Role for the molecular chaperones Zuo1 and Ssz1 in quorum sensing via activation of the transcription factor Pdr1

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Zuo1 functions as a J-protein cochaperone of its partner Hsp70. In addition, the C terminus of Zuo1 and the N terminus of Ssz1, with which Zuo1 forms a heterodimer, can independently activate the *Saccharomyces cerevisiae* transcription factor pleiotropic drug resistance 1 (Pdr1). Here we report that activation of Pdr1 by Zuo1 or Ssz1 causes premature growth arrest of cells during the diauxic shift, as they adapt to the changing environmental conditions. Conversely, cells lacking Zuo1 or Ssz1 overgrow, arresting at a higher cell density, an effect overcome by activation of Pdr1. Cells lacking the genes encoding plasma membrane transporters Pdr5 and Snq2, two targets of Pdr1, also overgrow at the diauxic shift. Adding conditioned medium harvested from cultures of wild-type cells attenuated the overgrowth of both *zuo1*Δ*ssz1*Δ and *pdr5*Δ*snq2*Δ cells, suggesting the extracellular presence of molecules that signal growth arrest. In addition, our yeast two-hybrid analysis revealed an interaction between Pdr1 and both Zuo1 and Ssz1. Together, our results support a model in which (*i*) membrane transporters, encoded by Pdr1 target genes act to promote cell–cell communication by exporting quorum sensing molecules, in addition to playing a role in pleiotropic drug resistance; and (*ii*) molecular chaperones function at promoters to regulate this intercellular communication through their activation of the transcription factor Pdr1.

Hsp40 | Zuotin | ABC transporter

Eukaryotes, from fungi to humans, have a conserved, Hsp70-based molecular chaperone system best known for its function near the exit site of the ribosome tunnel from which nascent polypeptide chains emerge (1, 2). The J protein of this system, called Zuo1/DNAJC2 in fungi/humans, is required for stimulation of the ATPase activity of its partner Hsp70, facilitating efficient interaction of Hsp70's peptide binding domain with client proteins. Although the majority of Zuo1/DNAJC2 is found in the cytosol, associated with ribosomes, reports from both fungi and metazoans (3–5) indicate that it also has functions off the ribosome and/or in the nucleus. Zuo1/DNAJC2 is found in a heterodimer with Ssz1/Hsp70L1 (2). Although Ssz1/Hsp70L1 shares sequence similarity with the Hsp70 family of proteins, no evidence points to its binding to client proteins. Rather, Ssz1, as the heterodimer partner of Zuo1, facilitates the ability of Zuo1 to function as a J protein—that is, to stimulate the ATPase activity of its partner Hsp70 (1, 6).

In addition to their roles tied to molecular chaperone activity, Ssz1 and Zuo1 have been shown to independently have the capacity to enhance the transcription of genes of the pleiotropic drug resistance (PDR) regulon (7, 8). The PDR regulon is predominately composed of genes encoding membrane transporters, such as Pdr5 and Snq2, and enzymes involved in lipid biosynthesis and membrane remodeling (9). As suggested by the PDR designation, this regulon was first identified because up-regulation renders cells resistant to a variety of toxic xenobiotics by increasing their export from cells. Two related Zn_2–Cys_6 cluster transcription factors (TFs), Pdr1 and Pdr3, are the major regulators of this regulon. Some PDR targets, such as *PDR5*, are transcribed by both Pdr1 and Pdr3; others, such as *YOR1*, are only activated by Pdr1 (9). Pdr1 and Pdr3 can activate PDR in response to different signals. Ssz1 activates Pdr1, but not Pdr3 (8); Pdr3, but not Pdr1, responds to the absence of the mitochondrial genome (10). However, both are activated by a variety of xenobiotics, which directly bind Pdr1/3, likely causing a conformational change exposing a C-terminal transcription activation domain (AD) (11).

Here we report the results of experiments designed to understand the physiological role of activation of the PDR regulon by Zuo1 or Ssz1. Activation of Pdr1 promotes the arrest of cell growth in the diauxic shift. Our results are consistent with a model in which Pdr1 is activated via direct interaction with Zuo1 and Ssz1, and the resulting up-regulation of transporters in the plasma membrane increases export of signaling molecules involved in cell:cell communication.

