

# Metabolic intermediates selectively stimulate transcription factor interaction and modulate phosphate and purine pathways

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Cells use strategic metabolites to sense the metabolome and accordingly modulate gene expression. Here, we show that the purine and phosphate pathways are positively regulated by the metabolic intermediate AICAR (5'-phosphoribosyl-5-amino-4-imidazole carboxamide). The transcription factor Pho2p is required for up-regulation of all AICAR-responsive genes. Accordingly, the binding of Pho2p to purine and phosphate pathway gene promoters is enhanced upon AICAR accumulation. In vitro, AICAR binds both Pho2p and Pho4p transcription factors and stimulates the interaction between Pho2p and either Bas1p or Pho4p in vivo. In contrast, SAICAR (succinyl-AICAR) only affects Pho2p–Bas1p interaction and specifically up-regulates purine regulon genes. Together, our data show that Bas1p and Pho4p compete for Pho2p binding, hence leading to the concerted regulation of cellular nucleotide synthesis and phosphate consumption.

[*Keywords*: Phosphate utilization; purine pathway; small molecules; transcription factor; yeast]

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To maintain homeostasis, cells must permanently “estimate” the concentration of critical metabolites and modulate activity of highly interconnected metabolic pathways. Interestingly, recent evidence has pointed to specific metabolites as major regulators of transcription processes. Small molecules can affect chromatin remodeling complexes (Shen et al. 2003; Steger et al. 2003) as well as the ability of transcription factors to activate transcription (Sze et al. 1992; Flynn and Reece 1999). Several yeast transcription factors respond to critical metabolic intermediates, the abundance of which directly reflects the flux through the metabolic pathway (Sellick and Reece 2005). Thereby, the flow in the metabolic pathway can be modulated by regulating the synthesis of all the enzymes of the pathway at the transcriptional level. This role for small molecules in transcriptional control of their own synthesis pathway is now clearly established and in few instances has been reconstituted in vitro (Sellick and Reece 2005).

We found previously that two metabolic intermediates, AICAR (5'-phosphoribosyl-5-amino-4-imidazole carboxa-

mid) and SAICAR (succinyl-AICAR), are involved in the transcriptional regulation of purine pathway genes (*ADE* genes) (Rebora et al. 2001, 2005). Activation of these purine regulon genes in response to purine limitation require the Bas1p and Pho2p transcription factors (Daignan-Fornier and Fink 1992). Based on genetic evidence, it was proposed that the increase of AICAR and/or SAICAR concentration was a molecular signal promoting Bas1p–Pho2p interaction, and consequently resulted in purine gene up-regulation (Rebora et al. 2001, 2005). Consistently, under purine limitation conditions, Pho2p is recruited to the *ADE* gene promoters in a Bas1p-dependent manner (Som et al. 2005). Importantly, Pho2p together with Pho4p, another transcription factor, activates the *PHO* regulon genes in response to phosphate limitation (Lenburg and O'Shea 1996), thus suggesting that regulation of purine and phosphate pathways could be interconnected.

Because small molecules such as AICAR and SAICAR play regulatory roles, their overabundance can be highly detrimental. Abnormal accumulation of AICAR and SAICAR is toxic for yeast cells (Rebora et al. 2005). Importantly, AICAR or SAICAR accumulation in human are associated with two monogenic disorders leading to severe mental retardation and autistic features (Stone

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et al. 1992; Marie et al. 2004). Recently, treatment of mice with AICAR has revealed a role for this molecule in improving endurance in the absence of training (Narkar et al. 2008). Therefore, AICAR accumulation has pleiotropic effects suggesting that its levels should be highly regulated. We took advantage of yeast genetics to investigate AICAR physiological roles in eukaryotes and identify underlying molecular mechanisms.

