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Dissecting the regulation of yeast genes by the osmotin receptor

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ABSTRACT

The Izh2p protein from *Saccharomyces cerevisiae* is a receptor for the plant antifungal protein, osmotin. Since Izh2p is conserved in fungi, understanding its biochemical function could inspire novel strategies for the prevention of fungal growth. However, it has been difficult to determine the exact role of Izh2p because it has pleiotropic effects on cellular biochemistry. Herein, we demonstrate that Izh2p negatively regulates functionally divergent genes through a CCCTC promoter motif. Moreover, we show that Izh2p-dependent promoters containing this motif are regulated by the Nrg1p/Nrg2p and Msn2p/Msn4p transcription factors. The fact that Izh2p can regulate gene expression through this widely dispersed element presents a reasonable explanation of its pleiotropy. The involvement of Nrg1p/Nrgp2 in Izh2p-dependent gene regulation also suggests a role for this receptor in regulating fungal differentiation in response to stimuli produced by plants.

The Izh2p protein from *Saccharomyces cerevisiae* was found to function as a receptor for a plant protein called osmotin [1]. This discovery is intriguing because osmotin belongs to the PR-5 family of defensins that possesses broad-spectrum antifungal activity and a better understanding of how plant PR-5 defensins affect fungi can be used affect to develop novel antifungal pharmaceuticals. Not surprisingly, there is considerable interest in characterizing the biochemical role of Izh2p and mapping the pathway through which it affects yeast physiology.

However, Izh2p has been implicated in a variety of biochemical processes ranging from iron and zinc homeostasis [2] to the metabolism of lipids and phosphate [3] to programed cell death [1]. To complicate the issue even further, Izh2p overexpression affects both a general stress responsive transcriptional reporter and the expression of a gene involved in filamentous growth [4]. Thus, *IZH2* is a pleiotropic gene that cannot yet be functionally assigned to any one particular biochemical pathway. The purpose of this study is to shed light on the origin of this pleiotropy by developing a clearer picture of how Izh2p regulates specific genes.

As a starting point, we used the fact that Izh2p overexpression represses the expression of two specific genes. This effect can be attributed to basal signaling capability of the receptor, which, when present at elevated levels, can activate its downstream signal transduction pathway [5]. Herein, we present an analysis of the promoter regions of these two Izh2p-regulated genes that indicates a CCCTC motif is responsible for their response to Izh2p. This motif has been shown to function as a binding site for the Nrg1p and Nrg2p transcriptional repressors [6] and may competitively bind the Msn2p and Msn4p transcriptional activators [7]. This short motif can be found in the promoters of hundreds of functionally divergent yeast genes, a fact that may help explain the pleiotropy of Izh2p and lead to a better understanding of how PR-5 defensins, through their interaction with Izh2p, affect fungal physiology.

Materials and methods

Yeast strains and growth conditions. MCY5326 wild type, MCY5338 (*msn2Δmsn4Δ*), MCY5378 (*nrg1Δnrg2Δ*) and MCY5385 (*msn2Δmsn4Δnrg1Δnrg2*) have been described previously [7]. All other yeast strains used in this study were purchased from Euroscarf (Frankfurt, Germany) and are in the BY4742 background. Strains were grown in either chelexed synthetic medium (CSM), low iron medium (LIM) or low zinc medium (LZM) the compositions of which have been previously described [2,4,8]. For CSM, which is a nutrient drop-out medium, metal-repletion is achieved by adding 10 μM of the respective metal. For LIM and LZM, which contain EDTA, the addition of 1 μM of each metal is considered metal-deficient, while 1 mM is considered metal-replete. For all media, 2% galactose is used as the carbon source to induce the expression of genes driven by the *GAL1* promoter.