Results

Activation of the PDR Regulon by Zuo1 Requires Pdr1. As a first step in our analysis of Zuo1 activation of PDR, we isolated a hyperactive *ZUO1* allele. Because the C-terminal 69 residues of Zuo1 are sufficient for induction of PDR (7), a library of randomly generated mutations within these 69 codons was screened for enhanced growth on plates containing the drug cycloheximide, relative to unmutagenized controls. From this screen we identified a single amino acid alteration, S427G. Cells expressing a fusion between a tandem affinity purification (TAP) tag and Zuo1's C-terminal 69 residues containing the S427G alteration (ZuoC*) displayed greater resistance to two mechanistically distinct drugs, cycloheximide and oligomycin, than cells expressing the wild-type (WT) fragment (ZuoC), even though the fusions were expressed at the same level (Fig. 1*A* and Fig. S1*A*). That the observed up-regulation was due to enhanced transcription was supported by the higher levels of mRNAs of Pdr5 and Yor1, known PDR target genes encoding drug transporters, in cells expressing ZuoC* compared with cells expressing ZuoC (Fig. S1*B*). In addition, ZuoC* expressing cells harboring a *PDR5* promoter–*lacZ* reporter had twice the β-galactosidase activity of ZuoC-expressing cells (Fig. S1*C*).

Given that Zuo1 and Ssz1 can independently activate PDR, we next tested whether Zuo1, like Ssz1, operated through the Pdr1 TF. PDR induction by ZuoC* in WT, *pdr1*Δ, and *pdr3*Δ cells was

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE31693).

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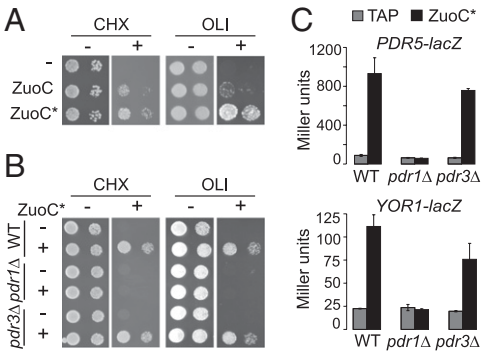

Fig. 1. Pdr1-dependent enhancement of drug resistance by the C terminus of Zuo1. WT cells were transformed with vector containing DNA encoding a TAP tag (–) or TAP tag fused to either amino acids 365–433 of WT Zuo1 (ZuoC) or ZuoC having the S427G alteration (ZuoC*). (*A* and *B*) S427G hyperactive alteration in C terminus of Zuo1. (*A*) Serial dilutions of WT cells harboring the indicated plasmids were spotted onto medium without (–) or with (+) cycloheximide (CHX) or oligomycin (OLI). (*B*) Serial dilutions of WT, *pdr1*Δ, and *pdr3*Δ cells transformed with either the control (–) or the ZuoC* plasmid (+) were spotted onto solid medium as indicated in *A*. (*C*) Cells harboring either the control (TAP) or TAP–ZuoC* (ZuoC*) plasmids were transformed with a second plasmid encoding either *PDR5* or *YOR1* promoter–*lacZ* fusion. The average β-galactosidase activity of three transformants of each was quantified as Miller units and plotted.

compared by assessing growth on plates containing drugs and by activation of *PDR5* and *YOR1* promoter–*lacZ* fusions. Similar levels of drug resistance were observed for WT and *pdr3*Δ cells, whereas no growth in the presence of drug was observed in *pdr1*Δ cells (Fig. 1*B*). ZuoC* activated the *PDR5–lacZ* and *YOR1–lacZ* reporters on the order of 10- and 4-fold, respectively, in WT cells and *pdr3*Δ cells. In contrast, no activation of *PDR5–lacZ* or *YOR1–lacZ* was observed in *pdr1*Δ cells expressing ZuoC* (Fig. 1*C* and Table S1). This Pdr1-dependent induction was not specific to the hyperactive allele, as induction by WT ZuoC was also dependent upon Pdr1 (Fig. S1*D*). Thus, we conclude that Zuo1 activation of the promoters of PDR genes, like that of Ssz1, is dependent on Pdr1.

PDR Regulon Is a Primary Target of Activation by Ssz1 and Zuo1. The results discussed above indicate that both Zuo1 and Ssz1 can activate PDR in a Pdr1-dependent manner. However, whether PDR is the primary pathway activated is not known. Therefore, we carried out whole-genome microarray analysis (Gene Expression Omnibus database accession no. GSE31693) to provide an unbiased assessment, using ZuoC* and a previously identified hyperactive variant $Ssz1_{S295F}$ (Ssz1*) (8). The gene expression pattern of cells expressing ZuoC* or Ssz1* were strongly correlated with an R^2 value of 0.9886, indicating that similar mRNAs were up- and down-regulated in these strains. The TF consensus motifs and gene ontology of the up- and down-regulated genes were identified (Table 1 and Tables S2 and S3). The major classes of genes up-regulated were part of the PDR regulon. The target genes of two other TFs, Msn2/4 and Gcr1, were also identified as significantly up-regulated. Although the relationship between Ssz1/Zuo1 and Gcr1, which has been linked to growth control and filamentation (12), is not obvious, previous microarray analysis revealed that xenobiotic compounds inducing PDR up-regulated Msn2/4 targets in a Pdr1/3 dependent manner (12). The major gene ontology classes that were enriched in up-regulated genes included those associated with the plasma membrane, budding, transporters, and polarized growth (Table S2). The major pathways down-regulated are involved in ribosome synthesis (Table S3), consistent with the identification of the PAC element as a down-regulated TF binding site (Table 1) and previous results assessing effects of xenobiotic compounds (12). Based on this analysis, we conclude that ZuoC* and Ssz1* activate a similar set of genes, a major class of which are PDR associated.