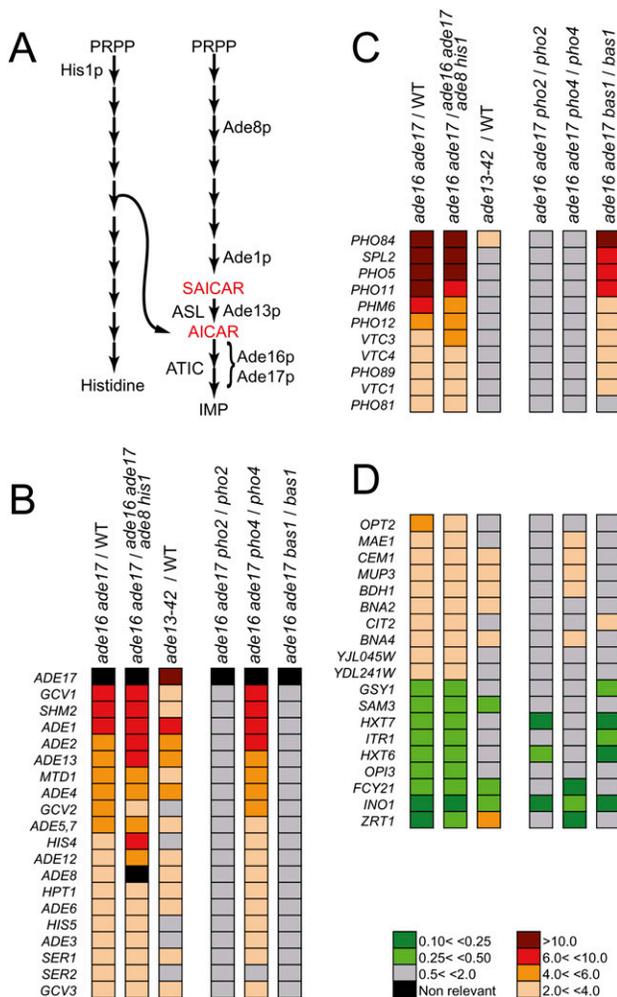
Here, we developed a set of yeast strains accumulating various amounts of AICAR. This well-controlled experimental design allowed us to uncover the effects of AICAR on transcription in a eukaryotic cellular model, and reveal that a small subset of yeast genes responds to AICAR. The vast majority of these genes require the transcription factor Pho2p and either Pho4p or Bas1p for their expression in response to AICAR. We show that both Pho2p and Pho4p directly bind AICAR in vitro. In vivo, AICAR promotes the interaction of Pho2p with both Pho4p and Bas1p, resulting in finely concerted activation of the purine biosynthesis and phosphate utilization pathways. Since purine nucleotide synthesis leads to significant phosphate consumption in yeast, coregulation of these pathways by both the transcription factor Pho2p and the small molecule AICAR provides an efficient mechanism to maintain purine–phosphate cellular homeostasis.

## Results

### *AICAR accumulation stimulates the expression of phosphate and purine regulon genes in a concentration-dependent manner*

We first designed a set of isogenic yeast strains carrying mutations affecting AICAR metabolism (Fig. 1A) and used high-performance ion chromatography to precisely measure intracellular AICAR concentration in these strains (Supplemental Fig. 1A). In an *ade16 ade17* double mutant lacking AICAR transformylase activity, AICAR accumulated (1.6 mM), while it was below detection level (<10  $\mu$ M) in both an isogenic wild-type strain and an *ade16 ade17 ade8 his1* mutant (referred to as “quadruple mutant” hereafter) that cannot synthesize AICAR due to the upstream blocks in both histidine and IMP (inosine 5'-monophosphate) biosynthesis pathways.

By comparing transcript abundance in this set of strains, we found that AICAR accumulation resulted in differential expression of 50 genes, all encoding metabolic enzymes, and largely composed of induced (41) rather than repressed (nine) genes (Fig. 1B–D, rows 1 and 2). As expected, the purine regulon, which includes IMP biosynthesis genes and other genes (*HIS4*, *SHM2*, *MTD1* . . .) known to be activated by the Bas1p and Pho2p transcription factors (Arndt et al. 1987; Daignan-Fornier and Fink 1992; Denis and Daignan-Fornier 1998), was strongly induced in the *ade16 ade17* mutant (Fig. 1B; Rebera et al. 2005). More surprisingly, several genes involved in phosphate utilization and its regulation (Ogawa et al. 2000) were among the most up-regulated genes in response to AICAR accumulation (Fig. 1C). The other



**Figure 1.** Transcriptional effects of AICAR and SAICAR accumulation. (A) Schematic representation of purine and histidine de novo pathways in yeast. (ASL) Adenylosuccinate lyase; (ATIC) AICAR transformylase IMP cyclohydrolase; (PRPP) 5-phosphoribosyl-1-pyrophosphate. (B–D) Functional classes of genes differentially expressed (more than twofold) in strains accumulating or not AICAR and/or SAICAR. Data were obtained by DNA microarray analyses as described in Breton et al. (2008) and Lemoine et al. (2006), and AICAR-responsive genes were divided into functional classes: purine regulon (B), phosphate regulon (C), and other metabolisms (D). Raw data are available at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13275>.

regulated genes did not belong to a clear unique metabolic pathway (Fig. 1D).

Importantly, in the *ade16 ade17* double mutant, not only AICAR but also the metabolite immediately upstream in the pathway (Fig. 1A), SAICAR, accumulated (0.3 mM) far above wild-type level (<10  $\mu$ M) (Supplemental Fig. 1A). To differentiate between the effects of SAICAR and AICAR on transcription, we used an *ade13* mutant, which accumulated SAICAR to a level comparable with the *ade16 ade17* mutant (0.1 mM range) but did not contain any detectable AICAR (Supplemental Fig.