Plasmids. All plasmids have been previously described as indicated by the references. p*FET3*-398 and p*FET3*-297 are episomal reporter plasmids in which *lacZ* is driven by different truncations of the *FET3* promoter (−398 to +3 and −297 to +3, respectively) [4]. Both constructs are induced by iron-deficiency via the Aft1p-transcription factor due to the presence of an iron-response

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element between −252 and −245 (see Supplemental Fig. 1A). p*ZRT1*-521, p*ZRT1*-361, p*ZRT1*-305, and p*ZRT1*-201 are integrating reporter plasmids in which *lacZ* is driven by different truncations of the *ZRT1* promoter (−521 to +3, −361 to +3, −305 to +3, and −201 to +3, respectively). The *ZRT1* promoter contains at least three functional zinc-response elements (ZREs: ZRE3, −445 to −434; ZRE1, −318 to −309; and ZRE2, −202 to −191) to which the Zap1p transcriptional activator binds during zinc-deficiency [9] (see Supplemental Fig. 1B). p*ZRT1-ZRT1*ET is a centromeric plasmid containing an HA-epitope tagged *ZRT1* open reading frame driven by approximately 600 base pairs of the native *ZRT1* promoter [10]. p*ZPS1-lacZ*, p*ZRC1-lacZ*, p*OLE1-lacZ*, and p*FET4-lacZ* contain ~1000 base pairs of the *ZPS1*, *ZRC1*, *OLE1*, and *FET4* promoters fused to *lacZ* [2,8]. Plasmids p*CYC1*(*ZRT1*ZRE1)-*lacZ* and p*CYC1*(*IZH1*ZRE)-*lacZ* consist of the *lacZ* gene driven by the minimal *CYC1* promoter into which fragments of the *ZRT1* or *IZH1* promoters containing functional zinc-response elements (ZREs) have been inserted [8] (see Supplemental Fig. 1C). p*GAL1-IZH2* contains the *IZH2* gene driven by the *GAL1* galactose-inducible promoter and is derived from the pRS316 expression vector [2]. p*GAL1-NRG2* contains a *GAL1*-driven TAP-tagged (Tandem Affinity Purification) *NRG2* construct and was purchased from OpenBiosystems.

Biochemical assays. β-Galactosidase assays [2] and total membrane protein preparations [4] were performed as previously described. Western blots on SDS–PAGE gels loaded with equal amounts of total membrane protein lysate were performed using standard chemiluminescence protocol with rabbit polyclonal anti-HA primary and goat anti-rabbit IgG-HRP conjugate secondary antibodies (Santa Cruz Biotechnology). Pattern searching for motifs in yeast promoters was performed using RSA tools (http://rsat.ul-b.ac.be/rsat/) by defining a promoter as 800 base pairs upstream of ATG excluding overlap with upstream genes.

Results and discussion

Izh2p negatively regulates the ZRT1 promoter

Because of its suspected involvement in zinc metabolism, we analyzed the effect of Izh2p overexpression on *ZRT1*, the gene that encodes the high-affinity zinc-uptake transporter. Izh2p overexpression repressed the ability of Zap1p to induce the expression of p*ZRT1*-521 and p*ZRT1*-361 during zinc-deficiency (Fig. 1A) and resulted in decreased accumulation of HA-tagged Zrt1p protein driven by ~600 base pairs of *ZRT1* promoter (Fig. 1B). These results indicate that Izh2p negatively regulates the *ZRT1* gene. Izh2p overexpression had no effect on basal expression of p*ZRT1*-521 (Fig. 1C) or inducible expression of p*ZRT1*-305 (Fig. 1A), indicating that the effect of Izh2p requires induction by Zap1p but does not globally affect the ability of Zap1p to activate genes.

Defining the Izh2p-response element

p*ZRT1*-305 is not repressed by Izh2p overexpression, indicating that Izh2p exerts its effects on a regulatory element between −361 and −305 of the *ZRT1* promoter. We previously found that Izh2p similarly represses the Aft1p-inducible expression of the *FET3* gene by affecting a regulatory element between −398 and −297 of the *FET3* promoter [4]. We scanned these regions of the *FET3* and *ZRT1* promoters for similar sequences and found a conserved ACCCTC motif t (Fig. 2A).