Table 1. Transcription factor motifs enriched in ZuoC* and Ssz1* expression arrays

		t value[†]		
Name	Motif	ZuoC*	Ssz1*	ZuoC*+Ssz1*
PDR1/3	TCCGYGGA	**4.62**	**3.82**	**4.21**
PDR like	TCCGYGGR	**4.43**	**3.79**	**4.11**
Msn2/4	CCCCT	3.50	**4.76**	**4.26**
GCR1	CWTCC	**3.82**	**4.06**	**4.00**
PAC	CGATGAG	−2.26	**-4.08**	−3.34

[†]*t* values considered significant are indicated in bold.

Zuo1 and Ssz1 Interact with Pdr1 in a Yeast Two-Hybrid Assay. Because the PDR regulon was a target of Zuo1 and Ssz1, we decided to test for their interaction with Pdr1 using a two-hybrid system. We generated a fusion of the Gal4 DNA binding domain (GBD) to Pdr1 to use as bait. To avoid confusion in interpretation of results, we used a Pdr1 fragment lacking its DNA binding domain (DBD) and AD, yielding GBD–$Pdr1_{\Delta DBD\Delta AD}$. As prey, the Gal4 AD (GAD) was fused to ZuoC* or $Ssz1^*_{1–407}$, generating GAD–ZuoC* and GAD–$Ssz1^*_{1–407}$. Positive signals were obtained. Cells expressing GBD–$Pdr1_{\Delta DBD\Delta AD}$ and GAD–ZuoC* or GAD–$Ssz1^*_{1–407}$, but not GAD alone, grew in the absence of histidine, indicating activation of the *Gal1p–HIS3* reporter. The *Gal7p–lacZ* reporter was activated, as cells expressing GAD–ZuoC* and GAD–$Ssz1^*_{1–407}$ had sixfold and fivefold higher β-galactosidase activity, respectively, than cells expressing GAD alone (Fig. 2*A* and Table S4).

When setting up the two-hybrid system, we intended also to carry out two-hybrid testing using ZuoC and Ssz1 fused to GBD as bait. However, GBD–ZuoC strongly auto-activated reporter genes in the absence of the GAD. GBD–ZuoC expressing cells grew in the absence of histidine and adenine, indicating activation of both *Gal1p–HIS3* and *Gal2p–ADE2* reporters. β-galactosidase activity was 50-fold higher in cells expressing GBD–ZuoC than GBD alone (Fig. 2*B* and Table S5). In addition, cells expressing GBD–ZuoC* grew more robustly on plates lacking histidine and adenine and showed approximately twofold higher β-galactosidase activity than those expressing GBD–ZuoC. We confirmed that the enhanced activity of GBD-ZuoC* was not due to increased expression (Fig. S2). We reasoned that an interaction between GBD–ZuoC and Pdr1 might eliminate the requirement for the GAD leading to auto-activation. Consistent with this idea, GBD–ZuoC cells containing an extra plasmid copy of *PDR1* grew more robustly on medium lacking histidine and adenine and had twofold higher β-galactosidase activity than cells having a single *PDR1* gene. In addition, *pdr1*Δ cells expressing GBD–ZuoC did not grow on medium lacking histidine and adenine. β-galactosidase activity was reduced to 19 units compared with 48 units in cells containing Pdr1. This residual activity indicates some Pdr1-independent activity. However, together, these results indicate that auto-activation of *GAL* promoters by GBD–ZuoC is largely dependent on Pdr1.

The fusion between GBD and $Ssz1^*_{1–407}$ also auto-activated the *GAL* reporter constructs in a Pdr1-dependent manner (Fig. 2*C* and Table S5), but to a lesser extent than the GBD–ZuoC fusion. No growth on selective plates was observed in *pdr1*Δ cells, and the β-galactosidase activity was at the basal level found in cells expressing GBD alone. Together, these two-hybrid results are consistent with an interaction of Zuo1 and Ssz1 with Pdr1. To support the idea that the activation of Pdr1 by Ssz1/Zuo1 likely occurs by direct action, chromatin immunoprecipitation (ChIP) was carried out by using strains expressing Ssz1*. The presence of