1B). In this mutant, the purine regulon, but not the phosphate regulon genes, were up-regulated (Fig. 1B,C, row 3). A set of *ade13* mutants accumulating various amounts of SAICAR further confirmed that these two regulons respond differently to SAICAR (Fig. 2A). Importantly, no other blocks in the IMP biosynthesis pathway resulted in up-regulation of *PHO5* or *PHO84* expression (Supplemental Fig. 2A,B). Therefore, expression of the *PHO* regulon cannot be induced by accumulation of either SAICAR alone or any upstream intermediates. Finally, we used an *ade16 ade17 ade1 ade13* mutant that is totally unable to synthesize SAICAR (Supplemental Fig. 1C) due to the *ade1* block in the de novo pathway combined to the *ade13* mutation that prevents reverse synthesis of SAICAR from AICAR (Fig. 1A). Using this strain, we established that AICAR alone was sufficient for

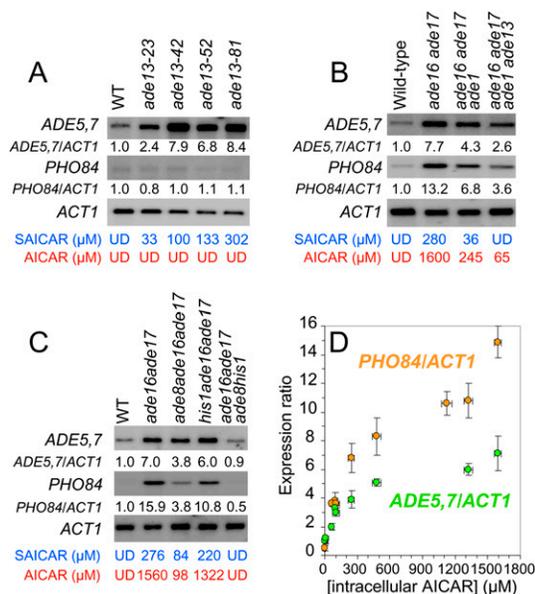
transcriptional up-regulation of the phosphate and purine regulon genes (Fig. 2B). Results shown in Figure 2B suggest that the transcriptional response was affected by intracellular AICAR concentration. Using several combinations of mutants in the purine and histidine pathways, we determined the AICAR dose-dependent response of *ADE5,7* and *PHO84* transcription (Fig. 2B–D). These results reveal that expression of these two genes respond to a wide range of intracellular AICAR concentrations.

#### *Pho2p occupancy at ADE and PHO promoters is strongly enhanced in the ade16 ade17 mutant*

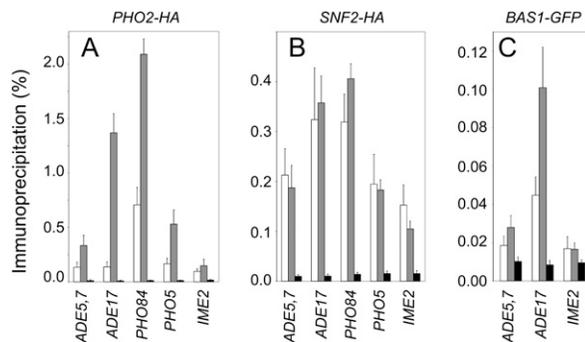
Activation of both purine and phosphate pathways involves Pho2p respectively combined with Bas1p or Pho4p, (Shao et al. 1996; Zhang et al. 1997). The requirement of these three transcription factors for AICAR-induced transcriptional response was assayed by introducing separately *bas1*, *pho2*, and *pho4* mutations in the *ade16 ade17* double mutant. The *PHO* regulon genes were totally dependent on Pho4p but not on Bas1p, while the purine regulon genes were fully dependent on Bas1p but not on Pho4p (Fig. 1B,C, rows 5 and 6; Supplemental Fig. 3A). Importantly, all but three AICAR-responsive transcripts were strongly dependent on Pho2p (Fig. 1B–D, row 4). Of note, all three Pho2p-independent genes are down-regulated in response to AICAR. This central role of Pho2p in this transcriptional response prompted us to investigate whether AICAR accumulation could modulate the presence of Pho2p at the endogenous *ADE* and *PHO* promoters. Chromatin immunoprecipitation (ChIP) revealed a much higher occupancy of the *ADE* and *PHO* promoters by Pho2p in the *ade16 ade17* mutant compared with the isogenic wild-type strain (Fig. 3A). In a similar experiment, AICAR accumulation had no effect on occupancy of the same promoters by Snf2p (Fig. 3B), which is required for *ADE5,7*, *ADE17*, *PHO84*, and *PHO5* expression (Sudarsanam et al. 2000; Neef and Klädde 2003; Koehler et al. 2007). We then investigated the occupancy of the *ADE* gene promoters by Bas1p and found that Bas1p was slightly more abundant on the *ADE* gene promoters when yeast cells accumulate AICAR (Fig. 3C). Thus, Pho2p is clearly required for the AICAR transcriptional response, and its presence at up-regulated gene promoters is strongly enhanced under conditions where AICAR accumulates. Importantly, we found that Pho2p protein level was not significantly different in the *ade16 ade17* mutant compared with the isogenic wild-type (Supplemental Fig. 3B).

