Inositol phosphates are key signaling molecules affecting a large variety of cellular processes. Inositol-polypolyphosphate multikinase (IPMK) is a central component of the inositol phosphate biosynthetic routes, playing essential roles during development. IPMK phosphorylates inositol 1,4,5-trisphosphate to inositol tetrakisphosphate and subsequently to inositol pentakisphosphate and has also been described to function as a lipid kinase. Recently, a catalytically inactive mammalian IPMK was reported to be involved in nutrient signaling by way of mammalian target of rapamycin and AMP-activated protein kinase. In yeast, the IPMK homologue, Arg82, is the sole inositol-trisphosphate kinase. Arg82 has been extensively studied as part of the transcriptional complex regulating nitrogen sensing, in particular arginine metabolism. Whether this role requires Arg82 catalytic activity has long been a matter of contention. In this study, we developed a novel method for the real time study of promoter development. IPMK phosphorylates inositol 1,4,5-trisphosphate to phosphatidylinositol 3,4,5-trisphosphate (9, 10). The function of IPMK is highly conserved in all eukaryotes, and its importance is underlined by the embryonic lethality observed in homozygous knock-out mice (8). It has also been demonstrated that IPMK acts as a phosphatididylinositol 3-kinase (P13K) in vivo, converting phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (9, 10). In yeast, the IPMK homologue Arg82 (or Ipk2) is the sole inositol-trisphosphate kinase (11). It was originally identified in a screen for mutants unable to grow on alternative nitrogen sources (arginine/ornithine) (12). Arg82 participates in the regulation of genes involved in arginine synthesis and degradation. It does so by stabilizing the trimeric complex, Arg80-Arg81-Mcm1, which binds to specific promoters possessing the consensual sequences designated “arginine boxes” (13). A long running controversy surrounds the requirement for Arg82 catalytic activity in regulating arginine metabolism, particularly the role of putative inositol phosphates produced by Arg82 (14, 15).

Very little is known about how IPMK itself is regulated. A recent publication indicates that regulation of IPMK may take place by altering its nuclear/cytoplasmic localization (16). In addition, it has recently been discovered to interact with two

**Background:** The involvement of inositol phosphates produced by the yeast inositol-polypolyphosphate multikinase, Arg82, in transcription is controversial.

**Results:** Catalytically inactive Arg82 restores the regulation of arginine-dependent genes in an ARG82 knock-out.

**Conclusion:** Inositol phosphates do not regulate arginine-dependent gene expression.

**Significance:** Independently of its enzymatic activity Arg82 controls arginine-responsive genes.

Inositol phosphates are family of soluble molecules which play a central role in cell signaling (1–3). The large variety of inositol phosphate species stems from the attachment of one or more phosphate groups to the six carbon myo-inositol ring. Inositol hexakisphosphate (also known as phytic acid) presents orthophosphate groups in all six carbons. More complex forms of inositol phosphates also exist, e.g. inositol pyrophosphates where one or several carbons hold pyrophosphate groups (4). The array of soluble inositol polyphosphate molecules depends on the activity of several inositol kinases and phosphatases (5). The many inositol phosphate species present in the cytosol and nucleus of eukaryotic cells constitute a metabolically interconnected grid, regulating almost every aspect of cell physiology. The foremost member of such network is the second messenger inositol 1,4,5-trisphosphate, which is involved in the release of calcium from intracellular stores. This mechanism provides one of the best characterized examples of signal transduction (6).

The phosphorylation of inositol 1,4,5-trisphosphate into more complex forms is achieved by inositol kinases of which inositol-polypolyphosphate multikinase (IPMK) is one of the most relevant. IPMK phosphorylates at positions 3 and 6, converting inositol 1,4,5-trisphosphate to inositol tetrakisphosphate and then inositol tetrakisphosphate to inositol pentakisphosphate (7). The function of IPMK is highly conserved in all eukaryotes, and its importance is underlined by the embryonic lethality observed in homozygous knock-out mice (8). It has also been demonstrated that IPMK acts as a phosphatididylinositol 3-kinase (P13K) in vivo, converting phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (9, 10). In yeast, the IPMK homologue Arg82 (or Ipk2) is the sole inositol-trisphosphate kinase (11). It was originally identified in a screen for mutants unable to grow on alternative nitrogen sources (arginine/ornithine) (12). Arg82 participates in the regulation of genes involved in arginine synthesis and degradation. It does so by stabilizing the trimeric complex, Arg80-Arg81-Mcm1, which binds to specific promoters possessing the consensual sequences designated “arginine boxes” (13). A long running controversy surrounds the requirement for Arg82 catalytic activity in regulating arginine metabolism, particularly the role of putative inositol phosphates produced by Arg82 (14, 15).

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□ This article contains supplemental Figs. S1–57.

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**References**

1. Bosch, D., and Saiardi, A. (2012) Arg82-dependent regulation of the arginine-dependent transcriptional complex regulating nitrogen sensing, in particular arginine metabolism. J. Biol. Chem. 287, 38347–38355.

