Regulation of Amino Acid, Nucleotide, and Phosphate Metabolism in *Saccharomyces cerevisiae*

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ABSTRACT Ever since the beginning of biochemical analysis, yeast has been a pioneering model for studying the regulation of eukaryotic metabolism. During the last three decades, the combination of powerful yeast genetics and genome-wide approaches has led to a more integrated view of metabolic regulation. Multiple layers of regulation, from suprapathway control to individual gene responses, have been discovered. Constitutive and dedicated systems that are critical in sensing of the intra- and extracellular environment have been identified, and there is a growing awareness of their involvement in the highly regulated intracellular compartmentalization of proteins and metabolites. This review focuses on recent developments in the field of amino acid, nucleotide, and phosphate metabolism and provides illustrative examples of how yeast cells combine a variety of mechanisms to achieve coordinated regulation of multiple metabolic pathways. Importantly, common schemes have emerged, which reveal mechanisms conserved among various pathways, such as those involved in metabolite sensing and transcriptional regulation by noncoding RNAs or by metabolic intermediates. Thanks to the remarkable sophistication offered by the yeast experimental system, a picture of the intimate connections between the metabolomic and the transcriptome is becoming clear.

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doi: 10.1534/genetics.111.133306

Manuscript received March 20, 2011; accepted for publication August 1, 2011

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IN addition to being the building blocks of proteins, amino acids have a central role in general metabolism. A major achievement of yeast research has been the determination of the complete metabolic pathways for amino acid utilization as carbon and nitrogen sources, amino acid biosynthesis, and the conversion of amino acids to other metabolites including nucleotides. Key reviews on these processes, of almost biblical stature, by Cooper (1982a) and Jones and Fink (1982) are notable since they summarized and integrated results from both biochemical and genetic analyses and thereby provided a solid framework to incorporate findings that have been highlighted in subsequent major reviews (Hinnebusch 1992; Johnston and Carlson 1992; Magasanik 1992). Extensive, albeit not fully complete, information regarding the metabolic networks involving amino acids and nucleotides is available in well-established databases with excellent user interfaces, *e.g.*, the Saccharomyces Genome Database (SGD) (Hong *et al.* 2008) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Aoki-Kinoshita and Kanehisa 2007).

In cells, catabolic nitrogen source utilization and anabolic amino acid and nucleotide biosynthetic pathways function in parallel. These competing processes must be coordinated to enable cells to manifest a proper response to nutrient availability. A requisite for coordination of metabolism is the ability to monitor concentrations of nutrients in the extracellular environment and within cells (for review see Zaman *et al.* 2008). Plasma membrane-localized sensors that respond to the availability of diverse sets of nutrients, including many nitrogen sources, have recently been identified. These environmental sensors operate together with networks of intracellular sensing systems that are spread and function in the cytosol, vacuole/endosome, mitochondria, peroxisome, and nucleus. Furthermore, catabolic and anabolic pathways generate multiple metabolic intermediates that significantly contribute to the complexity of the chemical composition of cells. These metabolic intermediates are not necessarily inert, and there are examples of intermediates providing information (signals) regarding the metabolic status of cells and exerting regulatory effects. Yeast cells can clearly integrate multiple nutrient-based signals derived from spatially separated sensing systems.

Here we focus on regulatory mechanisms and highlight newly attained information regarding aspects of both catabolic and anabolic processes affecting amino acid and nucleotide metabolism. In addition, because nucleotide synthesis is phosphate consuming, regulation of phosphate uptake and utilization is included. Specific examples have been chosen to illustrate how multiple layers of metabolic control are coordinated. Briefly, yeast cells possess suprapathway mechanisms that, in response to metabolic changes, can reprogram large-scale patterns of gene expression. Suprapathway control is exerted at both the transcriptional and the translational levels. In contrast to these general modes of control, cells can also respond very precisely by regulating the activity of specialized transcription factors that bind a particular metabolite and in response activate or repress the expression of specific sets of genes. These mechanisms are complemented by post-translational modes of regulation, which provide cells

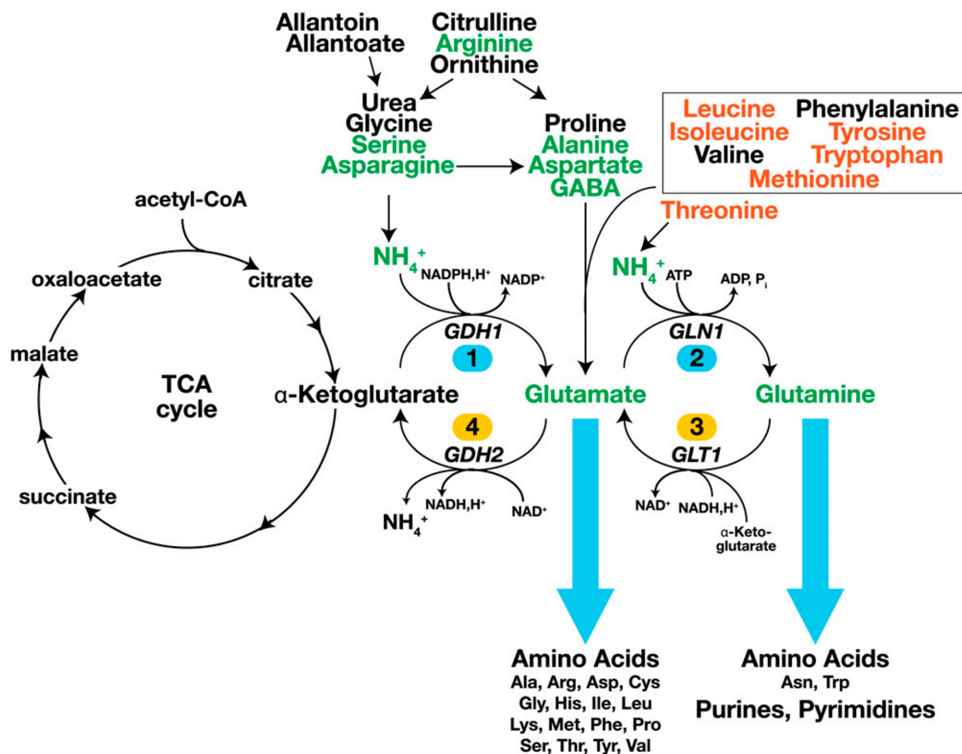

Figure 1 Schematic diagram of the main pathways of nitrogen metabolism. The entry routes of several nitrogen sources into the central core reactions are shown. The class A preferred and class B nonpreferred nitrogen sources are in green and red text, respectively. The nitrogen of preferred nitrogen sources is incorporated into glutamate, and the resulting carbon skeletons are shunted into pyruvate and α-ketoglutarate. Nitrogen from branched-chain amino acids, aromatic amino acids, and methionine (within box) is transferred to α-ketoglutarate by transaminases forming glutamate; the resulting deaminated carbon skeletons are converted to noncatabolizable and growth-inhibitory fusel oils (Hazelwood *et al.* 2008). Nitrogenous compounds are synthesized with nitrogen derived from glutamate or glutamine as indicated (blue arrows). Central anabolic reactions 1 and 2 are catalyzed by NADPH-dependent glutamate dehydrogenase (*GDH1*) and glutamine synthetase (*GLN1*). Central catabolic reactions 3 and 4 are catalyzed by NADH-dependent glutamate synthase (*GLT1*) and NAD^+-linked glutamate dehydrogenase (*GDH2*). For detailed descriptions of the pathways, the reader is referred to the SGD (http://pathway.yeastgenome.org/) or KEGG (http://www.genome.jp/kegg/pathway.html) databases.

with the means to rapidly adjust the catalytic properties of enzymes, modulating the degradation rates of enzymes and permeases and regulating the flow of metabolites in and out of intracellular organelles.