#### *AICAR activation of the PHO regulon is not associated with constitutive nuclear localization of Pho4p*

Transcriptional regulation of the *PHO* regulon in response to extracellular phosphate availability occurs through a well-characterized regulatory cascade involving synthesis of inositol heptakisphosphate (IP7) by Vip1p (Lee et al. 2007) and subsequent inhibition of nuclear relocalization of Pho4p (Komeili and O'Shea 1999). Wild-type strain and the *ade16 ade17* double mutant were indiscernible for



**Figure 2.** Expression of *PHO84* and *ADE5,7* is stimulated by AICAR in a concentration-dependent manner. (A) SAICAR accumulation stimulates *ADE5,7* but not *PHO84* transcription. *ADE5,7* and *PHO84* expression was monitored by Northern blotting on exponentially growing cells. *ADE5,7/ACT1* and *PHO84/ACT1* ratios were determined using a phosphorimager and were set at 1 for wild-type cells. Ratio values correspond to the average of at least three independent Northern blot analyses. Variations to the mean were <10%. AICAR and SAICAR concentrations determined by chromatography, correspond to an average of two independent injections done with at least three independent metabolite extractions. Variations to the mean were below 10%. (UD) Undetectable, the minimum detection level in our conditions corresponding to concentrations below 10–20 μM for both AICAR and SAICAR. (B) *ADE5,7* and *PHO84* transcription is stimulated in response to AICAR alone. (C) *PHO84* and *ADE5,7* expression is tightly correlated to intracellular AICAR concentration. (D) Correlation between intracellular AICAR concentration and expression of *PHO84* or *ADE5,7*. Data correspond to a compilation of transcript and AICAR measurements presented in the figure and data not shown. Error bars correspond to variation to the mean on each axis. The *PHO84/ACT1* ratio was set at 1 for wild-type cells.



**Figure 3.** Pho2p occupancy at *ADE* and *PHO* promoters is enhanced in the *ade16 ade17* mutant. ChIP assays were performed on the indicated promoters using antibodies against the HA tag on Pho2p (A) and Snf2p (B) and the GFP tag on Bas1p (C). In all cases, results are presented as percent of immunoprecipitation (immunoprecipitation signal/input signal) in strain either accumulating AICAR (gray bars) or not (white bars), or in the untagged control strain (black bars). In this experiment, *IME2*, which is not AICAR-responsive, was used as a control. Error bars correspond to variation to the mean.

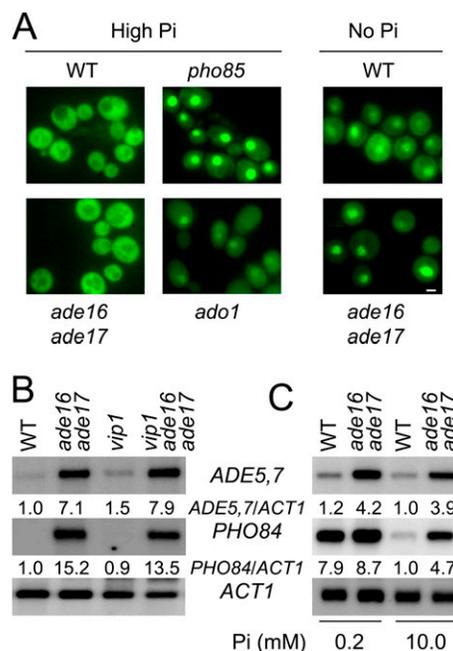
Pho4p localization. Indeed, under phosphate-replete conditions, Pho4-GFP was found both in the cytoplasm and the nucleus of the two strains (Fig. 4A; Supplemental Fig. 4A). The existence of a nuclear pool of Pho4p under these conditions is further attested by the significant expression of *PHO84* compared with that measured in a *pho4* deletion mutant (Supplemental Fig. 3A). In contrast, other mutations, such as *pho85* or *ado1*, leading to constitutive expression of the *PHO* regulon, caused a constitutive nuclear localization of Pho4p (Fig. 4A), as reported previously by others (O'Neill et al. 1996; Huang and O'Shea 2005). Finally, in both wild-type and *ade16 ade17* strains, Pho4p relocalized to the nucleus when cells were shifted to phosphate-free medium (Fig. 4A). Together, these results establish that AICAR effect on the *PHO* regulon is not associated with a constitutive relocalization of Pho4p to the nucleus and thus does not involve the low-phosphate—IP7-sensing—pathway (Lee et al. 2007). Consistently, activation of the *PHO* pathway in the *ade16 ade17* mutant was not dependent on synthesis of IP7 by Vip1p (Fig. 4B), indicating that AICAR and IP7 act through separated signaling pathways. However, under low-phosphate conditions where Pho4p is constitutively nuclear, *PHO84* expression was not further up-regulated by the *ade16 ade17* mutation (Fig. 4C) nor by purine limitation (Supplemental Fig. 4B). Thus, although AICAR and IP7 signaling pathways are separated, the resulting transcriptional outcome is not strictly additive.