2. The abbreviations used are: IPMK, Arg82, and Ipk2, inositol-polypolyphosphate multikinase; Kc1, inositol-hexakisphosphate and inositol-heptakisphosphate kinase; PLC and Plc1, phospholipase C; TOR, target of rapamycin; Arg3, ornithine carbamoyltransferase; Arg5,6, acetyleulatamine kinase; HSV1-TK, herpes simplex virus 1 thymidine kinase; FIAU, fialuridine.
Catalytically Inactive Arg82 Restores Arginine Metabolism

### TABLE 1

| Strain      | Relevant genotype     | Source/Ref. |
|-------------|-----------------------|-------------|
| BY4741      | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | 21          |
| arg82Δ      | BY4741 arg82::kanMX4  | 21          |
| plc1Δ       | BY4741 plc1::kanMX4   | 21          |
| kcs1Δ       | BY4741 kcs1::kanMX4   | 21          |
| arg82Δkcs1Δ | BY4741 arg82::kanMX4 plc1::Leu2 | 10          |
| arg82Δkcs1Δ | BY4741 arg82::kanMX4 kcs1::kanMX4 | This study |

### EXPERIMENTAL PROCEDURES

**Strains**—Saccharomyces cerevisiae strains used in this study are isogenic to BY4741 and described in Table 1. Knock-out mutants arg82Δ, plc1Δ, kcs1Δ, and arg82Δ plc1Δ have been described earlier (10, 21); arg82Δ kcs1Δ was generated for this study by crossing kcs1Δ and arg82Δ strains.

**Plasmids**—ARG82 (YDR173C) was amplified from S. cerevisiae BY4741 genomic DNA with SpeI/HindIII restriction sites and ligated into the T Easy vector (Promega). Subsequently, it was used as a template to generate ARG82-DK/AA (D131A, K133A) and ARG82-SDST/ADAA (S95A, S97A, T98A) mutations via QuikChange site-directed mutagenesis (Stratagene). Arabidopsis thaliana Ipk2β was cloned from genomic cDNA (a gift from C. Azevedo) with BamHI/PstI restriction sites inserted into a T Easy vector and used as a template to generate Atlpk2β-D/A (D100A) (QuikChange). The constructs were subcloned into p425-MET25, a vector with a methionine-regulated promoter (22). Human IPMK and a kinase-dead variant, IPMK-DK (D144A, K146A), were subcloned from human vectors (a gift from A. Resnick) with BamHI/Sall restriction sites into p425-MET25. Point mutations were confirmed by sequencing, and plasmid presence was confirmed by complementation of the leucine auxotrophy. pYES-HSV1-TK plasmid was generated by cloning the herpes simplex virus I thymidine kinase (HSV1-TK) gene obtained from pKHTK (23) into a pYES2 plasmid (Invitrogen) with HindIII/BamHI sites. A SpeI kinase (HSV1-TK) gene obtained from pKHTK (23) into a plasmid was used as a template to generate ARG82-DK/AA

**Experimental Procedures**

**Growth Tests in Solid Medium**—Strains were precultured overnight in synthetic complete medium (yeast nitrogen base without amino acids; Formedium) with the addition of auxotrophic amino acids and 110 mM glucose. The next day, cells were washed three times with water to remove traces of ammonium sulfate and then diluted to an optical density (OD) of 1. This dilution was used to make 6 × 10-fold dilutions, 5 μl of which were spotted on agar plates containing synthetic complete medium with ammonium, arginine, or ornithine as the sole nitrogen source. Plates were incubated at 30 °C for 48 h.

**Growth Tests in Liquid Medium**—Strains were cultivated overnight as described above. The next day, fresh medium was added to each overnight culture, which was then grown for 4 h. Cells were harvested, washed three times with minimal synthetic medium (yeast nitrogen base without amino acids or ammonium sulfate; Formedium), diluted to an OD of 0.05, and inoculated into minimal synthetic medium plus 110 mM glucose and either 38 mM ammonium (5 g/liter), 5.7 mM arginine (1 g/liter), or 15 mM ornithine (2 g/liter) as the sole nitrogen source. Cultivations were performed in a 384-well plate with a volume of 20 μl/well. The plate was shaken for 1 min every 2 min, and cell density was measured every 30 min using a plate reader with temperature control set at 30 °C (Infinite F200, Tecan). Cultivations were carried out for 25–70 h depending on the experimental conditions.

**Growth Rate Quantification**—To calculate the maximum growth rate, the raw OD was treated as described by Warringer et al. (24, 25). First, OD was normalized, and the initial value at time 0 was subtracted from all of the following values. Second, to account for a nonlinear correlation between OD and cell density at high cell densities, OD values were calibrated according to the following formula: Calibrated OD = OD + 0.8324 × OD². Third, growth curves were smoothed to reduce contributions from noise by averaging (nonweighted averaging) over three consecutive measurements (no averaging of initial value). Fourth, artifacts arising from nonbiological events were avoided by removing measurements yielding negative slopes. The growth rate was calculated by el(log) transforming smoothed growth curves. Slopes were calculated between every pair of values spaced 90 min apart along the curve. No slopes were calculated from the eight initial time points to filter for digitization effects. Of the seven highest slopes, the top two were discarded, and a mean was calculated from the remaining five.