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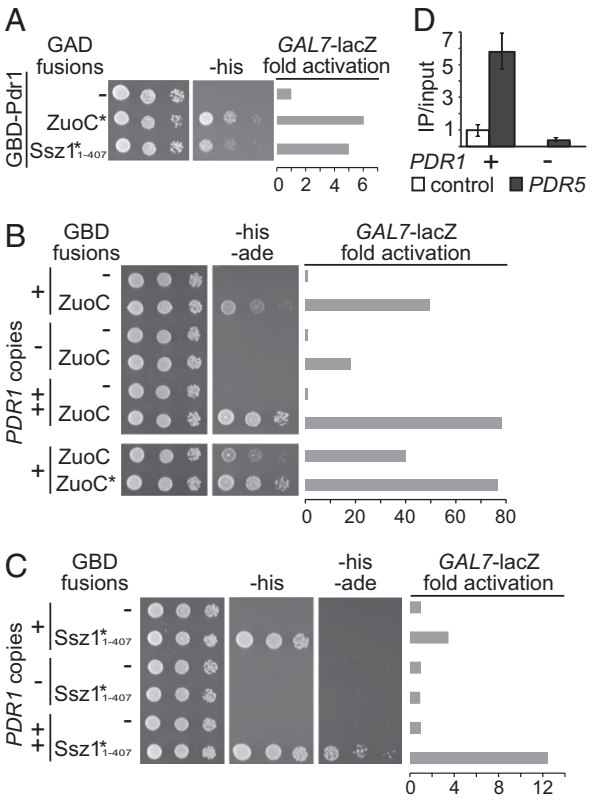

Fig. 2. Interaction of ZuoC, ZuoC*, and Ssz1*$_{1–407}$ with Pdr1. (*A*–*C*) Two-hybrid. (*A*) Interaction between Pdr1 and Ssz1/Zuo1. WT cells were transformed with a plasmid containing DNA encoding GBD fused to residues 76–965 of Pdr1 (GBD–Pdr1) and a second plasmid containing either DNA encoding the GAD (–) or GAD fused to ZuoC* or Ssz1*$_{1–407}$. Serial dilutions of cells containing the indicated plasmids were spotted onto medium lacking histidine (–his). The average β-galactosidase activity of three transformants of each was measured and reported as fold activation of experimental/control (GAD fusion/GAD). (*B* and *C*) ZuoC, ZuoC*, or Ssz1*$_{1–407}$ tethered to the GBD activate *GAL* promoters in a Pdr1-dependent manner. Cells carrying varying copies of *PDR1* were created by transforming WT cells with either empty vector (+) or a centromeric plasmid containing *PDR1* (++) or transforming *pdr1Δ* cells with empty vector (–). These cells were transformed with a second plasmid containing DNA encoding GBD (–), or GBD fused to codons for residues 365–433 of WT Zuo1 (ZuoC), ZuoC having the S427G alteration (ZuoC*), or 1–407 of Ssz1 containing the S295F alteration (Ssz1*$_{1–407}$). Serial dilutions of cells harboring the indicated plasmids were spotted onto medium lacking histidine (–his) or histidine and adenine (–his –ade). The average β-galactosidase activity of two to three transformants of each was measured and reported as fold activation of experimental/control (GBD fusion/GBD). (*D*) ChIP. DNA precipitated by Ssz1-specific antibodies from strains having (+) or lacking (–) *PDR1* and expressing Ssz1* was analyzed by qPCR using primer pairs designed to amplify the *PDR5* promoter (*PDR5*) and a region 1.5-kb upstream (control). The IP signal relative to input DNA was calculated for each location. The mean and SEs of the resulting IP/Input from three PCR measurements are plotted.

Ssz1 at the *PDR5* promoter and, as a control, a region 1.5 kb upstream, was evaluated by precipitating DNA using Ssz1-specific antibodies followed by quantitative PCR (qPCR). Increased levels of Ssz1 were detected at the *PDR5* promoter compared with the upstream region relative to a control strain deleted for *SSZ1* (Fig. 2*D*). This increase was dependent on the presence of Pdr1, because no precipitation above background levels was found in *pdr1Δ* cells. Together, these results support the idea that Ssz1/Zuo1 function at the *PDR5* promoter in a Pdr1-dependent manner.