Amino Acids

Nitrogen source utilization: the flow of nitrogen to amino acids, purines, and pyrimidines

Yeast cells react to the nitrogen content of the growth environment by controlling nitrogen source uptake and by regulating catabolic and anabolic processes. As reviewed by Cooper (1982a) and schematically depicted in Figure 1, yeast can use a variety of nitrogenous compounds as sole sources of nitrogen for growth. Although some strain variability exists, all L-amino acids, with the exception of lysine, histidine, and cysteine, can support growth as the sole nitrogen source (Table 1). However, each amino acid supports a distinct rate of growth; in media with glucose as the main carbon source, generation times vary from ∼2 h (*e.g.*, asparagine, glutamine, and arginine) to >4 h (*e.g.*, methionine and tryptophan). The ability to use amino acids and other nitrogenous compounds requires their internalization, and accordingly, yeast cells possess multiple permeases to facilitate their transport across the plasma membrane (Table 4). Notably, the presence of external amino acids induces the expression of several broad-specificity permeases; hence, amino acids induce their own uptake. This transcriptional response is mediated by the plasma membrane localized Ssy1-Ptr3-Ssy5 (SPS) sensor (reviewed in Ljungdahl 2009). Once internalized, nitrogenous compounds can be used directly in biosynthetic processes, be deaminated to generate ammonium, or serve as substrates of transaminases that transfer amino groups to α-ketoglutarate to form glutamate (reviewed in Cooper 1982a; Magasanik 1992; Magasanik and Kaiser 2002). In cells grown on glucose, ammonium can be assimilated by two anabolic reactions, *i.e.*, the synthesis of glutamate from ammonium and α-ketoglutarate catalyzed by the NADPH-dependent glutamate dehydrogenase (*GDH1*) (reaction 1) (Figure 1), and the synthesis of glutamine from ammonium and glutamate by glutamine synthetase (*GLN1*) (reaction 2). In cells grown on ethanol as a carbon source, a Gdh1 isozyme encoded by *GDH3* is expressed and contributes to the assimilation of ammonium (Avendano *et al.* 1997; DeLuna *et al.* 2001). When glutamine is the sole nitrogen source, the NADH-dependent glutamate synthase (*GLT1*) is required to catalyze the synthesis of glutamate (reaction 3). The catabolic release of ammonia from glutamate (reaction 4) is catalyzed by the NAD^+-linked

Table 1 Compilation of literature values: generation times and glutamate and glutamine pool sizes in cells grown on various sole nitrogen sources

Nitrogen source	Generation time (hours.minutes) strain background					μmol 100 mg^{-1} dry weight[d]	
	Σ1278b[a]	Σ1278b[b]	S288c[b]	S288c[c]	Y48[d]	Glu	Gln
	Preferred class A[a]: high-moderate active NCR/high-moderate active UPR/inactive GAAC						
NH_4^+	2.00	2.28	2.24	1.52	2.08	7.4	2.8
Asn	2.00	2.53	2.14	1.49	2.42	5.2	3.4
Gln	2.05	2.24	2.12	2.14	2.16	22.7	43.1
Ser	2.15	2.40	2.33	2.23	5.53	7.0	1.5
Asp	2.10	2.51	2.55	2.19	2.57	5.1	2.9
Ala	2.30	2.43	3.00	3.28	4.33	2.9	0.6
Arg	2.25	3.22	2.49	2.06	2.11	8.2	1.5
Glu	2.15	2.16	2.29	2.29	2.38	37.9	15.6
	Intermediate[a]: slight active NCR/moderate active UPR/inactive GAAC						
Orn	4.30	3.26	3.13	6.56	3.42	12.2	1.8
Pro	3.15	4.28	4.28	4.57	4.33	27.4	3.1
Val	3.00	3.32	3.24	4.05	8.20	4.5	1.0
Phe	3.20	2.33	3.44	3.39	2.51	5.3	0.9
	Intermediate[a]: inactive NCR/inactive-slight active UPR/inactive GAAC						
Urea	3.35	2.38	2.44	—	—	—	—
Cit	4.30	3.06	3.28	—	3.42	2.6	22.1
	Non-preferred class B[a]: inactive NCR/slight active UPR/active GAAC						
Leu	3.25	4.47	4.31	6.18	3.14	10.1	1.5
Ile	3.55	4.48	3.42	6.18	9.06	5.4	0.5
Met	4.05	—	5.29	5.47	5.33	10.7	6.9
Tyr	4.10	2.44	5.26	5.20	4.21	9.1	1.1
Thr	4.20	2.34	5.12	5.47	10.00	2.3	0.6
Trp	4.45	4.01	4.20	6.18	8.20	6.4	1.0

[a] Godard *et al.* (2007).
[b] Cooper (1982a).
[c] Niederberger *et al.* (1981).
[d] Watson (1976).

glutamate dehydrogenase (*GDH2*). This latter reaction is also required to provide ammonium for the synthesis of glutamine when glutamate is the sole nitrogen source. The central importance of glutamate and glutamine in biosynthesis of nitrogenous compounds is illustrated in Figure 1 (blue arrows); ~85% of the total cellular nitrogen is incorporated via the amino nitrogen of glutamate, and the remaining 15% is derived from the amide nitrogen of glutamine (Cooper 1982a).

Nitrogen source: quality of amino acids

The various nitrogen sources used by yeast are often qualitatively referred to as being preferred (good) or nonpreferred (poor). This less-than-precise classification has been empirically based on two criteria. The first criterion is how well the individual compounds support growth when present as sole source of nitrogen. The second criterion reflects the finding that preferred nitrogen sources generally repress processes required for the utilization of nonpreferred nitrogen sources (reviewed in Cooper 1982a; Magasanik 1992; Magasanik and Kaiser 2002). Nitrogen regulation of transcription is a general suprapathway response that is commonly referred to as nitrogen catabolite repression (NCR). NCR primarily functions to ensure that cells selectively use preferred nitrogen sources when they are available, and in the absence of a preferred nitrogen source, the general derepression of NCR-regulated genes enables cells to indiscriminately scavenge alternative, nonpreferred nitrogen sources. The classification of nitrogen sources is not absolute, and their repressive effects can vary significantly between different yeast strain backgrounds. For example, ammonium and, to a lesser extent glutamate are repressing nitrogen sources for Σ1278b-derived strains, whereas, for many S288c-derived strains, they are not, even though these nitrogen sources promote high rates of growth (Magasanik and Kaiser 2002). Genetic analyses have shown that the phenotypic differences between these genetic backgrounds are multifactorial and not fully understood (Magasanik and Kaiser 2002; Georis *et al.* 2009a).

Godard *et al.* (2007) carefully analyzed the patterns of gene expression in prototrophic wild-type cells (Σ1278b) growing in media containing glucose as the carbon source and different sources of sole nitrogen, including 16 individual amino acids. Importantly, the patterns of gene expression were monitored in cells from logarithmically expanding cultures fully adapted to growth with each individual

nitrogen source. This analysis revealed several significant findings. First, the yeast cultures grew at variable rates characteristic for the nitrogen source (Table 1); however, in the comparisons with gene expression patterns, no significant variations in the levels of general stress response genes were detected. Consequently, cells clearly adapt to the quality of the nitrogen source to achieve a balanced state of growth. Second, the pattern of gene expression in urea-grown cells could be used as the reference for comparisons; urea supports intermediate growth and, notably, the major transcriptional regulatory systems, *i.e.*, NCR, general amino acid control (GAAC), and the unfolded protein response (UPR), as well as the SPS-sensing system, are not active. Third, the ability of cells to sense the presence of extracellular amino acids via the SPS-sensing pathway and to prioritize their uptake is relatively independent of nitrogen source. Fourth, several of the nitrogen sources could unambiguously be classified as follows: class A, preferred nitrogen sources—nitrogen-sensitive gene expression is repressed (NCR is active), the UPR is moderately active, and GAAC is inactive; conversely, class B, nonpreferred nitrogen sources—nitrogen-sensitive gene expression is derepressed (NCR inactive), UPR is less active, and GAAC is highly active (Table 1). Finally, as pointed out by Godard *et al.* (2007), the utilization of the preferred amino acids as nitrogen sources yields carbon skeletons that are readily integrated in metabolism (Figure 1). Six of the seven preferred amino acids are substrates of transaminases or deaminases that yield pyruvate (alanine and serine), tricarboxylic acid (TCA) cycle intermediates oxaloacetate (asparagine and aspartate) or α-ketoglutarate (glutamate and glutamine). The nonpreferred class B amino acids are subject to transamination resulting in carbon skeletons that are converted via the Ehrlich pathway to noncatabolizable and growth-inhibitory fusel oils (Hazelwood *et al.* 2008).