**Calculation of Differences in Promoter Expression**—To compare the influence of promoter expression on different nitrogen sources using the HSV1-TK assay, we developed the following method. The maximum slope of the growth curve for either ammonium or arginine was calculated using the above described method (25). The tangents to the slopes were drawn, and the corresponding intersection point was determined (y1 = y2) (supplemental Fig. S1). The area of the triangle formed was calculated using the following formula: Area = 1⁄2(h × b) where h (height) corresponds to the x axis distance between the points at which the lines intersected (x1) with the last time point in the experiment (x2) and b (base) corresponds to the y axis absorbance segment (y2 − y1) obtained at the last time point (x2). Because arg82Δ showed a novel decrease in growth in arginine with respect to ammonium, we introduced a correction in the calculations. The area of the triangle formed by ammonium and arginine growth in DMSO was added to that calculated.
ARG82 Activity Plays a Minor Role during Growth in Ornithine—We took advantage of the ability of arg82Δ to grow relatively well in ammonium but poorly in ornithine to test whether its catalytic activity played a role in nitrogen regulation. We transformed arg82Δ with a plasmid expressing a version of Arg82 devoid of enzymatic activity (two point mutations turned aspartate 131 and lysine 133 to alanines, therefore disrupting the inositol binding domain) (14) and tested solid and liquid growth in ammonium, arginine, and ornithine. As shown in Fig. 2, A and B, catalytically inactive Arg82 was able to substantially restore growth in arginine and ornithine. Note, however, that it did not fully complement growth on ornithine. This suggested that Arg82 devoid of its enzymatic activity plays a role in the adaptation of the cell to alternative nitrogen sources. The expression of plasmids was generally confirmed by growth in medium lacking the corresponding amino acid marker. We further confirmed that Arg82 was expressed in the cells by detecting a tagged version by means of Western blot (data not shown).

We tested whether the lack of soluble inositol phosphates may play a role during growth in alternative nitrogen sources. In budding yeast, knock-out of the only phospholipase C (Plc1) prevents the generation of soluble inositol phosphate species. We tested the growth of plc1Δ in solid medium and observed a growth reduction similar to that for the wild type when switching from ammonium to ornithine medium (Fig. 2C and supplemental Fig. S3). Deletion of KCSI, which blocks synthesis of most inositol pyrophosphates (diphasphoinositol pentakisphosphate and bisdiphasphoinositol tetrakisphosphate), did not affect the growth in ornithine. Finally, we tested the growth

ARG82 Activity Plays a Minor Role during Growth in Ornithine
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FIGURE 2. Growth of yeast strains in different nitrogen sources. Growth in minimal synthetic medium containing ammonium, arginine, or ornithine as the sole nitrogen source is shown. A, yeast strains transformed with a either an empty plasmid (p) or expressing catalytically active or inactive (inositol phosphate binding domain-disrupted) Arg82. Growth assays were performed in solid medium as indicated in Fig. 1A. B, quantification of the growth rate in arginine and ornithine with respect to the maximum achieved in ammonium for the indicated strains. The growth rate was quantified as the average of the highest slopes of five growth curves. Error bars correspond to S.D. C, yeast strains bearing deletions of genes encoding either inositol polyphosphate multikinase (Arg82), phospholipase C (Plc1), inositol-hexakis- and -heptakisphosphate 6-kinase (Kcs1), or a combination of the above. Growth assays were performed in solid medium as indicated in Fig. 1A.

ability of double knock-outs arg82Δ plc1Δ and arg82Δ kcs1Δ and found that growth corresponded to the arg82Δ phenotype.

Arg82 Homologues Expressed in arg82Δ Restore Growth in Ammonium but Not in Ornithine—It has been shown that mammalian and plant IPMK homologues are able to restore growth of arg82Δ in rich medium (27, 28). This is probably achieved through their catalytic activity because their sequence identities with respect to Arg82 are very low. Human IPMK has been shown to phosphorylate both soluble and lipid inositols, whereas its plant counterpart only metabolizes inositol phosphates. We tested whether any of these homologues would be able to restore growth in arginine or ornithine. As seen in Fig. 3, both rescued growth in ammonium and did so partially in arginine. However, they were unable to complement the arg82Δ growth phenotype in ornithine. This was not due to a defect in general growth because plant and particularly human IPMKs were able to restore normal growth in ammonium in liquid culture (supplemental Fig. S4). Instead, these results suggest that Arg82 structure is more important than its catalytic activity for regulating growth in ornithine.

A Novel Method to Measure Real Time Promoter Strength—The growth tests presented so far recapitulated the overall defects of arg82Δ without making a distinction between its general effect on well being or its specific role as transcriptional regulator. To understand the specific role that Arg82 catalytic activity might play in stabilizing the Arg80-Mcm1-Arg81 complex, we looked at the regulation of promoters containing arginine boxes (supplemental Fig. S5). We developed a new technique that allows measurements of promoter strength in vivo by assaying cell growth. To that end, we constructed a plasmid bearing the promoter of interest fused to the gene encoding HSV1-TK. HSV1-TK has greater affinity than the eukaryotic thymidine kinase for nucleoside analogues like acyclovir or fialuridine, which are incorporated into DNA strands upon phosphorylation, blocking further extension (Fig. 4A). The growth rate of budding yeast will thus be inversely proportional to the strength of the promoter controlling HSV1-TK expression. FIAU was chosen as the nucleoside analog for its efficiency and relatively low cost.

The overall growth of a yeast strain carrying a plasmid with HSV1-TK under the control of a strong promoter, ADH1, is shown in Fig. 4B. The decrease in growth rate correlated well with the concentration of FIAU. To prove that FIAU per se is not toxic to yeast, we fused HSV1-TK to the GAL1 promoter, which is repressed in glucose medium but induced in galactose medium. As shown in Fig. 4C, 100 μM FIAU did not decrease the growth rate of yeast expressing GAL1-HSV1-TK on glucose but led to a 50% drop when galactose was the only carbon source.