In the *ZRT1* promoter, this motif directly overlaps ZRE1 (−318 to −309). To determine if this motif is sufficient to confer Izh2p-responsiveness onto a promoter, we measured the effect of Izh2p overexpression on p*CYC1*(*ZRT1*ZRE1)-*lacZ*. This construct was both zinc-responsive due to the presence of ZRE1 and Izh2p-repressible

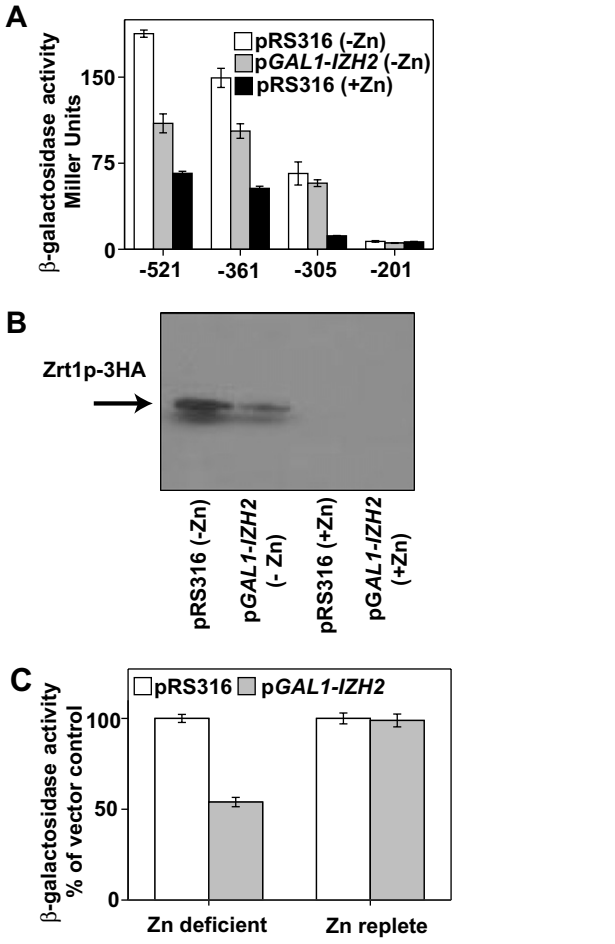

Fig. 1. Regulation of *ZRT1* by Izh2p. In all panels, cells were grown in zinc-replete (+Zn) or zinc-deficient (−Zn) LZM. Cells either carry pRS316 or p*GAL1-IZH2*. (A) The effect of Izh2p overexpression on various p*ZRT1-lacZ* fusion constructs. Numbers indicate the amount of upstream sequence contained in each construct. (B) Effect of Izh2p overexpression on the accumulation of HA-tagged Zrt1p expressed from the p*ZRT1-ZRT1*ET plasmid. (C) The effect of Izh2p overexpression on p*ZRT1*-521.

due to the presence of the ACCCTC motif (Fig. 2B). p*CYC1*(*IZH1*ZRE)-*lacZ*, another reporter containing a different ZRE, did not respond to Izh2p overexpression, confirming that Izh2p did not generally repress all reporters containing ZREs (Fig. 2B).

Thus, this ACCCTC motif, which we are calling the Izh2p-Response Element (IzRE) is sufficient to confer Izh2p-responsiveness onto promoters and we found putative IzREs in the promoters of over 600 yeast genes. We already possessed promoter-*lacZ* fusions constructs for three of these genes—*ZRC1*, *ZPS1*, and *OLE1* [2,8]. p*ZPS1-lacZ* and p*ZRC1-lacZ* are induced by zinc-deficiency and, as expected, their zinc-dependent induction was repressed by Izh2p overexpression (Fig. 2D). p*OLE1-lacZ* is inducible by iron-deficiency [2] and its iron-dependent induction was repressible by Izh2p overexpression (Fig. 2E). The IzRE in the *OLE1* promoter is in the opposite orientation relative to ATG, suggesting the orientation of the IzRE may not be important. We also previously showed that a fifth promoter construct, p*MUC1-lacZ*, is repressible by Izh2p overexpression [4], yet this promoter contains a variant TCCCTC motif, suggesting that the functional IzRE can tolerate changes at the first position. However, the p*FET4-lacZ* construct, which contains CCCCTC, is actually inducible by both zinc-deficiency and iron-deficiency [11], but unresponsive to Izh2p overexpression under either condition (Figs. 2D and E). Thus, the consensus IzRE is (A/T)CCCTC at this point, although more work is required to define all functional variations in the motif.