Activation of the PDR Regulon Promotes Early Growth Arrest at the Diauxic Shift. The ability of Zuo1 and Ssz1 to activate the PDR regulon in a Pdr1-dependent manner led us to ask whether such induction plays a physiological role. During our investigations, we noted that overnight cultures of cells expressing ZuoC* attained a lower cell number during the diauxic shift, the time during which glucose becomes depleted and growth arrests as cells transition to the utilization of nonfermentable carbon sources (13). We compared the growth of cells expressing ZuoC* with that of control cells. Overnight cultures of cells were diluted and grown in synthetic medium at 30 °C, with samples removed periodically over the next 25 h (Fig. 3*A*). The cultures grew at the same rate over the first 10 h. However, growth of ZuoC*-expressing cells transiently plateaued at an optical density at 600 nm (OD_{600}) of ∼2.8 after 13 h. Cells expressing the control vector continued dividing for an additional 2–3 h, plateauing at an OD_{600} of ∼3.3. To confirm that the lower OD_{600} in the cells expressing the PDR-inducing construct corresponded to a lower cell number, cells present in the cultures 15 h after dilution were counted. Cultures expressing ZuoC* had 19% fewer cells than those carrying the control vector. Early growth arrest was also seen in cultures of cells expressing Ssz1* (Fig. S3). To address whether this difference in cell number as cells arrest at the diauxic shift was dependent on Pdr1, we also tested a *pdr1Δ* strain. Both control and ZuoC*-expressing *pdr1Δ* cells reached the diauxic shift plateau at an OD_{600} of 3.3, similar to WT cells (Fig. 3*A*). Although the exact plateau OD_{600} reading varied slightly between batches of medium, the plateau value for the PDR activating strain was consistently lower than that of the three other strains. Thus, we conclude that the earlier transient growth arrest that occurs when ZuoC* is expressed is due to the activation of Pdr1.

Overgrowth of Cells Lacking Zuo1/Ssz1 Is Mitigated by Pdr1 Activation. Because Pdr1 activation by Zuo1 or Ssz1 leads to growth arrest at a lower cell density, we asked whether the absence of these two proteins affected the density at which growth plateaued. Although *zuo1Δssz1Δ* cells grew more slowly than WT cells in exponential phase, they entered the diauxic shift at a higher OD_{600} (4.0 compared with 3.5) (Fig. 3*B*). Consistent with the higher OD_{600}, 29% more cells were present in the *zuo1Δssz1Δ* culture than the WT culture after 25 h of growth, when both cultures had reached the diauxic shift. To test the effect of Pdr1 activity on the response of cells to the absence of Zuo1 and Ssz1, *zuo1Δssz1Δ* cells were treated 10 h after dilution with progesterone, a xenobiotic known to activate Pdr1 (11) (Fig. 3*B*). Overgrowth was mitigated; *zuo1Δssz1Δ* cells treated with progesterone plateaued at an OD_{600} of ∼3.4, similar to WT cells.

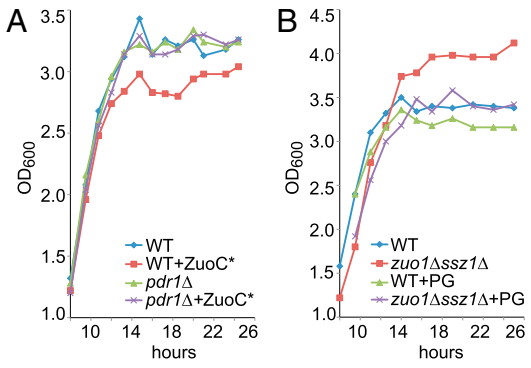

Fig. 3. Effect of Pdr1 activation on the cell density at which growth arrest occurs at the diauxic shift. Indicated cells were diluted to an OD_{600} of 0.2 in synthetic medium, and the OD_{600} was monitored for the subsequent 25 h. (*A*) WT or *pdr1Δ* cells were transformed with the control vector containing DNA encoding the TAP tag (no designation) or vector encoding TAP–ZuoC* (ZuoC*). (*B*) Two cultures of WT and *zuo1Δssz1Δ* cells were grown as above. One of each was treated with progesterone (PG) 10 h after dilution of the culture.

Addition of Conditioned Medium Alters Growth at the Diauxic Shift. The results presented above suggested to us that activation of Pdr1 by Zuo1 and Ssz1 plays a role in regulating the timing of growth arrest as cells approach the diauxic shift. Because Pdr1 regulates the expression of membrane transporters that extrude small molecules from cells, we decided to test the idea that the medium of WT cells contained molecules that signal growth arrest—that is, play a role in "quorum sensing". WT and *zuo1*Δ*ssz1*Δ cells were diluted to an OD_{600} of 0.2 and grown for 14 h. Cells were then pelleted. As controls, WT and *zuo1*Δ*ssz1*Δ cells were resuspended in medium in which they were grown (Fig. 4*A*), and further growth was monitored. As expected, *zuo1*Δ*ssz1*Δ cells attained an OD_{600} of ~4.1, whereas WT cells plateaued at 3.4. However, the growth of Δ*zuo1*Δ*ssz1* cells resuspended in "conditioned" medium from WT cells rapidly plateaued, only reaching an OD_{600} at the diauxic shift of 3.5. This cessation of growth is consistent with the presence of factors in the WT-conditioned medium that signal arrest.