ARG3 and ARG5,6 Promoters Are Deregulated in arg82Δ—We studied two promoters to genes of the arginine biosynthetic pathway, ARG3 and ARG5,6, encoding for ornithine carbamoyltransferase and acetylglutamate kinase, respectively, fused to HSV1-TK (29, 30). Because growth of arg82Δ in ornithine was minimal, and thus no differences could be appreciated before and after addition of FIAU (data not shown), we chose to study the response of the promoters in arginine where the effect on growth was less dramatic (Figs. 2 and 3). Expression of ARG3 and ARG5,6 increases during growth on ammonium and decreases on arginine. This was confirmed in our system: addition of FIAU to the medium led to a slower growth rate in the presence of ammonium with respect to arginine (supplemental Fig. S6, A and B). We quantified the differences between growth rates by calculating the area of the triangles generated by the tangents to the growth curves (see “Experimental Procedures”) and presented them in a column chart (Fig. 5A). The arg82Δ strain cannot properly regulate ARG3 and ARG5,6 promoters because it fails to repress them during
growth in arginine. Our system reflected this behavior by displaying a similar promoter response during growth in ammonium and arginine.

We compared the response of the TK system during growth in ammonium and arginine with the change in mRNA levels of ARG3 and ARG5,6. We observed a similar regulation, although the growth reduction upon ARG3-TK expression was less dramatic than the corresponding drop in mRNA levels (Fig. 5B).

We think that the portion of ARG3 promoter fused to HSV1-TK may not be fully responsive to arginine regulation. Thus, we concentrated on ARG5,6 promoter to study the rescue of arg82/H9004 by different IPMK forms.

Another caveat of the TK system is that the decrease in growth associated with using a nucleoside analog may alter the regulation of arginine-dependent genes. We address this by looking into the levels of ARG3 and ARG5,6 mRNA levels after treatment with 200 µM FIAU. As can be observed in Fig. 5C, gene regulation appeared in line with the untreated condition (DMSO). Also, HSV1-TK activity did not seem affected by the reduction in growth rate caused by FIAU (Fig. 5D).

**FIGURE 4. A novel method to measure promoter strength applied to the study of arginine-regulated genes.** A, the promoter of the gene of interest was fused to the HSV1-TK and expressed in a high copy plasmid. FIAU is phosphorylated by HSV1-TK and becomes a toxic compound once incorporated into DNA, reducing the growth rate. The table indicates the expected influence of FIAU on the growth rate of cells transformed with HSV1-TK fused to an active or inactive promoter. The rate of growth inhibition is proportional to the strength of the promoter studied. Yeast strains expressing HSV1-TK gene under the control of either ADH1 (B) or GAL1 (C) promoters are shown. ADH1 is active in the presence of glucose. GAL1 promoter is down-regulated in the presence of glucose and strongly up-regulated when galactose is the sole carbon source. Growth profiles in minimal medium containing ammonium as unique nitrogen source are shown. Error bars correspond to S.E.

**ARG5,6 Promoter Regulation Is Not Dependent on the Catalytic Activity of Arg82**—We looked into whether ARG5,6 regulation in arginine could be restored in an arg82Δ mutant by expressing the active or inactive Arg82. As shown in Fig. 6 and supplemental Fig. S7A, both constructs restored correct ARG5,6 promoter expression, indicating that the structure of Arg82 and not its activity is needed to stabilize the complex.

**Arg82 Homologues Are Unable to Restore Nitrogen Regulation to arg82Δ**—We also studied whether misregulation of the ARG5,6 promoter in arg82Δ could be rescued by expressing catalytically active, but structurally diverse, plant AtIpk2β and human IPMK homologues. Both Arg82 homologues were unable to restore regulation of ARG5,6 promoter (Fig. 6 and supplemental Fig. S7B). Instead, ARG5,6 promoter expression...
even seemed to be up-regulated, which could be due to off-target effects of expressing these IPMK homologues.

DISCUSSION

Different studies have shown that IPMK accumulates preferentially in the nucleus (10, 31, 32). This is a strong indication that some of its inositol phosphate products may be involved in regulating gene expression. In yeast, the role of Arg82 in transcription is well established (12, 13, 30); however, whether its catalytic activity plays a substantial role has long been contended (14, 15). Recent discoveries showing that enzymatically inactive mammalian IPMK participates in nutrient signaling (17, 19) prompted us to revisit this debate. Crucially, we assayed growth in liquid culture, which reflected a more accurate assessment of growth differences than in previous reports. First, we looked at the ability of arg82Δ/H9004 to grow in liquid mini-medium supplemented with ammonium or ornithine as the sole nitrogen source. We observed a 20% reduction in growth with respect to the wild type strain in ammonium and an 80% drop in ornithine. This contrasted with a stronger effect observed previously in solid medium (14, 15). We tested the ability of an Arg82 kinase-dead mutant to rescue the arg82Δ/H9004 phenotype and observed a partial restoration of growth in arginine and ornithine of 60 and 40%, respectively. These results support the idea championed by the Messenguy/Dubois laboratory whereby Arg82 kinase activity is not required for the control of arginine-regulated growth.