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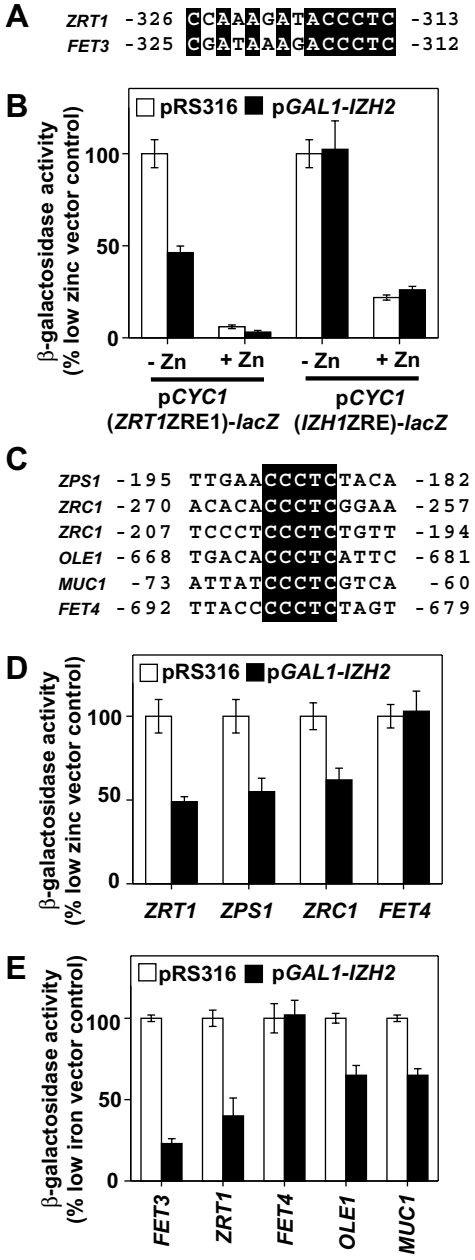

Fig. 2. Identification of the Izh2p-response element. In (B), (D), and (E), cells carry either pRS316 or p*GAL1-IZH2*. (A) Conserved regions of the *FET3* and *ZRT1* promoters. (B) The effect of Izh2p overexpression on either p*CYC1*(*ZRT1*ZRE1)-*lacZ* or p*CYC1*(*IZH1*ZRE)-*lacZ*. Cells are grown in LZM. (C) Location of putative Izh2p-response elements in various genes. (D,E) Effect of Izh2p overexpression on the ability to induce various promoter-*lacZ* constructs. Cells were grown in zinc-deficient (D) or iron-deficient (E) CSM.