Cells lacking the membrane transporters Pdr5 and Snq2, whose expression is regulated by Pdr1, have been reported to overgrow at the diauxic shift (14). Therefore, we extended our analysis to test whether these specific transporters play a role in signaling growth arrest. Cultures of WT and *pdr5*Δ*snq2*Δ cells approaching the diauxic shift were harvested at an OD_{600} of 2.5 and resuspended, either in the medium in which they were grown or the conditioned medium retrieved from the other culture (Fig. 4*B*). The overgrowth of the *pdr5*Δ*snq2*Δ cells when resuspended in the medium in which they were grown was more dramatic than that of *zuo1*Δ*ssz1*Δ cells; they attained an OD_{600} of 6.8 after 11 h of further incubation. When resuspended in conditioned medium from WT cells, however, growth of *pdr5*Δ*snq2*Δ cells plateaued at an OD_{600} of 3.2, only slightly higher than WT cells. Conversely, WT cells resuspended in *pdr5*Δ*snq2*Δ-conditioned medium continued to grow slowly, reaching an OD_{600} of 4 after 11 h. These results are consistent with the presence of a factor (s) in the WT-conditioned medium that promotes inhibition of cell division and that medium in which *zuo1*Δ*ssz1*Δ or *pdr5*Δ*snq2*Δ cells have been grown contain a lower concentration of this factor(s). As a preliminary assessment of the nature of these factors, medium harvested from WT cells was subjected to one of two treatments, boiling or dialysis, before adding to *pdr5*Δ*snq2*Δ. Consistent with the factor(s) being small molecules, cells resuspended in dialyzed medium overgrew, reaching an OD_{600} of 4.5 at 10 h after addition, compared with 5 and 3 for *pdr5*Δ*snq2*Δ and WT medium, respectively (Fig. S4).

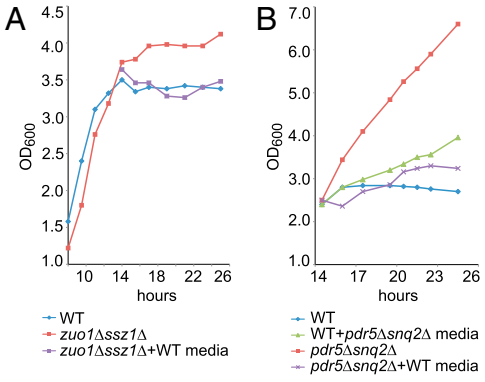

Fig. 4. Addition of conditioned medium alters growth at the diauxic shift. Indicated cells were diluted to an OD_{600} of 0.2. After 14 (*A*) or 15 (*B*) h, cultures were spun down, and the conditioned medium was harvested. Cells were resuspended in the indicated conditioned medium and growth monitored for the ensuing 11 (*A*) or 10 (*B*) h.

Conversely, boiled medium was as efficient as untreated medium in suppressing overgrowth.

Discussion

As discussed below, a picture emerges from the results presented here consistent with the ideas that: (*i*) Activation of Pdr1 by Zuo1 and Ssz1 plays a role in regulating cell growth upon nutrient depletion at the diauxic shift; and (*ii*) transporters of the plasma membrane, encoded by Pdr1 target genes, export quorum molecules sensed by other cells in the culture leading to growth arrest.

Activation of Pdr1 by Ssz1 and Zuo1. Expression of either the C-terminal 69 amino acids of Zuo1 or the ATPase domain of Ssz1 affects gene expression in very similar ways. Both cause the upregulation of genes of the PDR regulon in a Pdr1-dependent manner. The two-hybrid and ChIP data reported here suggest that this activation may be direct, involving an interaction of these proteins with Pdr1, leading to mobilization of its transcription AD that is normally sequestered by its endogenous inhibitory region. Such a mechanism is reminiscent of the activation of Pdr1 by xenobiotics, which bind Pdr1/Pdr3 in the internal "xenobiotic binding domain" (11), releasing the inhibition of the transcriptional AD.

The specificity of the activation of Pdr1 by Zuo1 and Ssz1 is intriguing. Although xenobiotics activate both Pdr1 and Pdr3 (11), Zuo1 and Ssz1 activate Pdr1, but not Pdr3. Conversely, Pdr3, but not Pdr1, is activated by a yet-to-be-defined signal present in cells lacking a functional mitochondrial genome (10). Analysis of the genome organization surrounding the *PDR1* and *PDR3* genes indicates that this gene pair is a product of the whole-genome duplication that occurred in an ancestral genome of *Saccharomyces cerevisiae* (15). The differences between Pdr1 and Pdr3 may represent specialization for physiologically important signaling events, whereas both TFs retained the basic property of being activated by xenobiotics.