FIGURE 5. Transcriptional response of ARG3 and ARG5,6 promoters and HSV1-TK activity. A, the down-regulation of ARG3 and ARG5,6 promoters was calculated as the decrease in growth rate in the presence of FIAU with respect to DMSO (see “Experimental Procedures”). The difference in promoter expression in arginine medium is depicted in relation to ammonium, which received an arbitrary value of 100. Error bars correspond to S.E. The statistical analysis was performed using a two-tailed and homoscedastic t test (***, p < 0.001; NS, not significant). B, comparison of mRNA expression of ARG3 and ARG5,6 from arg82Δ bearing either an empty plasmid or one containing ARG82. The relative mRNA level was calculated using as a reference the growth in ammonium, which received an arbitrary value of 1. The error bars correspond to S.D. Statistical analysis was performed as above. C, comparison of mRNA expression of ARG3 and ARG5,6 from cells growing in the presence or absence of FIAU. The strain corresponded to the wild type expressing TK under the control of ADH1. The relative mRNA level was calculated using as a reference the growth in ammonium. The error bars correspond to S.D. D, HSV1-TK activity from wild type cells expressing TK under the control of ADH1. The error bars correspond to S.D.

FIGURE 6. Transcriptional response of ARG3 and ARG5,6 promoters in an arg82Δ strain rescued with IPMK homologues. Quantification of ARG5,6 promoter strength was determined as indicated in Fig. 5A. arg82Δ was rescued with an Arabidopsis IPMK homologue (AtIpk2β), its kinase-dead (KD) variant (AtIpk2β-D/A), the human IPMK homologue (hIPMK), or a kinase-dead version (hIPMK-DK/AA). Error bars correspond to S.E. The statistical analysis was performed using a two-tailed and homoscedastic t test (***, p < 0.001; NS, not significant). Catalytically Inactive Arg82 Restores Arginine Metabolism
Catalytically Inactive Arg82 Restores Arginine Metabolism

The lack of a role for Arg82 activity may indicate that inositol phosphate intermediates are not involved in nitrogen regulation. To test this hypothesis, we looked at mutants unable to synthesize pools of inositol phosphates: *plc1Δ* in which production of all higher phosphorylated forms is abolished and *kcs1Δ* in which the synthesis of inositol pyrophosphates is substantially decreased. The mild growth defect observed in both mutants when cultured in media containing arginine and ornithine as compared with ammonium was similar to that displayed by the wild type strain. Hence, abolishing inositol phosphate synthesis did not affect growth in demanding nitrogen sources. It has been proposed that the transcriptional role of Arg82 could depend on its PI3K activity. This would be independent of PLC activity and hence would not be affected in the *plc1Δ* mutant (10). We tested this hypothesis by expressing in *arg82Δ* the plant and human IPMK homologues. Expression of the plant AtIpk2β, which lacks PI3K activity, and the human IPMK, which hosts both lipid and soluble inositol enzymatic activities, restored normal growth in ammonium but not in ornithine. Therefore, Arg82 is able to control arginine-regulated transcription independently of its activity, perhaps due to its scaffolding properties (13). It should be noted that the interaction between rat IPMK and AMP-activated protein kinase depends on the phosphorylation of a tyrosine in position 174, which is also conserved in yeast. On the contrary, interaction with TOR depends on the N-terminal region (amino acids 1–60), which is particularly variable among eukaryotes.

Several high throughput screens have indicated that the N-terminal end of Arg82 is phosphorylated at positions in close proximity (33–35). We tested whether these modifications have any implication for cell growth or transcriptional regulation of arginine-regulated promoters in vivo. The expression of a form of Arg82 that cannot be phosphorylated (*ARG8*-*SDST/ADAA*) in the *arg82Δ* mutant was able to complement growth in ornithine and restored nitrogen transcriptional regulation of *ARG5,6* promoter (data not shown). Thus, we conclude that Arg82 phosphorylation does not play a relevant role in vivo.

A number of publications have shown that expression of IPMK homologues (human, fly, plant (*Arabidopsis*, potato, rice, and salt cress), and fission yeast) in *arg82Δ* complement some of its growth defects (28, 36–42). This has been interpreted as a proof that the IPMK catalytic activity is involved in transcriptional regulation. Most of these experiments, however, addressed only growth in ammonium on solid medium. Given that total growth is a blunt measure of phenotypes caused by Arg82 deletion, we decided to look into transcriptional readouts. Available methods, such as Northern blot, RT–PCR, and enzymatic activity of genes under Arg80–Arg81–Mcm1 control, currently do not resolve the regulation of promoter expression in real time and in vivo. Instead, we developed a new technology to study promoter strength. We took advantage of the specificity of viral thymidine kinases toward nucleoside analogues, which once metabolized are toxic to the cell (43, 44). We constructed a plasmid bearing the promoter of interest fused to HSV1-TK and transformed it together with plasmids carrying IPMK homologues and Arg82 mutants into the *arg82Δ* strain. The promoter of Arg8, encoding acetylornithine aminotransferase, has been used to determine the capacity of Arg80-Arg81-Mcm1 to bind and regulate arginine boxes by means of RT-PCR (10). As the full extent of the promoter region is not known, our efforts to clone it in full failed. Indeed, we studied the promoters of two other genes strongly down-regulated in the presence of arginine, *ARG3* and *ARG5,6*. Both promoters behaved as expected using HSV1-TK and RT-quantitative PCR with *ARG5,6* being more responsive. Hence, the system was used to study the effect of a kinase-dead Arg82. The results of *ARG5,6* promoter regulation showed that Arg82 kinase activity is to a large extent dispensable. In addition, expression of plant or human IPMK was unable to restore wild type promoter functionality, indicating that enzymatic activity alone was insufficient. In conclusion, Arg82 kinase activity plays a minor role, if any, in the control of arginine-responsive promoters.