Biosynthesis of amino acids

As schematically depicted in Figure 2, yeast cells provided with an appropriate source of carbon and ammonium can synthesize all L-amino acids used in protein synthesis (Jones and Fink 1982). Ammonia is incorporated during the formation of glutamate from α-ketoglutarate (reaction 1) by NADPH-dependent glutamate dehydrogenase (*GDH1*), and glutamine from glutamate (reaction 2) by glutamine synthetase (*GLN1*) (reviewed in Magasanik 2003). The families of amino acids derived from a common molecule are readily identifiable and include the glutamate family (glutamate, glutamine, arginine, proline, and lysine); the aromatic family (phenylalanine, tyrosine, and tryptophan); the serine family (serine, glycine, cysteine, and methionine); the aspartate family (aspartate, asparagine, threonine, and the sulfur-containing amino acids cysteine and methionine); and the pyruvate family (alanine and the branched amino acids valine, leucine, and isoleucine). The histidine and nucleotide biosynthetic pathways are connected. The importance of glutamate and glutamine, and consequently the central core reactions in nitrogen metabolism, becomes apparent by highlighting their involvement in transamination reactions required in the synthesis of each amino acid (Cooper 1982a; Magasanik 1992; Magasanik and Kaiser 2002).

Nitrogen-regulated gene expression

NCR was first recognized as a physiological response in the early 1960s, and the literature regarding NCR is extensive; however, the primary mechanism underlying how cells sense the overall nitrogen status remains unknown (Cooper 2002; Magasanik and Kaiser 2002). This represents a major hole in understanding and a challenge for the future. The aim of the following discussion of NCR is to provide the basis for understanding the rapidly evolving concepts of how nitrogen source utilization pathways are regulated. The gene names defined as standard in the SGD will be used.

Although the nitrogen-sensing mechanism(s) operating upstream of NCR remain elusive, a rather comprehensive understanding of the downstream events of NCR can be outlined as follows. NCR-sensitive genes are controlled by a core set of regulatory components, including Ure2 and the four GATA transcription factors Gln3, Gat1, Dal80, and Gzf3. Gln3 and Gat1 function as activators of gene expression that are efficiently targeted to the nucleus under conditions that derepress the expression of NCR-sensitive genes. In contrast, Dal80 and Gzf3 act as repressors that constitutively localize to the nucleus. All four transcription factors possess zinc-finger DNA-binding motifs that bind core GATAAG consensus sequences present in the promoters of NCR-sensitive genes. The ability of the GATA factors to compete for *cis*-acting GATAAG sequence elements is influenced by nitrogen source availability and is even modulated by events within the nucleus (Georis *et al.* 2009b, 2011).

The expression of NCR-sensitive genes is constitutively depressed by mutations that inactivate Ure2 (Drillien and Lacroute 1972), indicating that Ure2 participates in repressing gene expression in cells grown in the presence of preferred nitrogen sources (Wiame *et al.* 1985; Courchesne and Magasanik 1988; Coschigano and Magasanik 1991). The derepression of NCR genes in the absence of Ure2 is largely dependent on Gln3; cells lacking *GLN3* are unable to derepress NCR-sensitive gene expression (Mitchell and Magasanik 1984; Minehart and Magasanik 1991). Cells carrying mutations that inactivate *URE2* are able to utilize nonpreferred nitrogen sources even in the presence of preferred ones, a finding that has been exploited to optimize industrial fermentations (Salmon and Barre 1998). The inactivation of Ure2 results in constitutive nuclear localization of Gln3. Microscopic analysis and subcellular fractionation studies suggest that a significant portion of Gln3 is membrane associated in cells grown in the presence of a preferred nitrogen source, which may have important consequences for the regulation of the Ure2–Gln3 interaction (Cox *et al.* 2002; Puria *et al.* 2008). Gat1 also targets the nucleus in cells grown in nonpreferred nitrogen sources (Kulkarni *et al.* 2006). However, in contrast to Gln3, Gat1 is not specifically excluded from the nucleus, and the loss of Ure2 does not greatly affect

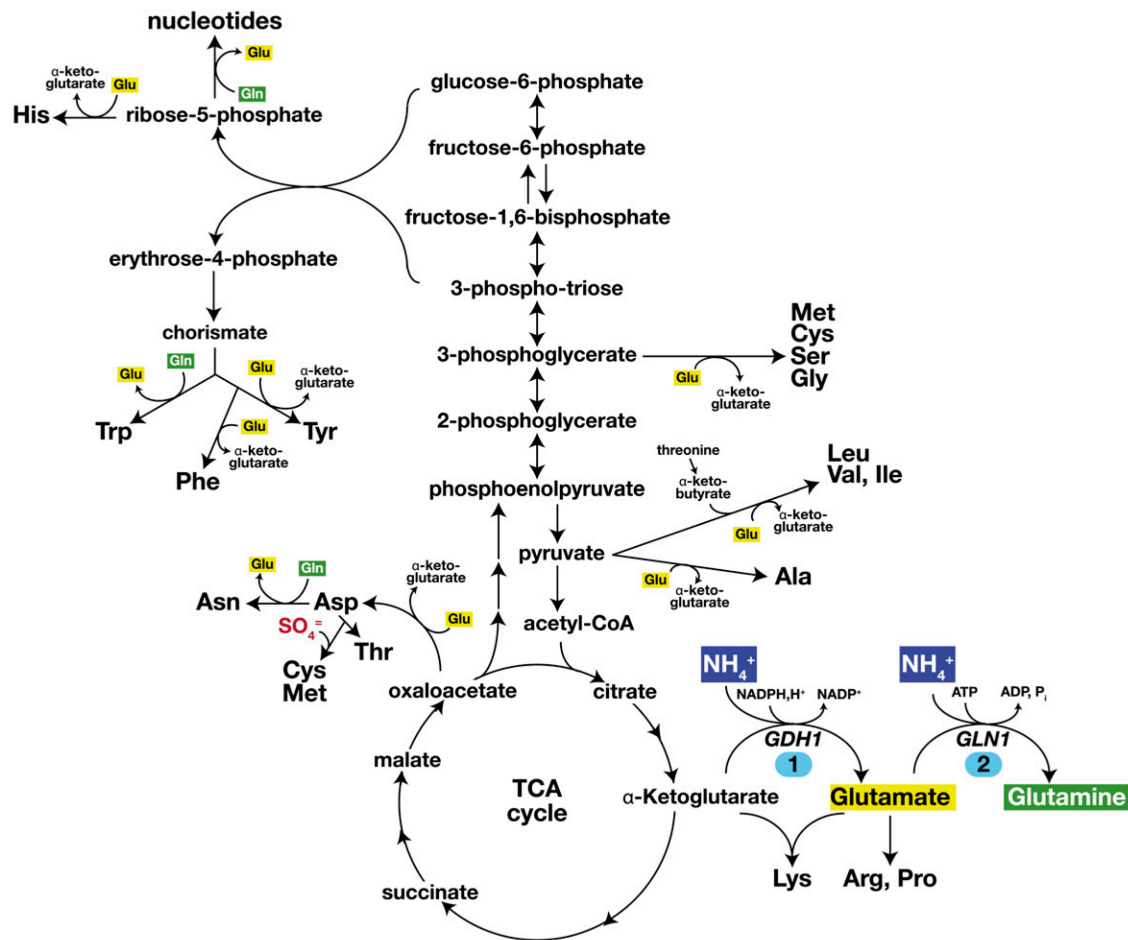

Figure 2 General scheme for the biosynthesis of amino acids from glucose and ammonia. Ammonia is incorporated during the formation of glutamate from α-ketoglutarate (reaction 1) by NADPH-dependent glutamate dehydrogenase (*GDH1*) and of glutamine from glutamate (reaction 2) by glutamine synthetase (*GLN1*). The transamination reactions transferring nitrogen from glutamate (yellow) or glutamine (green) are shown. For detailed descriptions of the pathways, the reader is referred to the SGD (http://pathway.yeastgenome.org/) or KEGG (http://www.genome.jp/kegg/pathway.html) databases.

Gat1 localization. Consequently, Gat1 localization appears largely independent of Ure2; other factors thus must be important in determining Gat1 function (Georis *et al.* 2008, 2009a,b). This notion is consistent with the finding that Gzf3 interacts directly with Gat1 in the nucleus, an interaction that regulates Gat1 promoter binding (Georis *et al.* 2009b).