It is also worth noting that domains of Zuo1 and Ssz1 are independently capable of activating Pdr1. Because Ssz1 and Zuo1 form a very stable heterodimer (16, 17), it is reasonable to posit that under normal physiological conditions, they activate Pdr1 as a complex. It is also possible that the heterodimer is destabilized in response to some yet-unknown signal, allowing them to act independently. Further work is required to understand the complexities of the relationship between the activation of Pdr1 by Zuo1 and by Ssz1.

Physiological Role of Activation of Pdr1 at the Diauxic Shift by Ssz1/Zuo1. Based on the results presented here, which link Pdr1 activation to the transition point of the diauxic shift, we propose a model in which the density at which yeast cells arrest growth, transitioning to an environment of less favorable nutrients, depends in part on the level of small molecules in the surrounding medium. The efflux of these quorum-sensing molecules from cells in which they were synthesized is mediated by plasma membrane transporters such as Snq2 and Pdr5. According to this model, the concentration of these regulatory molecules in the medium is dependent upon the level of such transporters. Consistent with this model, *pdr5*Δ*snq2*Δ cells arrest at a very high cell density. As expression of these transporters is regulated in part by Pdr1, activation by either Zuo1/Ssz1 or xenobiotics results in growth arrest at a lower cell density, because more autoregulatory molecules are exported from cells. Interestingly, a recent bioinformatics analysis of several genome-wide studies identified Pdr1 as one of handful of TFs predicted to be involved in quorum sensing (18).

Sensing of signals in response to cell density has been extensively studied in bacteria. In fact, cell:cell signaling related to regulating the cell density attained by bacterial cultures has been

linked to multidrug transporters in *Escherichia coli* (19). Studies on cell:cell communication in eukaryotic unicellular organisms are more limited. Nevertheless, several intercellular signal transduction pathways have been identified in fungi. In the dimorphic fungal human pathogen *Candida albicans* aromatic alcohols have been linked to nitrogen limitation and yeast-to-filamentous growth transition (20). The very limited number of reports of quorum sensing in *S. cerevisiae* is likely due to the fact that standard yeast strains, such as those used here, do not undergo filamentous growth because of a mutation in the *FLO8* gene, which is needed for invasive and filamentous growth (21). However, using a truly WT strain, Chen and Fink (22) uncovered a quorum-sensing signaling pathway responsive to aromatic alcohols that regulates filamentation in response to both cell density and nutrient availability. Interestingly, in this strain background, *ZUO1* was identified in a screen for genes involved in filamentous growth (23). Future studies in such truly WT strains may be required to determine whether the role of the Ssz1 and Zuo1 chaperones in quorum sensing relates to the regulation of filamentous growth and nitrogen metabolism, an unappreciated cellular response to glucose deprivation, or an unknown signaling pathway.

Universal Role of Zuo1:Ssz1 Orthologs in Transcriptional Regulation in Eukaryotes. The C-terminal regions of Zuo1 and Ssz1 in fungi differ from those of other eukaryotic orthologs (24). However, it is intriguing that Zuo1 orthologs in most other eukaryotes have Myb/SANT domains, well-established DBDs, at their C termini. These domains of Zuo1 orthologs have been implicated in regulation of asymmetric cell division in blue-green algae (25) and in nematodes (26). In yeast, our evidence supports the idea that molecular chaperones regulate cell:cell communication through modulation of the PDR pathway. Future study will be required to define the similarities and differences in mechanisms of action of these molecular chaperones in cell:cell communication in diverse organisms. Regardless, this conserved connection between the ribosome, the cellular center of protein synthesis, and the regulation of growth control/development raises the intriguing possibility of regulatory connections coordinating the regulation of protein synthesis and growth control/development.

Materials and Methods

Genetic Methods. Most yeast strains used were isogenic with DS10 (*his3-11, 15 leu2-3, 112 lys1 lys2 trp1Δ ura3-52*). *zuoΔ1::HIS3 ssz1Δ::LYS2* was published (27); *pdr5*(Δ)::*URA3 snq2*(Δ)::*TRP1*, *pdr1Δ::TRP1*, and *Δpdr3::HIS3* were made by transforming a PCR product generated from amplifying the *URA3* marker from pRS306 or *TRP1* marker from pRS304 or *HIS3* marker from pRS303 using primers that contain homology immediately upstream and downstream of the gene to be deleted. Integration was confirmed by PCR. *PDR5–lacZ* and *YOR1–lacZ* were described (24). TAP and TAP–ZuoC plasmids were created by PCR amplifying codons for an N-terminal TAP tag and Zuo1 residues 365–433 and cloning into pRS415GPD by using SpeI and BamHI (TAP) and BamHI and SalI (ZuoC). ZuoC* was created by site-directed mutagenesis using S427G mutagenic primers. Full-length Ssz1 (BamHI and SalI) was cloned into the pRS415 GPD vector by using BamHI and SalI. Ssz1* was created by introducing S295F into this vector using site-directed mutagenesis.