The novel strategy presented to measure promoter strength (fusion to HSV1-TK) offers the possibility of unlimited real time data collection by monitoring growth rate. Its use, combined with high density microtiter plates, may speed up the study of conditions altering gene expression. It also presents drawbacks associated with growth in plates (i.e., poor aeration makes it unsuitable for growth in non-fermentative substrates). Also, mutants with poor growth or conditions that dramatically reduce fitness or cell shape may not be used with this approach.

Indirect evidence indicates that Arg82 activity plays a relevant role in other aspects of nitrogen metabolism. For instance, arginine represents the largest nitrogen storage reserve in yeast, accumulating in vast quantities inside vacuoles (45) where it counteracts negatively charged polyphosphate polymers (46, 47). These are long inorganic phosphate polymers that constitute the main phosphate repository in yeast (48). Polyphosphate polymer metabolism is linked to inositol pyrophosphate synthesis of which Arg82 activity is a necessary step (49). Lysine is an alternative nitrogen storage amino acid in the absence of arginine. *arg82Δ* has been shown to display higher lysine levels than wild type (36). Another link between Arg82 and nitrogen metabolism is via the TOR signaling pathway. The yeast TOR pathway comprises two branches, and at least one of them is dedicated to monitoring nitrogen availability (20), although the amino acid-sensing module remains largely unknown (50). *arg82Δ*, like TOR complex 1, is hypersensitive to rapamycin, suggesting a common regulation (51, 52). It becomes clear that Arg82 influences multiple phenotypic traits; hence, its catalytic activity may affect transcriptionally independent aspects of amino acid metabolism.

The importance of Arg82 enzymatic activity for growth is highlighted by the near complete rescue seen by expressing the plant or human IPMK homologue. However, the lack of a requirement for Arg82 enzymatic activity to regulate arginine-dependent transcription suggests that this evolutionarily conserved enzyme has also acquired non-catalytic functions, such as the stabilization of the Arg80–Arg81–Mcm1 transcriptional complex (13). The non-enzymatic role of Arg82 can be compared with that played by pseudokinases. About 10% of the human kinome is constituted by kinases that have lost their protein phosphorylation ability (53); however, many pseudokinases keep their signaling functions often as scaffolds or chaperones toward proteins that likely were their original kinase substrate (54, 55).
Catalytically Inactive Arg82 Restores Arginine Metabolism

The importance of Arg82 to non-enzymatic regulated transcription of arginine boxes might be interpreted as an adaptation of an ancient more fundamental role played by this enzyme and by their enzymatic products in controlling transcription. These considerations are supported by the discovery that histone deacetylase polypeptides from Tetrahymena and Paramaecium possess an inositol-phosphatase kinase domain similar to Arg82 (56). In addition, inositol tetrakispshosphate (a product of IPMK) has recently been shown to aid in the interaction of histone deacetylase 3 with a transcriptional corepressor (nCOR2) (57). This opens up the possibility that the catalytic activity of IPMK could play a subsidiary role in protein-protein interaction influencing gene transcription. Finally, a recent report showed that IPMK phosphorylated phosphatidylinositol 4,5-bisphosphate bound to a nuclear protein, pointing to a new mechanism of gene regulation (58). It is likely that novel IPMK partners will emerge in the future, shedding light on the important roles that this enzyme plays in the nucleus.