With the notable exception of *GLN3*, the genes for the other three GATA factors (*GAT1*, *GZF3*, and *DAL80*) are expressed under the control of promoters containing multiple GATAAG sequences, and their expression is sensitive to NCR (Cunningham and Cooper 1991; Coffman *et al.* 1996; Rowen *et al.* 1997; Soussi-Boudekou *et al.* 1997). These factors participate in regulating each other's expression (cross-regulation), exhibiting either positive or negative regulation dependent on their corresponding roles. In certain instances, the factors regulate their own expression (Figure 3) (Coffman *et al.* 1997; Rowen *et al.* 1997; Soussi-Boudekou *et al.* 1997; Georis *et al.* 2009b).

In growing cells, *URE2* and *GLN3* expression are not tightly regulated in response to nitrogen (Coschigano and Magasanik 1991; Georis *et al.* 2009b). Consequently, the Ure2–Gln3 interaction provides cells with a stably expressed regulatory complex, or switch, that can be rapidly controlled to directly activate gene expression. The Ure2–Gln3 switch appears to function as the master controller, which, together with the overlapping regulatory activities of the GATA factors, enables cells to adjust GATA factor levels in a manner appropriate for prevailing nitrogen source availability (Zaman *et al.* 2008). Activation of the switch in cells grown in the presence of nonrepressing (nonpreferred) nitrogen sources results in the suprapathway induction of ∼90 genes (Table 2). Although several models have been proposed for the regulation of the Ure2–Gln3 switch, the current literature does not support a consensus view, and clearly, deciphering the mechanism(s) controlling the Ure2–Gln3 switch remains the Holy Grail of the NCR field.

Target of rapamycin (TOR) signaling and NCR are functionally distinct

It has also been proposed that TOR signaling directly regulates NCR by controlling the Ure2-mediated cytoplasmic retention of Gln3 (Beck and Hall 1999). Consistent with

this notion, cells treated with rapamycin, a specific inhibitor of the TORC1 complex (Loewith *et al.* 2002), exhibit derepressed expression of NCR-sensitive genes. Rapamycin treatment reduces levels of Gln3 phosphorylation, which correlates with its nuclear targeting. In apparent support of this model, the TORC1-regulated phosphatase Tap42-Sit4, negatively controlled by TORC1, has been shown to influence the extent of Gln3 phosphorylation (Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999; Bertram *et al.* 2000; Carvalho *et al.* 2001).

Although very important insights regarding NCR have been gained by examining rapamycin inhibition of TORC1 signaling, and without doubt TORC1 activity can influence NCR, this major signaling hub appears to operate independently, perhaps in parallel of the nitrogen sensor that "naturally" regulates NCR. Consistent with this notion, there is accumulating evidence that rapamycin exerts its effects in a manner that does not faithfully mimic nitrogen starvation (Cox *et al.* 2004; Crespo *et al.* 2004; Kulkarni *et al.* 2006; Georis *et al.* 2008, 2009a; Puria and Cardenas 2008; Puria *et al.* 2008; Tate *et al.* 2009, 2010). For example, in direct opposition to rapamycin treatment, a functional myc-tagged Gln3 construct becomes hyperphosphorylated during nitrogen and carbon starvation (Cox *et al.* 2004; Kulkarni *et al.* 2006), and the phosphorylation status of Gln3 does not affect its ability to bind Ure2 (Bertram *et al.* 2000). Also, Gln3 phosphorylation levels do not correlate with the presence of preferred or nonpreferred nitrogen sources, the intracellular localization of Gln3, or the capacity to support NCR-sensitive transcription (Cox *et al.* 2004; Tate *et al.* 2005; Kulkarni *et al.* 2006). Consequently, the mechanisms controlling Gln3 localization remain to be clarified.

Since the inactivation of TORC1 induces signals that impinge on the NCR-mediated transcriptional control pathway, it is imperative to distinguish between direct and indirect effects. There are several examples where this has been problematic. For example, in ammonium-grown cells, the mutational inactivation of *NPR1* results in Gln3-dependent derepression of NCR-sensitive genes (Crespo *et al.* 2004). The kinase activity of Npr1 is required for proper post-transcriptional control of several ammonium-sensitive permeases (Boeckstaens *et al.* 2007). On the basis of experiments indicating that Npr1 is rapidly dephosphorylated upon rapamycin treatment (Schmidt *et al.* 1998), the derepression of nitrogen-regulated genes was interpreted to support the placement of TORC1 in a pathway negatively controlling NCR (Crespo *et al.* 2004). However, further analysis has shown that the derepression of NCR-regulated genes is linked to the known requirement of Npr1 in facilitating efficient ammonium uptake, *i.e.*, the nitrogen source used in the initial studies. Indeed, NCR is active in *npr1* mutants when ammonium uptake is restored using buffered media (see Wiame *et al.* 1985) or in heterologous expression of a non-Npr1-regulated ammonium transporter from the fungus *Hebeloma cylindrosporum* (Feller *et al.* 2006). Consistently, the presence of preferred amino acids glutamine,

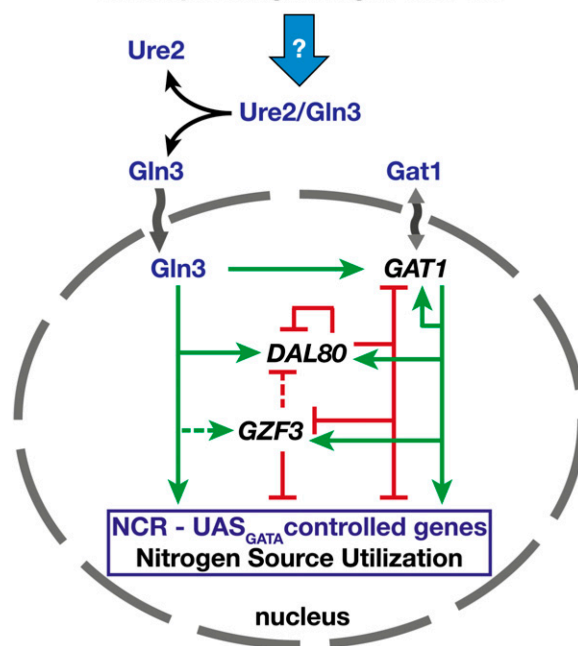

Figure 3 Model of GATA factor and NCR-controlled gene expression. The promoters of *GAT1*, *GZF3*, and *DAL80* contain multiple GATAAG sequences, and their expression is sensitive to NCR. These factors regulate each other's expression (cross-regulation) and in certain instances exhibit partial autogenous regulation. *GAT1* and *DAL80* expression is primarily dependent on Gln3 and Dal80; the expression of these factors is the highest in cells grown under nonrepressive conditions. Inactivation of *GZF3* results in the derepressed expression of several NCR-sensitive genes including *GAT1*, indicating that, in contrast to Dal80, Gzf3 is expressed at functionally significant levels and active in the presence of repressing nitrogen sources. Consistent with this latter finding, *GZF3* expression is induced by Gat1 under conditions when Gln3 is apparently inactive (Rowen *et al.* 1997). Gzf3 maintains low levels of *GAT1* expression by competing with Gat1 at GATAAG-binding sites; in essence, these two factors participate in an autoregulatory loop. Green lines and arrows indicate positive regulation; red lines and bars indicate negative regulation; and dashed lines reflect relatively weaker regulation. The model is modified from Coffman *et al.* (1997) and Georis *et al.* (2009a).

serine, or asparagine also represses NCR-sensitive genes in cells lacking Npr1 function (Tate *et al.* 2006). Also, Crespo *et al.* (2004) reported that inactivating mutations affecting the function of the E3-ubiquitin ligase Rsp5, or its associated proteins Bul1/Bul2, restores repression of NCR-regulated genes in cells lacking *NPR1*. In accordance with the current understanding of these components, and their role in governing the stability of plasma membrane permeases (for review see Lauwers *et al.* 2010), loss of Rsp5-mediated ubiquitylation prevents the degradation of nitrogen-sensitive permeases. Consequently, suppression of the *npr1* mutant phenotype is accounted for by the increased capacity of the *npr1 rsp5* double mutants to take up ammonium (Feller *et al.* 2006). These data demonstrate that Npr1 and TORC1 have indirect roles in regulating NCR, presumably by controlling the functional expression of ammonium permeases.