#### *AICAR accumulation stimulates Pho2p–Pho4p interaction in vivo*

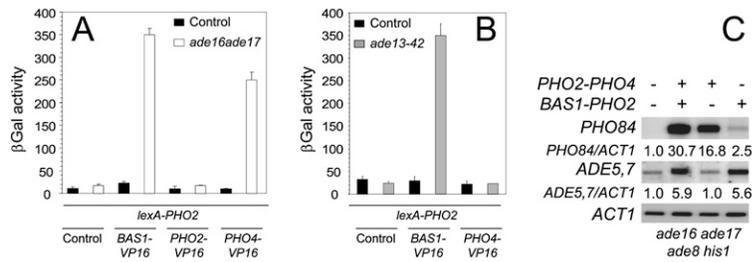
The fact that Pho4p is not constitutively localized in the nucleus of AICAR-accumulating cells suggests that the mechanism leading to up-regulation of the *PHO* regulon operates somewhere downstream from Pho4p nuclear

localization. We therefore developed an *in vivo* assay to establish whether AICAR accumulation could stimulate interactions between Pho4p and Pho2p. This assay, based on the two-hybrid method performed in parallel in yeast strains accumulating, or not, AICAR, revealed that Pho2p–Pho4p interaction was very low in the control strain but strongly stimulated in the *ade16 ade17* mutant accumulating AICAR (Fig. 5A). In the same experiment, we found that interaction between Pho2p and Bas1p was also enhanced by AICAR, while no Pho2p–Pho2p interactions could be detected (Fig. 5A). Importantly, AICAR accumulation had no effect on the individual transcription factors fused to *lexA* (Supplemental Fig. 5). Consistently with our transcriptome analysis (Fig. 1), Bas1p–Pho2p interaction was stimulated in the *ade13-42* mutant accumulating only SAICAR, as previously reported (Rebora et al. 2001), but this accumulation had no effect on Pho2p–Pho4p interaction (Fig. 5B).

In view of these results, a simple assumption is that forced interactions between transcription factors, via fusion chimeras, should bypass AICAR requirement for transcriptional activation *in vivo*. *PHO2–PHO4* and



**Figure 4.** AICAR activation of the *PHO* regulon is not associated with constitutive nuclear localization of Pho4p. (A) Pho4p localization was monitored by microscopy using an integrative Pho4p-3xGFP fusion. Cells were grown in High Pi medium, diluted in the same medium containing orthophosphate (10 mM) or not, and observed 16 h later. Bar, 2  $\mu$ m. (B) Vip1p is not required for expression of the *PHO* pathway in response to AICAR accumulation. *ADE5,7* and *PHO84* expression levels were determined by Northern blotting as in Figure 2A. (C) *PHO84* expression is not further up-regulated by the *ade16 ade17* mutation under low-phosphate conditions. Expression of *ADE5,7* and *PHO84* in wild-type and *ade16 ade17* mutants grown under low- or high-phosphate conditions was monitored by Northern blotting as in Figure 2A.



**Figure 5.** AICAR, but not SAICAR, stimulates Pho2p–Pho4p interaction. (A) Two-hybrid assay reveals that AICAR stimulates Pho2p interaction with its partners Bas1p and Pho4p.  $\beta$ GAL activities were measured in cells transformed with a *lexAop-LacZ* plasmid and the indicated plasmids. Error bars correspond to variations to the mean. (B) SAICAR stimulates Pho2p interaction with Bas1p but not with Pho4p.  $\beta$ GAL activities were measured in cells transformed with a *lexAop-LacZ* plasmid and the indicated plasmids. (C) *BAS1-VP16* and *PHO2-VP16* chimeras, respectively, restore *ADE5,7* and *PHO84* transcription in *ade16 ade17 ade8 his1* quadruple mutant cells.

*BAS1-VP16* fusions should, respectively, make *PHO* and *ADE* gene expression AICAR-independent. Indeed, a *PHO2-VP16* fusion expressed under the control of the *PHO2* promoter on a centromeric plasmid in the quadruple mutant, which cannot synthesize AICAR, led to strong expression of *PHO84* but had no effect on *ADE5,7* expression (Fig. 5C). Conversely, the *BAS1-VP16* fusion up-regulated *ADE5,7* expression but had little effect on *PHO84* (Fig. 5C).