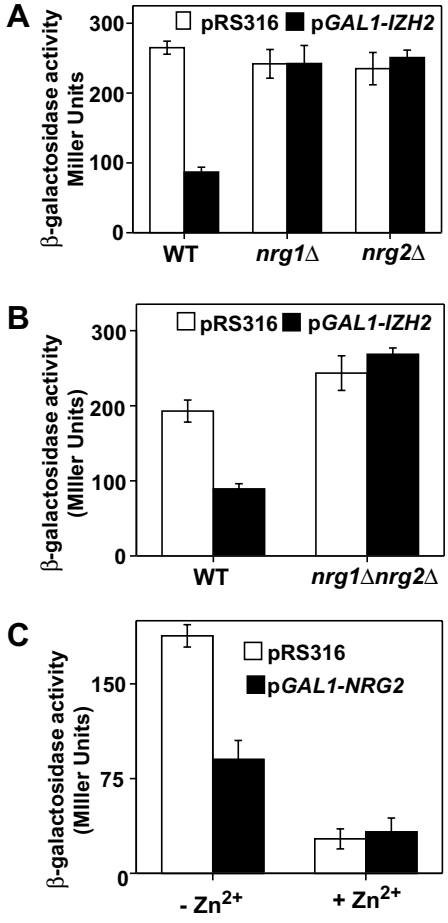

Fig. 3. Izh2p-dependent repression requires Nrg1p and Nrg2p. In all panels, cells carry either pRS316 or p*GAL1-IZH2* and were grown in LZM. (A) The repression of p*ZRT1*-521 by Izh2p overexpression in wild type (WT, BY4742) and isogenic mutant strains lacking either Nrg1p (*nrg1Δ*) or Nrg2p (*nrg2Δ*). (B) The repression of p*CYC1*(*ZRT1*ZRE1)-*lacZ* by Izh2p overexpression in wild type (WT, MCY5326) and an isogenic mutant strain lacking both Nrg1p and Nrg2p (*nrg1Δnrg2Δ*).

Nrg1p and Nrg2p as Izh2p-dependent repressors

Footprinting analysis revealed that CCCTC is a binding site for the Nrg1p/Nrg2p transcriptional repressors [6], suggesting that the IzhRE is an Nrg1p/Nrg2p binding site. Indeed, Nrg1p has already been identified as a repressor of the Izh2p-regulated *ZPS1* and *MUC1* genes [7] and we previously showed that Nrg1p/Nrg2p are required for Izh2p-dependent *FET3* repression [4]. Figs. 3A and B show that Nrg1p/Nrg2p were also required for Izh2p-dependent repression of p*ZRT1*-521 and p*CYC1*(*ZRT1*ZRE1)-*lacZ*. Consistent with the involvement of Nrg1p/Nrg2p in Izh2p-dependent repression, we also previously showed that overexpression of Nrg2p had the same effect on *FET3* as Izh2p. Herein, we show that overexpression of Nrg2p also repressed the Zap1p-dependent induction of p*CYC1*(*ZRT1*ZRE1)-*lacZ* (Fig. 3C). The fact that the IzRE and ZRE overlap in this construct suggests that Nrg2p competes with Zap1p, and thereby represses inducible expression. The precise mechanism through which Izh2p affects the ability of Nrg1p and Nrg2p to serve as transcriptional repressors is still under investigation.

Msn2p and Msn4p as Izh2p-dependent activators

Izh2p affects the ability to induce *FET3* and *ZRT1*, but has no effect on either the Aft1p or Zap1p activators of these genes. This suggests that Izh2p negatively regulates the activity of a co-activator. We previously showed that the Msn2p and Msn4p transcription factors were essential co-activators of *FET3* expression [4]. This is demonstrated in Fig. 4A, which shows that, while p*FET3*-398 required Msn2p for iron-dependent induction, p*FET3*-297 did not. Hence, in addition to the IzRE, there is an Msn2p-dependent upstream co-activating element in the *FET3* promoter between −398 and −297. This finding is intriguing because recent findings suggest Msn2p/Msn4p compete with Nrg1p/Nrg2p for similar binding sites, including CCCTC, in a subset of promoters [7]. Thus, Izh2p could function by activating Nrg1p/Nrg2p or by inactivating Msn2p/Msn4p. Clearly, Izh2p does not work solely by inactivating Msn2p/Msn4p because its overexpression still represses the zinc-dependent induction of p*ZRT1*-521 in a strain lacking Msn2p and Msn4p (Fig. 4B). In addi-