Table 2 NCR-controlled genes

Category	Experimentally verified[a]	Predicted[b]
Amino acid – nitrogen metabolism	*ASP3*[c], *BAT2*, *CAR1*, *DAL1*, *DAL2*, *DAL3*, *DAL7*, *DCG1*, *DUR1*,*-2*, *GDH2*, *GDH3*, *GLN1*, *PUT1*, *PUT2*, *UGA1*	*ARG4*, *CHA1*, *GDH1*, *GLT1*, *NIT1*, *SDL1*
Plasma membrane nitrogen uptake	*AGP1*, *CAN1*, *DAL4*, *DAL5*, *DUR3*, *GAP1*, *MEP1*, *MEP2*, *MEP3*, *PUT4*, *UGA4*	*DIP5*, *OPT1*, *OPT2.* , *PTR2*
Transcription factors	*DAL80*, *GAT1*, *GZF3*	*GCN4*, *MIG2*, *UGA3*
Vacuole function	*ATG14*, *CPS1*, *LAP4*, *PEP4*, *PRB1*	*AVT1*, *AVT4*, *AVT7*, *MOH1*, *VBA1*, *YHR138c*
Mitochondrial carrier proteins		*GGC1*
Regulatory proteins		*NPR2*, *PMP1*, *RTS3*, *YGK3*
Nucleotide salvage pathways		*AAH1*, *GUD1*, *NRK1*, *URK1*
Carbon metabolism		*ALD4*, *HXK1*
Other	*ECM38*, *VID30*, *YHI9*, *YGR125w*	*ECM37*, *LEE1*, *RNY1*, *RPS0B*, *RSM10*, *SLX9*, *UGX2*, *YDL237w*, *YDR090c*, *YGL196w*, *YJR011c*, *YLR149c*, *YLR257w*, *YOR052c*

[a] Forty-one known NCR-regulated genes as compiled by (Godard *et al.* (2007).
[b] Forty-four genes identified by (Godard *et al.* (2007).
[c] Four copies are present in genome reference strain S288c.

General amino acid control

As noted by Jones and Fink (1982), many enzymes in multiple amino acid biosynthetic pathways are induced in response to starvation for any amino acid. This supra-, cross-pathway regulation is termed general amino acid control, or GAAC (reviewed in Hinnebusch and Natarajan 2002; Hinnebusch 2005). Amino acid starvation can be rapidly induced by the addition of antimetabolites [*e.g.*, 3-amino-1,2,4-triazole, a competitive inhibitor of imidazoleglycerol-phosphate dehydratase (*HIS3*) that catalyzes the sixth step of histidine biosynthesis, and metsulfuron methyl, an inhibitor of acetohydroxyacid synthase (Ilv2) that catalyzes the first step of branched-chain amino acid biosynthesis] or by the removal of an amino acid required for growth of auxotrophic strains. GAAC is required for survival of cells grown on media prepared with amino acid compositions that elicit starvation through feedback inhibition of enzymes in shared pathways. For example, when cells are grown in the presence of both tyrosine and phenylalanine, mutants lacking GAAC cannot grow on media lacking tryptophan (Niederberger *et al.* 1981). In either of the starvation conditions just described, cells activate the expression of a large set of genes (>500) (Figure 4C), including representatives in every amino acid biosynthetic pathway, with the exception of cysteine (Table 3) (Natarajan *et al.* 2001; Hinnebusch and Natarajan 2002).

The transcriptional activator Gcn4, which binds to promoters of genes possessing the consensus UAS_{GCRE} sequence motif GAGTCA, mediates GAAC. *GCN4* expression is induced in starved cells at the translational level by a reinitiation mechanism involving four short upstream open reading frames (uORFs) (Mueller and Hinnebusch 1986). The analysis of how the GAAC pathway controls *GCN4* expression has defined the central mechanisms governing the initiation of protein synthesis in eukaryotes and has provided the basis for understanding translational control of gene expression (Hinnebusch 2005; Altmann and Linder 2010). The mechanistic details of GAAC regulation have advanced to a very precise level of understanding (Sonenberg and Hinnebusch 2009), and a detailed discussion is out of the scope of this review. However, in subsequent sections, the role of GAAC as integrated into the overall metabolic adjustments in growing and nonstarved cells will be discussed, as will its role in the transcriptional regulation of genes associated with amino acid biosynthesis.

An outline of the GAAC pathway and the global consequence of the induced expression of Gcn4 in metabolic regulation is presented in Figure 4. Briefly, upon amino acid starvation, multiple tRNAs become deacylated (Zaborske *et al.* 2009, 2010). Gcn2 has an auto-inhibited kinase domain that is allosterically activated in starved cells through binding of uncharged tRNAs to an adjacent histidyl-tRNA synthetase-like domain (Wek *et al.* 1989; Dong *et al.* 2000). The activated Gcn2 kinase phosphorylates the α-subunit of eIF2, leading to reduced levels of ternary complex (TC; eIF2-GTP-Met-$tRNA_i^{Met}$). The diminished levels of TC decrease the efficiency of scanning ribosomes to reinitiate translation, increasing the proportion of ribosomes that reinitiate translation at *GCN4*. In addition to translational control, *GCN4* transcription is induced under conditions that derepress NCR (Godard *et al.* 2007), and starvation leads to decreased degradation of Gcn4 by the proteasome (Kornitzer *et al.* 1994).

Nitrogen utilization and amino acid biosynthetic pathways are coordinately regulated

The comparisons of the transcriptional response in exponentially growing cells adapted to growth in the presence of a single nitrogen source make the global analyses carried