#### AICAR binds to Pho2p and Pho4p in vitro

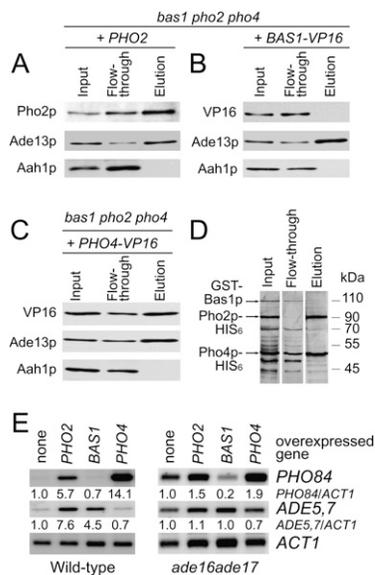
To address the question as to how AICAR affects Pho2p–Bas1p and Pho2p–Pho4p interactions, we expressed each of the three transcription factors individually and, once purified from yeast, assayed their respective ability to bind an AICAR affinity column. Pho2p (Fig. 6A) and Pho4p (Fig. 6C) were effectively retained on the AICAR column, whereas Bas1p was clearly not retained (Fig. 6B). Moreover, adenylosuccinate lyase (Ade13p), the enzyme responsible for AICAR synthesis (positive control), selectively bound the AICAR column (Fig. 6A–C), while, as expected, the adenine deaminase (Aah1p) used as a negative control was not retained on the column (Fig. 6A–C). At this point, we could not rule out that binding of Pho2p and Pho4p to AICAR required accessory factors or post-translational modification. To address this issue, we further performed the AICAR-binding experiment with the three transcription factors independently purified from *Escherichia coli*. Clearly, Pho2p and Pho4p were still efficiently retained while Bas1p was not (Fig. 6D), demonstrating that binding of Pho2p and Pho4p to AICAR does not require additional yeast proteins.

The transcriptional response to AICAR is strongly dependent on Pho2p combined with either Pho4p or Bas1p. This implies that if Pho2p is limiting, these two transcription factors should compete for Pho2p binding in vivo. To assess this assumption, we independently over-expressed each of the transcription factors in a wild-type strain and monitored the expression of genes of the purine and *PHO* pathways. Clearly, *PHO84* expression was enhanced by overexpression of Pho2p and even more efficiently by overexpression of Pho4p (Fig. 6E, left panel). Similarly, *ADE5,7* expression was strongly activated by overexpression of Pho2p and Bas1p to a lesser extent (Fig. 6E, right panel). Thus, all three transcription factors appear limiting under these conditions. Importantly, in condi-

tions of AICAR accumulation, in an *ade16 ade17* mutant, overexpression of Bas1p strongly down-regulated *PHO84* expression (Fig. 6E, right panel) further revealing an effective competition between Pho4p and Bas1p transcription factors for Pho2p binding.

#### Discussion

Here, we found that yeast cells take profit of an intermediate metabolite to modulate the expression of different metabolic pathways. Our data reveal a new role for the purine metabolic intermediate AICAR, which not only regulates its own pathway but also modulates expression of the phosphate utilization pathway. Importantly, while we found that a wide range of AICAR concentrations could be sensed (Fig. 2D), the highest transcriptional variations were found at low AICAR concentrations that are closer to physiological conditions. Interestingly, the AICAR dose response curves of both *ADE* and *PHO* genes had similar shapes suggesting a common mechanism. We propose that the transcription factor Pho2p is the major player in the AICAR response because it is required for up-regulation of all the AICAR-responsive genes, it binds AICAR in vitro, and its presence is enhanced at the target gene promoters under AICAR accumulation conditions. Importantly, the presence of Bas1p was also enhanced (about twofold) on the *ADE* gene promoters when yeast cells accumulate AICAR. However, this enrichment was much lower than the one observed for Pho2p. These results are very similar to those reported by Rolfes and coworkers (Som et al. 2005), who found a twofold increase in the binding of Bas1p when yeast cells are shifted from adenine-replete to adenine-limited medium. Som et al. (2005) also reported a Bas1p-dependent recruitment of Pho2p under derepressing (low adenine) conditions. A simple explanation for enhanced binding of both Bas1p and Pho2p to the *ADE* promoters in the *ade16 ade17* mutant is that AICAR favors Bas1p–Pho2p interaction and promotes cooperative binding of Bas1p and Pho2p. While such a cooperative effect has not been observed in vitro (Rebora et al. 2001), cooperative binding of Bas1p and Pho2p was proposed to occur in vivo based on ChIP analysis (Som et al. 2005). Together, our results and those published by the Rolfes laboratory (Som et al. 2005) suggest that AICAR promotes both cooperative binding of Bas1p and Pho2p



**Figure 6.** Pho2p and Pho4p but not Bas1p can bind AICAR in vitro. (A–C) Pho2p (A), Bas1p (B), and Pho4p (C) binding on an AICAR affinity resin. A triple *bas1 pho2 pho4* mutant strain was transformed with plasmids allowing expression of *PHO2*, *BAS1-VP16*, or *PHO4-VP16*. Protein extraction and binding to an AICAR affinity resin were performed as described in the experimental procedures section. Proteins from input, flowthrough and eluted fractions were separated on SDS-PAGE and revealed by Western blotting using anti-Pho2p, anti-VP16, anti-Ade13p, and anti-Aah1p. (D) Purified His<sub>6</sub>-Pho2p and His<sub>6</sub>-Pho4p bind the AICAR affinity resin. His<sub>6</sub>-Pho2p, His<sub>6</sub>-Pho4p, and GST-Bas1p proteins individually purified from *E. coli* were mixed and tested for their ability to bind an AICAR affinity resin. Proteins from input, flowthrough and eluted fractions were separated by SDS-PAGE and revealed by silver staining. Additional bands correspond to His<sub>6</sub>-Pho2p and GST-Bas1p degradation products as revealed by anti-Pho2p and anti-GST antibodies (data not shown). (E) Effect of *BAS1*, *PHO2*, or *PHO4* overexpression on *PHO84* and *ADE5,7* transcription in wild-type and *ade16 ade17* double mutant strains. Northern blot analyses were done as in Figure 2A on cells transformed with *BAS1*, *PHO2*, or *PHO4* (each expressed under its own promoter on a 2  $\mu$  plasmid).

and further recruitment (or retainment) of Pho2p on the *ADE* gene promoters.