To obtain a hyperactive *ZUO1* allele, mutagenic PCR with high magnesium was used to generate PCR products in the codons encoding the C-terminal 69 residues. These products were then used in a QuikChange reaction to generate a library of plasmids in pRS315 $Zuo1_{\Delta 285–364}$ (28). The library was transformed into DS10, and candidates able to grow in the presence of 1 μg/mL cycloheximide were selected. Plasmid from each candidate was recovered, sequenced, retransformed, and then assayed for induction of the *PDR5–lacZ* reporter.

To assay drug resistance, approximately equal numbers of cells were subjected to 10-fold serial dilutions and spotted on medium: selective minimal glucose, containing either 0, 0.7, or 1 μg/mL cycloheximide or YPGE (1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol) containing 0 or 1.5 μg/mL oligomycin. Plates were incubated at 30 °C for 2–3, 4, or 7–11 d on minimal, YPGE, or YPGE + oligomycin medium, respectively, before photographing. β-galactosidase assays were performed as described (7); the average of three independent transformants was reported.

Tiled Microarray. A 20-μg sample of total RNA (prepared by pooling equal quantities of three biological replicates) was digested with RQ1 DNase (Promega). Reactions were extracted with phenol/chloroform/isoamyl alcohol, followed by chloroform and then precipitated. RNA quality was assessed by using the Agilent 2100 Bioanalyzer. 10 μg RNA was used to prepare labeled cDNA using SuperScript II (Invitrogen) followed by the one-color Cy3 labeling kit (NimbleGen). Labeled sample was hybridized to a 12 × 137K NimbleGen *S. cerevisiae* array (05543835001) by using the manufacturer's recommended conditions. Arrays were scanned by using the MS 200 Microarray Scanner and MS 200 Data Collection Software. Probes were averaged, and samples were quantile normalized with a log2 transformation and analyzed by using ArrayStar (DNAstar). T-profiler (http://www.t-profiler.org/) was used to identify TF binding motifs and gene ontogeny (29).

Two-Hybrid and ChIP. Strain background PJ69, a derivative of W303, was used. GBD and GAD plasmids and the two-hybrid methods are published (30). *pdr1Δ::TRP1* was created by amplifying the integrated *pdr1Δ::TRP1* cassette from DS10 *pdr1Δ::TRP1* genomic DNA and transforming into PJ69. GBD–ZuoC and GBD–$Ssz1^*_{1–407}$ plasmids were created by PCR amplification of DNA encoding Zuo1 residues 365–433 and Ssz1 residues 1–407 containing the S295F mutation and cloning into pGBDU-C1 using BamHI and PstI. GBD–ZuoC* was created by site-directed mutagenesis using S427G primers. GBD–$Pdr1_{\Delta DBD}$ was created by PCR amplification of DNA encoding Pdr1 residues 76–1063 and cloning into pGBD-C1 using ClaI and SmaI. $pdr1_{\Delta DBD\Delta AD}$ was created by removing DNA encoding residues 965–1063. GAD–ZuoC* and GAD–$Ssz1^*_{1–407}$ were created by PCR amplification of each and cloning into pGAD-C1 using BamHI and SacI. Cells were grown for 2 d at 30 °C on minimal medium lacking uracil, leucine, or tryptophan to select for the respective plasmids and lacking either histidine and adenine or histidine and containing 2 mM 3-aminotriazole to detect activation of *Gal2p–ADE2 and Gal1p–HIS3* reporters. At least two independent transformants were measured for β-galactosidase activity of the *GAL7–lacZ* reporter and averaged.

ChIP was carried out as described (31). Strains used were deleted for *ZUO1* to ensure that Ssz1 was not ribosome-associated and carrying Ssz1* on pRS316. Briefly, cultures at 0.6 OD_{600} were treated with formaldehyde to induce cross-linking. Lysates were sonicated to shear genomic DNA and then incubated with Ssz1-specific antibody followed by precipitation with Protein A-agarose beads. After washing, cross-linking was reversed by incubation overnight at 65 °C. DNA was then precipitated and subjected to qPCR. The background signal from a control experiment using cells lacking *SSZ1* was subtracted from experimental values obtained. Target DNA in the sample was quantified by generating a standard curve with a fourfold dilution series of the input DNA for each sample.

Growth Curves. Overnight cultures were diluted to an OD_{600} of 0.2 into synthetic complete medium (0.67% yeast nitrogen base with ammonium sulfate and without amino acids, 2% dextrose, supplemented with all amino acids). Leucine was omitted in the case of TAP vectors. 5-mL cultures were grown at 30 °C with shaking, and OD_{600} was measured between 8 and 25 h after dilution. Each experiment was performed a minimum of five times. Progesterone (Sigma) was diluted to 0.1 M in ethanol and added to the yeast culture after 10 h of growth for a final concentration of 100 μM.

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