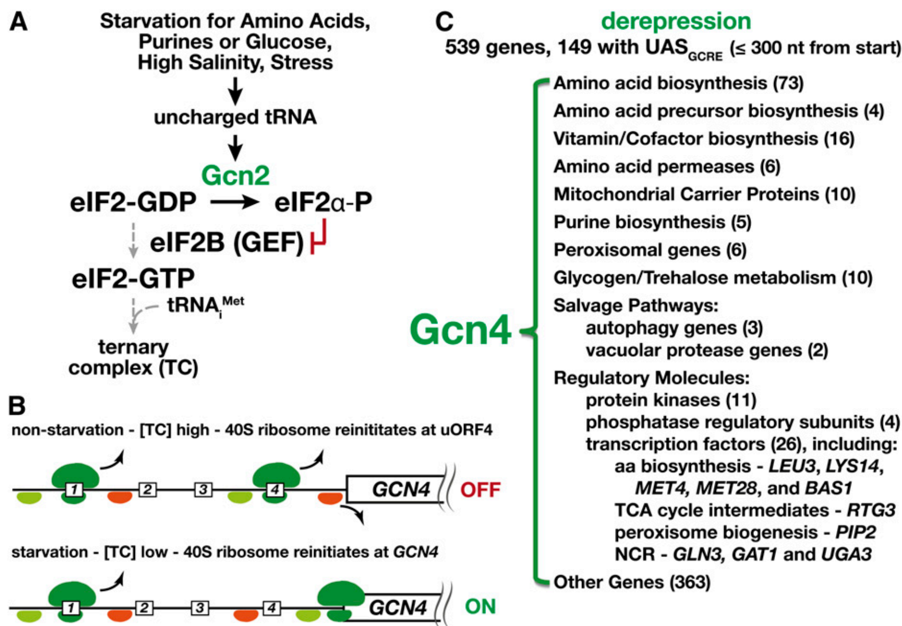

Figure 4 Schematic depiction of the GAAC pathway and the global affect of Gcn4-dependent transcription. (A) GAAC is activated when the levels of any amino acid become limiting, leading to alterations in the pools of charged tRNAs (Zaborske *et al.* 2009, 2010). Uncharged tRNAs bind and activate the Gcn2 kinase, which phosphorylates Ser-51 of the α-subunit of the translation initiation factor eIF2 (Wek *et al.* 1989; Dong *et al.* 2000; Qiu *et al.* 2001). The phosphorylated eIF2α exhibits an enhanced affinity for the GTP-GDP exchange factor eIF2B (GEF), competitively inhibiting the rate of nucleotide exchange, resulting in a reduction in the rate of TC eIF2-GTP-Met-tRNAi formation (gray dashed arrows). (B) The gene encoding the transcription factor Gcn4, the effector of GAAC, is transcribed as an mRNA with four small open reading frames in the 5′-untranslated region (uORF; boxes 1–4) (Mueller and Hinnebusch 1986). As a scanning 40S ribosome with a TC (light green) encounters the first initiator codon of uORF1, the GTP bound to the TC is hydrolyzed to GDP, releasing the eIF2-GDP, and the 60S ribosome is recruited and translation initiates (80S ribosome, dark green). Translation terminates at the uORF1 stop codon, and the 60S ribosome dislocates; the 40S ribosome continues to scan but is unable (red) to initiate translation until it reacquires a TC. Under non-inducing conditions with a high level of TC, the 40S ribosome regains competence (light green) to initiate translation at uORF4. The translation of uORF4 interferes with initiation at *GCN4*. Under GAAC-inducing conditions, due to a low level of ternary complex, the 40S ribosome regains competence after passing uORF4 and initiates translation at *GCN4*. (C) Gcn4 binds to promoters of genes possessing the consensus UAS_{GCRE} sequence motif GAGTCA. Activation of GAAC leads to major reprograming of transcription (>500 genes are induced and >1000 are repressed) (Natarajan *et al.* 2001). The number of induced genes (parentheses) in the categories of proteins relevant to amino acid and nucleotide metabolism is indicated. As indicated in A, a variety of stimuli have been shown to result in increased levels of Gcn4 (Hinnebusch and Natarajan 2002). Some of these responses impinge directly on Gcn2 (Cherkasova *et al.* 2010; Zaborske *et al.* 2009, 2010) and some function independently, apparently in parallel. Notably, Gcn4 stability is increased under amino acid starvation (Kornitzer *et al.* 1994; Shemer *et al.* 2002; Bomeke *et al.* 2006; Aviram *et al.* 2008; Streckfuss-Bomeke *et al.* 2009).

out by Godard *et al.* (2007) unique. Specifically, the absence of gross experimental impositions, such as a shift in nitrogen source, mutational inactivation of genes, or use of inhibitors, provides novel insight as to how multiple general modes of regulation, *i.e.* UPR and GAAC, are integrated with NCR to coordinate the pathways regulating nitrogen source utilization. The UPR is required to modulate processes promoting efficient protein folding in the endoplasmic reticulum (ER); in response to increased levels of folding intermediates or the presence of misfolded proteins in the ER, the UPR is activated to restore folding homeostasis (reviewed in Bernales *et al.* 2006). Accordingly, UPR activation in cells using preferred nitrogen sources presumably reflects an increased demand for ER lumenal chaperones to support the higher rates of protein synthesis in rapidly growing cells. Finally, this study demonstrated that SPS-sensor-controlled gene expression is induced in the presence of high concentrations of most amino acids. This latter finding indicates that cells maintain the expression of broad-specificity amino acid permeases to ensure an enhanced amino acid uptake capacity.

The derepression of GAAC gene expression in cells conditioned for growth using nonpreferred nitrogen sources is significant and clearly supports the notion that GAAC is not merely a starvation response, but is integral to the proper regulation of the amino acid biosynthetic capacity of growing cells. The pronounced activation of GAAC in cells grown in amino acids classified as class B nonpreferred nitrogen sources (Table 1) (Godard *et al.* 2007) suggested that Gcn2 is activated. This was tested by examining the growth of strains lacking *GCN2*, and consistently, the *gcn2*Δ strains exhibited reduced growth in the presence of nonpreferred nitrogen sources. These results clearly indicate that Gcn2 is important to achieving a balanced logarithmic mode of growth on these nitrogen sources. Consistently, it has been demonstrated that shifting cells from ammonium to nonpreferred nitrogen sources leads to alterations in the pools sizes of charged tRNA, leading to Gcn2-dependent phosphorylation of eIF2α (Staschke *et al.* 2010). Finally, *GCN4* mRNA abundance also correlated with derepression of NCR-sensitive genes (Godard *et al.* 2007). Thus, cells using nonpreferred nitrogen sources modulate Gcn4 levels at both the transcriptional and the translational levels in a manner that synergistically increases NCR- and GAAC-regulated gene expression. In the absence of a preferred nitrogen source, the combined derepression of NCR- and GAAC-regulated gene expression reflects the ability of cells to reprogram patterns of gene expression to optimize the catabolic utilization of nonpreferred amino acids and to simultaneously modulate protein synthesis in a manner that prioritizes processes leading to increased amino acid uptake and biosynthesis.

Table 3 General Amino Acid Control (GAAC) - Gcn4-controlled genes linked to amino acid biosynthesis

Pathway	Genes
Histidine	*HIS1*, *HIS2*, *HIS3*, *HIS4*, *HIS5*, *HIS7*
Glutamate/Glutamine	*GLT1*, *GDH2*
Proline	*PRO2*
Arginine	*ARG1*, *ARG2*, *ARG3*, *ARG4*, *ARG5*, *6*, *ARG7*, *ARG8*, ***ARG80***, *CPA1*, *CPA2*
Lysine	*LYS1*, *LYS2*, *LYS4*, *LYS5*, *LYS9*, *LYS12*, ***LYS14***, *LYS20*, *LYS21*
Aromatic (Phe, Trp, Tyr)	*ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8*, *ARO9*, *ARO10*, *TRP2*, *TRP3*, *TRP4*, *TRP5*
Serine/Glycine	*SER1*, *SER3*, *SER33*, *ICL1*, *AGX1*, *GCV1*, *GCV2*, *GCV3*, *LPD1*, *SHM2*
Aspartate	*AAT2*
Asparagine	*ASN1*, *ASN2*
Threonine	*HOM2*, *HOM3*, *THR1*, *THR4*
Methionine	*SUL1*, *SUL2*, *MET1*, *MET2*, *MET3*, ***MET4***, *MET10*, *MET13*, *MET14*, *MET16*, *MET17*, *MET22*, ***MET28***
Branched chain (Leu, Ile, Val)	*ILV1*, *ILV2*, *ILV3*, *ILV6*, *LEU1*, *LEU4*, *BAT1*, *BAT2*, ***LEU3***
Alanine	*ALT1*
TCA cycle	*ACO2*, *IDP1*, *CIT3*, ***RTG3***
NCR	***GLN3***, ***GAT1***

Data are from (Natarajan *et al.* (2001). Genes in boldface type encode transcription factors.

Integration of general and specific modes of regulation

The metabolic pathways in yeast are tuned to nutrient availability. Amino acid utilization and biosynthetic pathways rely on multiple modes of regulation to coordinate metabolic events. Here aspects of the arginine, lysine, methionine, and serine metabolic pathways will be discussed as paradigm examples to illustrate important concepts and the diversity of regulatory mechanisms. In these pathways, both general (NCR and GAAC) and specific modes of transcriptional control operate and function together to achieve nuanced responses. Integration of signals to generate the correct transcriptional response often occurs at the level of the promoters of regulated genes and requires the ordered combinatory assembly of multisubunit complexes. Arginine metabolism provides good examples of end product (arginine) inhibition at the enzyme (allosteric control), transcriptional (direct binding to the ArgR/Mcm1 transcription factor), and translational (*CPA1* expression) levels. Lysine biosynthesis illustrates how end-product inhibition coupled with the direct transcriptional regulation by the pathway intermediate α-aminoadipate semialdehyde (α-AAS) permits the fine-tuning of biosynthetic pathways. Recent advances regarding the regulation of methionine biosynthesis indicates that polyubiquitylation is not necessarily linked to metabolite-induced degradation of the transcriptional activator Met4. Finally, the expression of *SER3*, encoding an enzyme in the serine biosynthetic pathway, provides an example of the recently uncovered role of small noncoding RNAs in governing gene expression.

Arginine metabolism: The arginine utilization (Cooper 1982b) and biosynthetic pathways (Jones and Fink 1982) are outlined and described in Figure 5. If available, arginine is transported into cells by the arginine permease Can1 (Hoffmann 1985), after which it is transported into the vacuole by the Vba2 transporter (Ohsumi and Anraku 1981; Sato *et al.* 1984; Shimazu *et al.* 2005). Greater than 90% of free arginine within cells, *i.e.*, arginine not incorporated in protein, is compartmentalized within the vacuole (Messenguy *et al.* 1980; Kitamoto *et al.* 1988; Ohsumi *et al.* 1988). The mechanisms enabling cells to properly coordinate and regulate levels of arginine in cytoplasmic pools are not well understood. However, it is well established that noncompartmentalized arginine exerts a positive effect on the expression of genes encoding enzymes required for arginine utilization and a repressive effect on genes required for arginine biosynthesis (Dubois *et al.* 1978).