We found that AICAR accumulation stimulates interaction of Pho2p with both Bas1p and Pho4p. Utilization of a common transcription factor together with a common activating metabolite results in competition for activation of the purine and phosphate pathways and thereby probably contributes to fine tuning of both pathways. Intriguingly, accumulation of the upstream metabolic intermediate SAICAR only stimulates Pho2p–Bas1p interaction and specifically up-regulates the Bas1p-responsive genes. The physiological reasons for such a differential response to AICAR and SAICAR are unclear, but could reflect the dual role of adenylosuccinate lyase, the SAICAR metabolizing enzyme, in both the IMP and AMP biosynthesis pathways (Guetsova et al. 1997). This enzyme is feedback inhibited by its products, and therefore

SAICAR levels could reflect both the flux in the de novo pathway and the AMP/GMP balance, while AICAR concentration would mostly reflect the flux in the de novo pathway. Importantly, we found that AICAR accumulation mimics purine starvation in multiple respects, leading to induction of the *PHO* genes only under phosphate replete conditions (Fig. 4C; Supplemental Fig. 4B), resulting in increased Pho2p at the *ADE* gene promoters (Fig. 3A; Som et al. 2005) and stimulating Bas1p–Pho2p interaction (Fig. 5A; Zhang et al. 1997). Thus, our results strongly support AICAR as a secondary messenger of cellular purine availability in yeast. Whether this role could be extended to other eucaryotes remains to be established. In mammalian cells, AICAR is widely used as an “artificial” activator of the AMP-dependent protein kinase (AMPK) (Towler and Hardie 2007) and as such was shown to have anti-diabetic properties in rodents (Buhl et al. 2002) and mimic some effects of exercise (Narkar et al. 2008). The yeast homolog of AMPK is encoded by the *SNF1* gene. To our surprise, none of the genes described as strongly regulated by Snf1p (Young et al. 2003) were found in the 50 AICAR-responsive genes. While it has not been established whether AICAR can stimulate Snf1p in vitro or in vivo, the direct interaction of AICAR with both Pho2p and Pho4p in vitro indicates that the regulation of their target genes in response to AICAR is most probably not dependent on AICAR activation of Snf1p.

Because purine nucleotides are abundant molecules, their synthesis leads to significant phosphate consumption. There is growing evidence that yeast cells have evolved complex regulatory mechanisms to coordinate phosphate utilization and nucleotide synthesis. Recently, two purine metabolism mutants affecting the adenosine kinase (*ADO1*) and the adenylate kinase (*ADK1*) genes were shown to strongly stimulate expression of Pho5p (Auesukaree et al. 2005; Huang and O’Shea 2005). In both mutants, this effect was associated to the relocalization of Pho4p to the nucleus under high-phosphate conditions (Huang and O’Shea 2005) and in the case of *adk1*, the up-regulation was inversely correlated to ATP levels independently of AICAR (Gauthier et al. 2008). Together, our data indicate that the *PHO* pathway regulation is highly responsive to the cellular purine level through sensing of both the final product (ATP) and an intermediate of the pathway (AICAR). The regulation by AICAR is clearly independent of the previously described phosphate-sensing response, since it does neither affect nuclear localization of the transcription factor Pho4p nor require the Vip1p-dependent synthesis of IP7. Instead, we found that AICAR accumulation stimulates Pho2p–Pho4p and Pho2p–Bas1p interactions, thereby unraveling a novel mechanism for a small molecule to regulate transcription.

Overall our data support the idea that cells use a subset of critical metabolites to permanently sense their metabolome and maintain homeostasis. It becomes clear that various mechanisms have evolved for metabolite sensing from “ancestral” riboswitches to protein–protein signaling. Identification of such key cellular metabolites and their effectors will significantly contribute to our understanding of cellular homeostasis.

## Materials and methods

### Strains and media

Strains used in this study are described in the Supplemental Material. Phosphate-free SD medium was prepared with yeast nitrogen base without phosphate (Bio101).

### Two-hybrid assay

For the two-hybrid assay, *bas1 pho2 pho4 ade16 ade17, ade13-42*, and isogenic control cells were transformed with a *lexAop-LacZ* plasmid, and with either a *lexA-BAS1* (Zhang et al. 1997), or a *lexA-PHO2* (Zhang et al. 1997) plasmid and with a plasmid allowing expression of either *BAS1-VP16* (Pinson et al. 2000), *PHO2-VP16* (Barbaric et al. 1998), or *PHO4-VP16* (Barbaric et al. 1998).  $\beta$ Gal activities were measured in transformants as previously described (Daignan-Fornier and Bolotin-Fukuhara 1988).

