, Co^{2+}, Ni^{2+}, and *AFT1* deletion. Metalloregulation of *IZH4* depends on the Mga2p hypoxia-responsive transcription factor. All three metal treatments, as well as *aft1* deletion, are proven inducers of the hypoxic response (12). *IZH2* has also been shown to be a weak target of Mga2p. The involvement of Hly3-CheA from *A. brasilense* in hypoxia sensing emphasizes the importance of this finding and suggests a conservation of function across species.

Furthermore, we confirmed earlier reports suggesting that *IZH2* responds to exogenous myristate. Regulation of the *IZH* genes by fatty acids and hypoxia strongly suggests an alternative role in lipid metabolism. Preliminary phenotypic analysis shows that *izh2* mutants are resistant to the sterol-binding antibiotic, nystatin (3), suggesting that *izh2* mutants have altered membrane sterol composition. Furthermore, *IZH3* transcription is induced and *IZH4* transcription is repressed by defects in the ergosterol biosynthetic pathway (20). These observations, combined with the fact that some vertebrate orthologs function as receptors for structurally related steroids, identify ergosterol metabolism as a likely biochemical pathway in which to place the Izh proteins.

A role for the *IZH* genes in ergosterol metabolism helps explain the need for long precultures to see strong zinc-dependent *izh* mutant phenotypes. Even when cultures are vigorously shaken, yeast rapidly deplete the growth medium of O_2 and cultures become increasingly anaerobic over time (30). Anaerobically grown yeast are auxotrophic for ergosterol (31); therefore, prolonged culturing may result in decreased sterol biosynthesis. If *izh* mutations exacerbate or ameliorate this situation they can have significant effects on sterol composition in preculture.

An anecdotal relationship between membrane sterol content and zinc metabolism has already been established. Depletion of cholesterol content in MCF-10A breast epithelial cells by using mevastatin significantly diminished the uptake of zinc but not 2-deoxyglucose (32). Nystatin treatment also inhibited ^{65}Zn uptake in human fibroblasts (33) and induced the Zap1p regulon in *S. cerevisiae* (34). These studies suggest that changes in membrane sterols can specifically affect the permeability of certain ions. Therefore, it is possible that the sole role of the *IZH* genes is in ergosterol metabolism and that their effects on zinc tolerance are an indirect consequence of this role.

However, the induction of *IZH1* and *IZH2* by Zap1p under zinc deficiency, as well as the specific decrease in Zap1p activity in cells overexpressing Izh proteins, suggests a deeper connection between these genes, sterols, and zinc metabolism. A possible clue to the nature of this connection comes from the mSRs. Both transcriptional and posttranslational steroid signaling can be linked to zinc metabolism. The first obvious connection is that classical nuclear hormone receptors are zinc-binding proteins (35). That the most highly conserved residues in the mSR superfamily are potential metal-binding residues suggests that mSRs may indeed be metalloproteins as well. In addition, corticosteroids are known inducers of both zinc uptake and the expression of metallothionein (MT), a zinc-buffering protein (36). Sex hormones have been shown to induce zinc uptake as well as to regulate the expression of ZIP-family zinc transporters (37, 38). Lastly, zinc has been shown to regulate the aldosterone-mediated intracellular acidification of cultured kidney cells involving PKC (39). These studies suggest an intimate relationship between steroid signaling and zinc metabolism.

It is important to note here that posttranslational estrogen and glucocorticoid receptor signaling has been shown to involve a kinase cascade that includes PDK, a 3-phosphoinositide-dependent kinase (40) that phosphorylates a variety of substrates, including PKC (41). Yeast possess two orthologs of PDK, *PKH1* and *PKH2*, which also act upstream of PKC (42). Intriguingly, *IZH1* and *IZH4* share promoter regions with the *PKH1* and *PKH2* genes, respectively. In yeast, such divergently transcribed genes often encode proteins in the same biochemical pathway (43). This finding suggests that there is a functional relationship between the *IZH* genes and the PDK cascade. This hypothesis is bolstered by the involvement of the PDK cascade in steroidal signaling in mammalian cells. It is also important to note here that signaling via both PDK (44) and PKC (45) is thought to be regulated directly by alterations in cellular zinc, a fact that connects this pathway to zinc homeostasis.

In conclusion, we propose three possible functions for the Izh proteins. First, these proteins may function solely in sterol metabolism. In this capacity they may influence the permeability of the plasma membrane and, consequently, the homeostasis of cations such as zinc. It is also possible that the Izh proteins function as transporters for zinc used in a signaling capacity, a possibility that may explain their regulation by Zap1p and their effect on Zap1p activity. The role of zinc in signal transduction is controversial. However, evidence for such a role is mounting. Perhaps the most compelling evidence is the finding that the CDF-1 zinc transporter in *C. elegans* positively regulates Ras-mediated signaling (46), another pathway that can transmit signals to PKC (47). It is possible that zinc, perhaps through Izh-like proteins, plays a widespread role in cellular kinase cascades. A third possibility is that the Izh proteins are involved in a signal transduction cascade that is independent of zinc, and that Zap1p is a downstream target of this pathway.

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