The positive and negative effects on arginine-dependent gene expression are mediated by the multimeric ArgR/Mcm1 complex that binds upstream regulatory motifs in the promoters of arginine-sensitive promoters. The ArgR/Mcm1 complex minimally consists of Arg80, Arg81, Arg82, and Mcm1. Arg80 and Mcm1 are MADS box proteins that are known to coregulate gene expression by facilitating the cooperative binding of diverse sequence-specific factors to cognate promoters (Messenguy and Dubois 2003). Mcm1 and Arg80 form heterodimers that bind arginine-regulated promoters more efficiently than Mcm1 or Arg80 homodimers (Amar *et al.* 2000). The N-terminal domain of Arg81 contains a region related to bacterial arginine repressors, and mutations affecting the conserved residues alter the arginine concentration required for DNA-binding activity of ArgR/Mcm1. Consequently, Arg81 is a good candidate for being the arginine sensor that regulates the activity of the complex (Amar *et al.* 2000). Consistent with a sensor/regulator function, Arg81 facilitates the arginine-dependent recruitment of the Arg80/Mcm1 dimers to promoters. Arg82 is thought to stabilize Arg80/Mcm1 dimers. Arg82 exhibits inositol polyphosphate kinase activity; however, this activity is dispensable for proper ArgR-dependent repression of *ARG* genes (Dubois *et al.* 2000). In contrast, the kinase activity of Arg82 is required for proper Gat1-mediated derepression of NCR-sensitive genes and for phosphate-mediated repression of *PHO* genes (El Alami *et al.* 2003).

The promoters of four arginine-sensitive promoters are schematically depicted in Figure 6; *CAR1* (P_{CAR1}) and *CAR2*

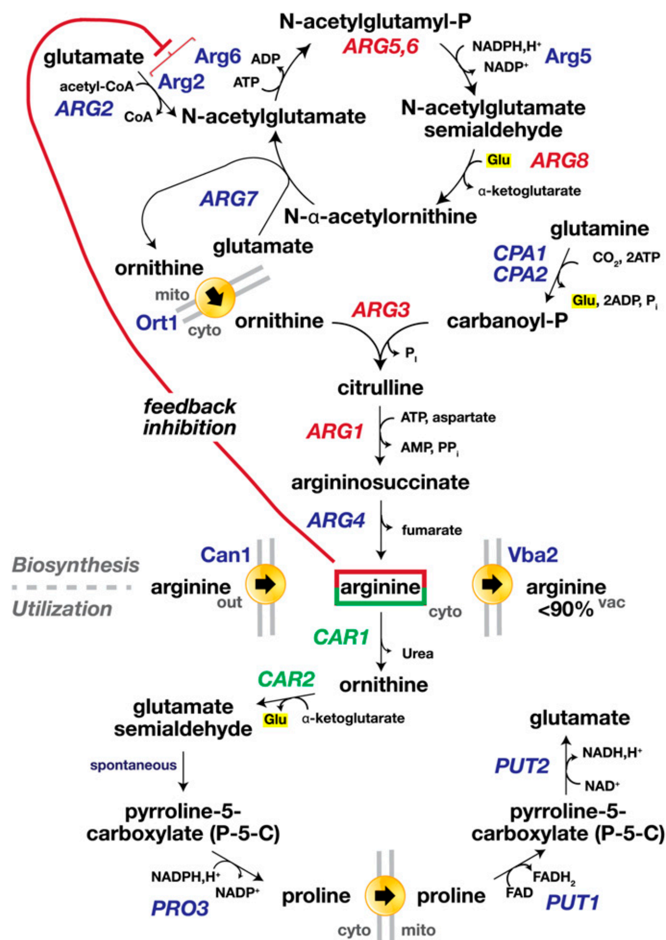

Figure 5 Arginine metabolic network. Arginine is primarily transported into cells by the arginine permease Can1 (Table 4), and once internalized, the bulk of arginine is transported into the vacuole by the Vba2 transporter (Table 6). Cytoplasmic arginine exerts positive (green) and negative (red) effects on gene expression encoding enzymes required for arginine utilization and catabolism, respectively. Both positive and negative regulation relies on the ArgM/Mcm1 complex, which in an arginine-dependent manner participates in activating the expression of the genes in green and repressing the genes in red. (Arginine utilization; bottom) Arginine is degraded to form glutamate. Arginine is initially degraded in the cytoplasm to form proline; this requires the concerted action of arginase (*CAR1*) and ornithine aminotransferase (*CAR2*) to form glutamate γ-semialdehyde, which spontaneously converts to Δ^1-pyrroline-5-carboxylate (P5C). P5C is converted to proline by the *PRO3* gene product. Cytoplasmic proline is transported into the mitochondria where it is converted back to P5C by proline oxidase (*PUT1*). Finally, the mitochondrial P5C is converted to glutamate by the *PUT2* gene product. Whereas *CAR1* and *CAR2* are positively regulated by the presence of arginine (discussed below), the expression of *PUT1* and *PUT2* is induced by proline (Marczak and Brandriss 1989; Siddiqui and Brandriss 1989). Proline binds directly to the transcription factor Put3, a member of the well-studied Zn(II)$_2$Cys$_6$ binuclear cluster family of transcriptional regulators (Des Etages *et al.* 2001). The activation of Put3 requires no additional components and can be induced by certain proline analogs with an unmodified pyrrolidine ring (Sellick and Reece 2003). Detailed structural analysis indicates that proline directly controls the regulatory properties of transcriptional activator, providing a clear demonstration of how metabolite recognition and transcriptional control can be directly coupled (Sellick and Reece 2005). (Arginine biosynthesis; top) The first five steps of biosynthesis take place in the mitochondria (*ARG2*, *ARG5*,*-6*, *ARG8*, *ARG7*) and result in the synthesis of ornithine. *ARG5*,*-6* encode the enzymes that catalyze the second and third steps and are translated into a pre-protein that is imported into mitochondria, where it is cleaved, resulting in separate proteins, *i.e.*, *N*-acetylglutamate kinase (Arg6) and *N*-acetylglutamyl-phosphate reductase (Arg5) (Boonchird *et al.* 1991). The first two enzymes in the pathway, *N*-acetylglutamate synthase (Arg2) and *N*-acetylglutamate kinase (Arg6), bind each other, forming a complex that is necessary for their stability and for feedback inhibition by arginine (Abadjieva *et al.* 2000, 2001; Pauwels *et al.* 2003). The ornithine synthesized in mitochondria is transported to the cytoplasm via the mitochondrial carrier protein Ort1 (Table 5), and the remaining steps are carried out in the cytoplasm. Carbomoyl phosphate reacts with ornithine to form arginine in three steps (*ARG3*, *ARG1*, *ARG4*). Carbomoyl phosphate is synthesized from CO_2, ATP, and the amide nitrogen of glutamine in a reaction catalyzed by the arginine-specific carbomoyl phosphate synthetase, a heterodimeric enzyme composed of a small regulatory subunit (*CPA1*) and a catalytic subunit (*CPA2*).

(P_{CAR2}) are positively controlled, whereas *ARG1* (P_{ARG1}) and *ARG3* (P_{ARG3}) are negatively controlled by arginine. P_{CAR1} contains up to 14 different *cis*-acting promoter elements, of which at least 11 are functionally active and contribute to regulate *CAR1* expression (Smart *et al.* 1996; Dubois and Messenguy 1997). Only three of these promoter elements will be discussed, *i.e.*, the repressing URS1 motif, the UAS_i required for arginine induction, and the UAS_{NTR} required for NCR-controlled transcription. Expression of *CAR1* is largely dependent on overcoming the strongly repressing effect of URS1; mutations that modify the URS1 lead to constitutive expression in the absence of arginine induction. Under non-inducing conditions, Ume6 binding to URS1 recruits the components of the histone deacetylase complex Sin3–Rpd3–Sap30, which results in a repressed state (Messenguy *et al.* 2000). Upon nitrogen starvation, the repression at URS1 is released and Ume6 interacts with the ArgR/Mcm1 complex, presumably enhancing the binding of this complex to the three UAS_i motifs in a manner that facilitates induced expression. To achieve full derepression of *CAR1*, Gln3 and Gat1 binding to GATA sequences in the UAS_{NTR} elements is required (Smart *et al.* 1996). Finally, *CAR1* expression is nonspecifically induced by the addition of micromolar amounts of the amino acids (Dubois and Wiame 1976; Godard *et al.* 2007). Although the precise mechanism responsible for this effect has not been established, mutations inactivating the SPS-sensing pathway prevent this nonspecific induction (Klasson *et al.* 1999). The regulatory mechanisms controlling *CAR2* expression appear similar to *CAR1* (Figure 6); however, P_{CAR2} is not under NCR control (Deschamps *et al.* 1979), but rather is responsive to allophanate, a degradation product of urea. Consistently, P_{CAR2} lacks an UAS_{NTR}, but instead has an upstream inducing sequence motif (UIS_{ALL}) required for allophanate induction (Park *et al.* 1999). The allophanate-regulated factor Dal82 and its coactivator Dal81 bind the UIS_{ALL}.