### GST-Bas1p, Pho2p-His<sub>6</sub>, and Pho4p-His<sub>6</sub> cloning, expression, and purification

GST-Bas1p, Pho2p-His<sub>6</sub>, and Pho4p-His<sub>6</sub> plasmids construction details are described in the Supplemental Material. Protein expression and purification were done as described (Brazas and Stillman 1993; Pinson et al. 1998).

### AICAR affinity column

An AICAR resin was obtained by coupling AICAR to an epoxy-activated sepharose 6B resin (GE Healthcare). For affinity binding with yeast extracts, a *bas1 pho2 pho4* strain was transformed with plasmids allowing expression of either Pho2p, Bas1p-VP16, or Pho4p-VP16. Yeast protein extracts were prepared by vortex grinding with glass beads (5 min in Na<sub>2</sub>HPO<sub>4</sub>/HCl at pH 7.2, 100 mM NaCl) followed by 2-min sonication at maximum intensity (FB15050; Thermo-Fischer Scientific). Cellular debris were eliminated by successive centrifugations (5000g, 5 min, 4°C, and 25,000g, 30 min, 4°C). The supernatant was dialyzed at 4°C against buffer A [Na<sub>2</sub>HPO<sub>4</sub>/HCl at pH 7.2, 10 mM NaCl]. The extract was then incubated with the AICAR affinity resin for 1 h and the suspension was loaded onto a poly-prep chromatography column (Biorad) and rinsed with buffer A. Proteins were eluted with a NaCl gradient in buffer A as described by the manufacturer (GE Healthcare). Ade13p, Aah1p, Bas1p-VP16, Pho4p-VP16, and Pho2p were revealed by Western blotting with anti-Ade13p (1/50,000) (Escusa et al. 2006), anti-Aah1p (1/10,000) (Escusa et al. 2006), anti-VP16 (1/2000; Abcam) and anti-Pho2p antibodies (1/5000; O.S. Gabrielsen). For binding of *E. coli* purified proteins to AICAR affinity resin, GST-Bas1p, Pho2p-His<sub>6</sub> and Pho4p-His<sub>6</sub> were mixed (2 nmol each) in buffer A and then treated as for yeast protein extracts.

### Microscopy

Three tandem copies of GFP were fused in frame at the C terminus of *PHO4* in wild-type, *pho85, ado1, ade16, ade17* strains. Details of construction are described in the Supplemental Material. For imaging, strains were grown in liquid free Pi SD casa WAU medium, either supplemented (High Pi condition) or not (No Pi) with KPi 10 mM, and imaged as described in Laporte et al. (2008)

### ChIP assays

ChIP experiments were performed as described previously (Bernard et al. 2008). Yeast strains (Y4790, Y4757, Y4751,

Y4836, and Y4838) were grown in SDcasaWAU medium OD<sub>600</sub> ~1.0. Cells were fixed for 30 min with formaldehyde 1%, NaCl 10 mM, EDTA 0.1 mM, EGTA 0.05 mM, and Tris/HCl 5 mM (pH 8). Cross-linking was stopped by the addition of Glycine (125 mM). Fifty-microliter cultures were used for each ChIP. Cells were lysed in a Fastprep-24 (four runs of 20 sec at 6.5 m/sec). Extracts were sonicated to shear chromatin to an average DNA fragment size of 0.5–1 kb using a bioruptor sonicator (Diagenode), twice (seven cycles 30 sec on/30 sec off) at maximum power. GFP- and HA-tagged proteins were immunoprecipitated by incubating chromatin extracts with 2  $\mu$ L of polyclonal anti-GFP antibody (A-11122, Invitrogen) or 15  $\mu$ L of monoclonal anti-HA antibody (Sc-7392, Santa Cruz Biotechnologies), respectively. Immunoprecipitations were carried out overnight at 4°C in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, Tris/HCl (pH 8). Immune complexes were collected using ChIP Adembeads protein-A (Ademtech, #04240), washed, and recovered according to manufacturer instructions. ChIP quantifications were performed by real time PCR using a MX3000P cycler (Stratagene) and Absolute qPCR Sybr Green Mix (ABGene). Primers used are described in Supplemental Table 3. A 10-fold dilution series of genomic DNA was used to calibrate the quantification. DNA was quantified in the input and immunoprecipitated samples and the ratio was calculated (percent of immunoprecipitation). Immunoprecipitations were performed in duplicate.

### Northern blots, microarray analyses, and metabolites quantification

Northern Blot, DNA microarray analyses, and intracellular metabolites determination by high-performance ionic chromatography were performed as described in Denis et al. (1998), Breton et al. (2008), and Diaz-Ruiz et al. (2008), respectively. The microarray data were normalized without background subtraction by the global Lowess method performed with the Goulphar software (Lemoine et al. 2006).

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