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REFERENCES

1. Alcázar-Román, A. R., and Wente, S. R. (2008) Inositol polyphosphates: a new frontier for regulating gene expression. Chromosoma 117, 1–13
2. Monserrate, J. P., and York, J. D. (2010) Inositol phosphate synthesis and the nuclear processes they affect. Curr. Opin. Cell Biol. 22, 365–373
3. Resnick, A. C., and Saiardi, A. (2009) in Wiley Encyclopedia of Chemical Biology (Begley, T. P., ed) Vol. 2, pp. 349–359, John Wiley & Sons, Inc., New York
4. Burton, A., Hu, X., and Saiardi, A. (2009) Are inositol pyrophosphates signalling molecules? J. Cell Biol. 200, 8–15
5. Irvine, R. F., and Schell, M. J. (2001) Back in the water: the return of the Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 98, 15724–15729
6. Bang, S., Kim, S., Dailey, M. J., Chen, Y., Moran, T. H., Snyder, S. H., and Kim, S. F. (2012) AMP-activated protein kinase: nature’s energy sensor. Nat. Chem. Biol. 8, 512–518
7. Kim, S., Kim, S. F., Maag, D., Maxwell, M. J., Resnick, A. C., Juluri, K. R., Chakraborty, A., Koldobskiy, M. A., Cha, S. H., Barrow, R., Snowman, A. M., and Snyder, S. H. (2011) Amino acid signaling to mTOR mediated by inositol phosphatase multikinase. Cell Metab. 13, 215–221
8. Loewith, R., and Hall, M. N. (2011) Target of rapamycin (TOR) in nutrient signaling and growth control. Genetics 189, 1177–1201
9. Saiardi, A., Sciambi, C., McCaffery, J. M., Wendland, B., and Snyder, S. H. (2002) Inositol pyrophosphates regulate endocytic trafficking. Proc. Natl. Acad. Sci. U.S.A. 99, 14206–14211
10. Muirberg, D., Müller, R., and Funk, M. J. (1994) Regulable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. 22, 5767–5768
11. Borrelli, E., Heyman, R., Hsi, M., and Evans, R. M. (1988) Targeting of an inducible toxic phenotype in animal cells. Proc. Natl. Acad. Sci. U.S.A. 85, 7572–7576
12. Warringer, J., and Blomberg, A. (2003) Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in Saccharomyces cerevisiae. Yeast 20, 53–67
13. Warringer, J., Ericson, E., Fernandez, L., Nerman, O., and Blomberg, A. (2003) High-resolution yeast phenomics resolves different physiological features in the saline response. Proc. Natl. Acad. Sci. U.S.A. 100, 15724–15729
14. Yaghoubi, S. S., and Gambhir, S. S. (2006) Measuring herpes simplex virus thymidine kinase reporter gene expression in vitro. Nat. Protoc. 1, 2137–2142
15. Chang, S. C., Miller, A. L., Feng, Y., Wente, S. R., and Majerus, P. W. (2002) The human homolog of the rat inositol phosphate multikinase is an inositol 1,3,4,6-tetrakisphosphate 5-kinase. J. Biol. Chem. 277, 43836–43843
16. Stevenson-Paulik, J., Odom, A. R., and York, J. D. (2002) Molecular and biochemical characterization of two plant inositol phosphatase 6-/3-/5-kinases. J. Biol. Chem. 277, 42711–42718
17. Bechet, J., Greenmon, S., and Wiame, J. M. (1970) Mutations affecting the repressibility of arginine biosynthetic enzymes in Saccharomyces cerevisiae. Eur. J. Biochem. 12, 31–39
18. Dubois, E., and Messenguy, F. (1991) In vitro studies of the binding of the ARG5 proteins to the ARG5.6 promoter. Mol. Cell. Biol. 11, 2162–2168
19. El Bakkoury, M., Dubois, E., and Messenguy, F. (2000) Recruitment of the Saccharomyces cerevisiae MCM1 in the regulation of arginine metabolism in vivo. Mol. Cell. Biol. 20, 5258–5266
20. Saiardi, A., and Messenguy, F. (1987) Regulation of arginine metabolism in Saccharomyces cerevisiae: expression of the three ARG5 genes and cellular localization of their products. Gene 55, 277–285
21. Resnick, A. C., Snowman, A. M., Kang, B. N., Hurt, K. J., Snyder, S. H., and Saiardi, A. (2005) Inositol phosphatase multikinase is a nuclear PI3-kinase with transcriptional regulatory activity. Proc. Natl. Acad. Sci. U.S.A. 102, 12783–12788
22. Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol phosphatase kinases. Curr. Biol. 9, 1323–1326
23. Bercy, J., Dubois, E., and Messenguy, F. (1987) Regulation of arginine metabolism in Saccharomyces cerevisiae: expression of the three ARG5 regulatory genes and cellular localization of their products. Gene 55, 277–285
24. Dubois, E., Dewaste, V., Erneux, C., and Messenguy, F. (2000) Inositol phosphatase kinase activity of Arg82/ArgRIII is not required for the regulation of the arginine metabolism in yeast. FEBS Lett. 486, 300–304
25. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science 287, 2026–2029
26. Meyer, R., Nalaskowski, M. E., Ehm, P., Schröder, C., Naj, X., Brehm, M. A., and Mayr, G. W. (2012) Nucleocytoplasmic shuttling of human inositol phosphate multikinase is influenced by CK2 phosphorylation. Biol. Chem. 393, 149–160
27. Bang, S., Kim, S., Dailey, M. J., Chen, Y., Moran, T. H., Snyder, S. H., and Kim, S. F. (2012) AMP-activated protein kinase: a newly identified family of higher inositol polyphosphate kinases and their enzymatic products in controlling transcription. J. Biol. Chem. 287, 2026–2029
28. Komatsu, M., Imai, T., and Yokota, Y. (2007) Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. U.S.A. 104, 10364–10369
Catalytically Inactive Arg82 Restores Arginine Metabolism

34. Albuquerque, C. P., Smolka, M. B., Payne, S. H., Bafna, V., Eng, J., and Zhou, H. (2008) A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Mol. Cell. Proteomics* 7, 1389–1396

35. Wu, R., Haas, W., Dephoure, N., Huttlin, E. L., Zhai, B., Sowa, M. E., and Gygi, S. P. (2011) A large-scale method to measure absolute protein phosphorylation stoichiometries. *Nat. Methods* 8, 677–683

36. Caddick, S. E., Harrison, C. J., Stavridou, I., Johnson, S., and Brearley, C. A. (2007) Molecular characterization of a novel inositol polyphosphate metabolic pathway initiated by inositol 1,4,5-trisphosphate 3-kinase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 27654–27661