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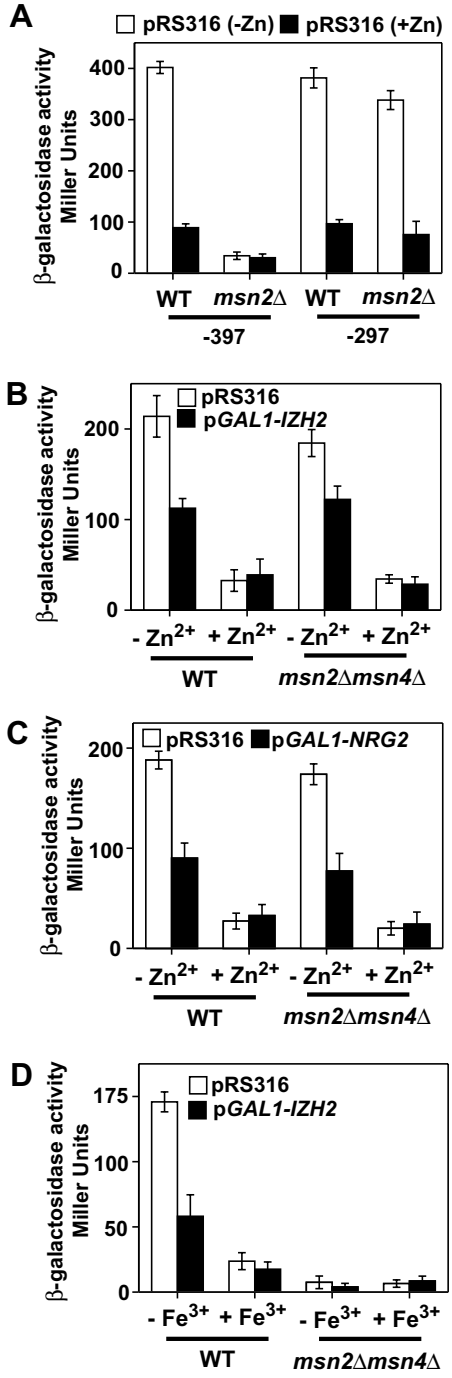

Fig. 4. Msn2p and Msn4p are essential co-activators of *ZRT1*. In all panels, cells carry either pRS2316 or p*GAL1-IZH2* and were grown in iron- or zinc-deficient CSM. (A) The effect of deletion of Msn2p (*msn2Δ*, BY4742 background) on the iron-responsiveness of two truncations of the p*FET3*-398 and p*FET3*-297 reporters. (B, C, and D) The effect of deletion of Msn2p and Msn4p (*msn2Δmsn4Δ*, MCY background) on the zinc- and Izh2p-responsiveness of p*ZRT1*-521 (B), the zinc- and Nrg2p-responsiveness of p*CYC1*(*ZRT1*ZRE1)-*lacZ* (C), and the iron- and Izh2p-responsiveness of p*ZRT1*-521 (D).

tion, Nrg2p overexpression still represses p*CYC1*(*ZRT1*ZRE1)-*lacZ* in the *msn2Δmsn4Δ* strain (Fig. 4C). Thus, activation of Nrg1p/Nrg2p is sufficient for Izh2p-dependent gene repression.

Finally, it is intriguing that the Aft1p-dependent induction of *FET3* is constitutively repressed in an *msn2Δmsn4Δ* strain [4], but the zinc-dependent induction of *ZRT1* is not. These results suggest that Msn2p/Msn4p plays no role in *ZRT1* expression. However, recent genome-wide transcriptional analysis suggested that *ZRT1* is induced by iron-deficiency [12] and has a putative FeRE in its promoter between −364 and −358 (see Supplemental Fig. 1B). Fig. 4D shows that p*ZRT1*-521 was both inducible by low iron and repressible by Izh2p overexpression in a manner that depends on the presence of Msn2p/Msn4p.

Summary

These results show that Nrg1p/Nrg2p are repressors of both *FET3* and *ZRT1*, while Msn2p/Msn4p are co-activators. Moreover, Msn2p/Msn4p likely compete with Nrg1p/Nrg2p for binding to the IzRE. Izh2p affects gene expression by influencing the balance of this competition. Since Nrg1p/Nrg2p and Msn2p/Msn4p regulate hundreds of genes, their involvement in Izh2p-dependent gene regulation provides an explanation for the pleiotropy of this receptor. More importantly, however, their involvement provides a tantalizing clue to the physiological function of this receptor. Nrg1p/Nrg2p are negative regulators of fungal filamentation [7], suggesting that Izh2p, through Nrg1p/Nrg2p, could inhibit the yeast to filament transition. This is supported by a recent study in which *IZH2* was identified as a gene that, when overexpressed, repressed filamentous growth [13]. Thus, it is possible that plant PR-5 defensins are designed to influence fungal developmental programs by activating Izh2p. This would represent a new paradigm in plant-fungal interactions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.002.

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