37. El Alami, M., Messenguy, F., Scherens, B., and Dubois, E. (2003) Arg82p is a bifunctional protein whose inositol polyphosphate kinase activity is essential for nitrogen and PHO gene expression but not for Mcm1p chaperoning in yeast. *Mol. Microbiol.* 49, 457–468

38. Seeds, A. M., Bastidas, R. J., and York, J. D. (2005) Molecular definition of a novel inositol polyphosphate metabolic pathway initiated by inositol 1,4,5-trisphosphate 3-kinase activity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 27654–27661

39. Suzuki, M., Tanaka, K., Kuwano, M., and Yoshida, K. T. (2007) Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): implications for the phytic acid biosynthetic pathway. *Gene* 405, 55–64

40. Xia, H. J., Brearley, C., Elge, S., Kaplan, B., Fromm, H., and Mueller-Roeber, B. (2003) *Arabidopsis* inositol polyphosphate 6-/3-kinase is a nuclear protein that complements a yeast mutant lacking a functional ArgR-Mcm1 transcription complex. *Plant Cell* 15, 449–463

41. Yang, L., Tang, R., Zhu, J., Liu, H., Mueller-Roeber, B., Xia, H., and Zhang, H. (2008) Enhancement of stress tolerance in transgenic tobacco plants constitutively expressing AtIPK2β, an inositol polyphosphate 6-/3-kinase from *Arabidopsis thaliana*. *Plant Mol. Biol.* 66, 329–343

42. Zhu, J. Q., Zhang, J. T., Tang, R. J., Lv, Q. D., Wang, Q. Q., Yang, L., and Zhang, H. X. (2009) Molecular characterization of *ThiPK2*, an inositol polyphosphate kinase gene homolog from *Thelungiella halophila*, and its heterologous expression to improve abiotic stress tolerance in *Brassica napus*. *Physiol. Plant.* 136, 407–425

43. Hsieh, C. H., Liu, R. S., Wang, H. E., Hwang, J. J., Deng, W. P., Chen, J. C., and Chen, F. D. (2006) *In vitro* evaluation of herpes simplex virus type 1 thymidine kinase reporter system in dynamic studies of transcriptional gene regulation. *Nucl. Med. Biol.* 33, 653–660

44. Jonsson, J. J., and McVor, R. S. (1991) Herpes simplex virus thymidine kinase enzymatic assay in transient transfection experiments using thymidine kinase-deficient cells. *Anal. Biochem.* 199, 232–237

45. Davis, R. H. (1986) Compartmental and regulatory mechanisms in the arginine pathways of *Neurospora crassa* and *Saccharomyces cerevisiae*. *Microbiol. Rev.* 50, 280–313

46. Dürr, M., Urech, K., Boller, T., Wemken, A., Schwencke, J., and Nagy, M. (1979) Sequestration of arginine by polyphosphate in vacuoles of yeast (*Saccharomyces cerevisiae*). *Arch. Microbiol.* 121, 169–175

47. Saiardi, A. (2012) How inositol pyrophosphates control cellular phosphate homeostasis? *Adv. Biol. Regul.* 52, 351–359

48. Rao, N. N., Gómez-García, M. R., and Kornberg, A. (2009) Inorganic polyphosphate: essential for growth and survival. *Annu. Rev. Biochem.* 78, 605–647

49. Lonetti, A., Szijgyarto, Z., Bosch, D., Loss, O., Azavedo, C., and Saiardi, A. (2011) Identification of an evolutionary conserved family of inorganic polyphosphate endopolyphosphatases. *J. Biol. Chem.* 286, 31966–31974

50. De Virgilio, C., and Loewith, R. (2006) Cell growth control: little eu- karyotes make big contributions. *Oncogene* 25, 6392–6415

51. Chan, T. F., Carvalho, J., Riles, L., and Zheng, X. F. (2000) A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR). *Proc. Natl. Acad. Sci. U.S.A.* 97, 13227–13232

52. Ikai, N., Nakazawa, N., Hayashi, T., and Yanagida, M. (2011) The reverse, but coordinated, roles of Tor2 (TORC1) and Tor1 (TORC2) kinases for growth, cell cycle and separase-mediated mitosis in *Schizosaccharomyces pombe*. *Open Biol.* 1, 110007

53. Taylor, S. S., and Kornev, A. P. (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem. Sci.* 36, 65–77

54. Scheff, E. D., Eswaran, J., Bunkoczi, G., Knapp, S., and Manning, G. (2009) Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly conserved kinase fold, and a putative regulatory binding site. *Structure* 17, 128–138

55. Fukuda, K., Gupta, S., Chen, K., Wu, C., and Qin, J. (2009) The pseudokinase of ILK is essential for its binding to α-parvin and localization to focal adhesions. *Mol. Cell* 36, 819–830

56. Smith, J. J., Torigoe, S. E., Masson, J., Fish, L. C., and Wiley, E. A. (2008) A class II histone deacetylase acts on newly synthesized histones in *Tetrahyena Eukaryot. Cell.* 7, 471–482

57. Watson, P. J., Fairall, L., Santos, G. M., and Schwabe, J. W. (2012) Structure of HDAC3 bound to co-repressor and inositol tetraphosphate. *Nature* 481, 335–340

58. Blind, R. D., Suzawa, M., and Ingraham, H. A. (2012) Direct modification and activation of a nuclear receptor-PIP2 complex by the inositol lipid kinase IPMK. *Sci. Signal.* 5, ra44