Regulation of the functional expression of the arginine biosynthetic pathway genes is complex. The transcription of *ARG1*, *ARG3*, *ARG4*, *ARG5*, *6*, and *ARG8* is repressed by arginine via the ArgR/Mcm1 complex (Messenguy and Dubois 2003; Godard *et al.* 2007), and in addition, nine of the genes (*i.e.*, *ARG1*–*ARG8*, *CPA1*, and *CPA2*) are targets of GAAC

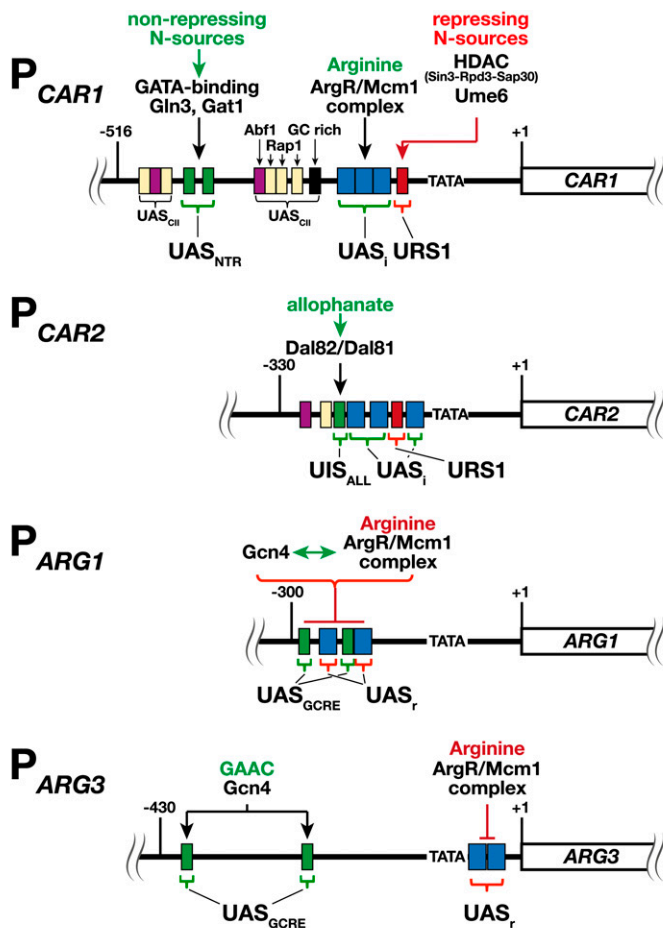

Figure 6 Schematic diagram of the arginine-sensitive promoters P_{CAR1}, P_{CAR2}, P_{ARG1}, and P_{ARG3}. P_{CAR1} and P_{CAR2} are induced, whereas P_{ARG1} and P_{ARG3} are repressed by arginine in an ArgR/Mcm1-dependent manner. The promoter elements, *i.e.*, the sites for specific DNA-binding proteins, are color coded as follows: red, URS1 (Ume6 binding); blue, UAS_i and UAS_r (ArgR/MCM1 binding); black, GC rich; light yellow, Rap1; purple, Abf1; green, UAS_{NTR} (P_{CAR1}; GATA factor Gln3 and Gat1 binding), UIS_{ALL} (P_{CAR2}; Dal82/Dal81 binding), or UAS_{GCRE} (P_{AGR1} and P_{ARG3}; Gcn4 binding). Coordinates are relative to the translation start sites.

(Natarajan *et al.* 2001). Chromatin-immunoprecipitation experiments, used to probe ArgR/Mcm1 repressor binding to the *ARG1* promoter P_{ARG1} (Figure 6), have revealed that Gcn4 binding to P_{ARG1} strongly enhances the subsequent arginine-dependent assembly of ArgR/Mcm1 repressor complexes (Yoon *et al.* 2004). Arg80/Mcm1 heterodimers lacking Arg81 and Arg82 are efficiently recruited to P_{ARG1} in a Gcn4-dependent and an Arg81-independent manner either in the presence or the absence of exogenous arginine. The presence of arginine stimulates the recruitment of Arg81 and Arg82. These findings suggest that Gcn4 facilitates the binding of an Arg80/Mcm1 heterodimer to UAS_r motifs and that, under conditions of arginine excess, arginine binding promotes the subsequent assembly of a functional holo-ArgR/Mcm1 repressor complex. Conversely, during arginine starvation Mcm1 exerts a positive role in *ARG1* transcription. Mcm1 binding to P_{ARG1} enhances Gcn4 binding and recruitment of the positively acting SWI/SNF ATP-dependent chromatin-remodeling complex (Yoon and Hinnebusch 2009; Hong and Yoon 2011). In summary, Gcn4 and Mcm1 function cooperatively, and arginine availability controls the repressor or activator functions of the ArgR/Mcm1 complex at P_{ARG1}.

The role of Gcn4 binding at P_{ARG1} under GAAC-inducing conditions in the absence of arginine has been intensively studied (Govind *et al.* 2005; Kim *et al.* 2005). In response to amino acid starvation, the binding of Gcn4 to the P_{ARG1} UAS_{GCRE} is facilitated by its interactions with the Cyc8–Tup1 complex (Kim *et al.* 2005). Gcn4 binding initiates the nearly simultaneous recruitment of SAGA histone acetylase (HAT), SWI/SNF, and Mediator components [Mediator facilitates interactions between specific coactivation complexes and RNA polymerase II (Pol II)] (Govind *et al*. 2005). These coactivators, together with RSC (ATP-dependent chromatin-remodeling complex), coordinate the rapid recruitment of TATA-binding protein (TBP)–TFIID and Pol II to the promoter, stimulating preinitiation complex assembly and elongation through *ARG1* (see Roeder 2005 for a review on transcriptional coactivators). The finding that amino acid starvation-induced Gcn4 binding results in the rapid recruitment of coactivators suggests cooperative or synergistic interactions between these factors (Govind *et al.* 2005). Consistent with this notion, the SAGA HAT subunit Gcn5 is required for wild-type kinetics of SWI/SNF recruitment, and RSC function is needed for optimal SAGA recruitment. Also, Mediator is strongly required for activator recruitment of both SAGA and SWI/SNF.

Deletion of *CYC8* confers sensitivity to metsulfuron methyl, an inhibitor of isoleucine/valine biosynthesis, suggesting that Cyc8-Tup1 is broadly required in facilitating Gcn4-dependent activation of GAAC-controlled genes. The positive role of Cyc8 in GAAC is consistent with its requirement in activating Rtg3-dependent *CIT2* transcription in response to mitochondrial dysfunction (Conlan *et al.* 1999). In the absence of Cyc8, the diminished binding of Gcn4 to P_{ARG1} is severe enough to reduce the recruitment of SAGA, Mediator, TBP, and RNA Pol II (Kim *et al.* 2005). The overexpression of *GCN4* does not suppress these defects, raising the possibility that Gcn4 may enhance its own binding to the UAS_{GCRE} by recruiting Cyc8-Tup1. Together, these findings clearly demonstrate the important role of Cyc8 in the induction of amino acid biosynthetic gene expression.

Two sequence motifs involved in arginine-mediated repression of *ARG3* lie immediately downstream of the TATA box in P_{ARG3} (de Rijcke *et al.* 1992). To mediate ArgR/Mcm1-dependent repression, these motifs must be located close to the transcription initiation start site; however, they remain functional even when the TATA box is moved to a downstream location. The placement of the arginine response element in P_{ARG3} is consistent with ArgR/Mcm1 binding interfering with the assembly (or functioning) of the transcriptional preinitiation complex. Interestingly, the displacement of the UAS_r to a far upstream position 5′ of the most proximal UAS_{GCRE} abolishes its repressive effect. In this

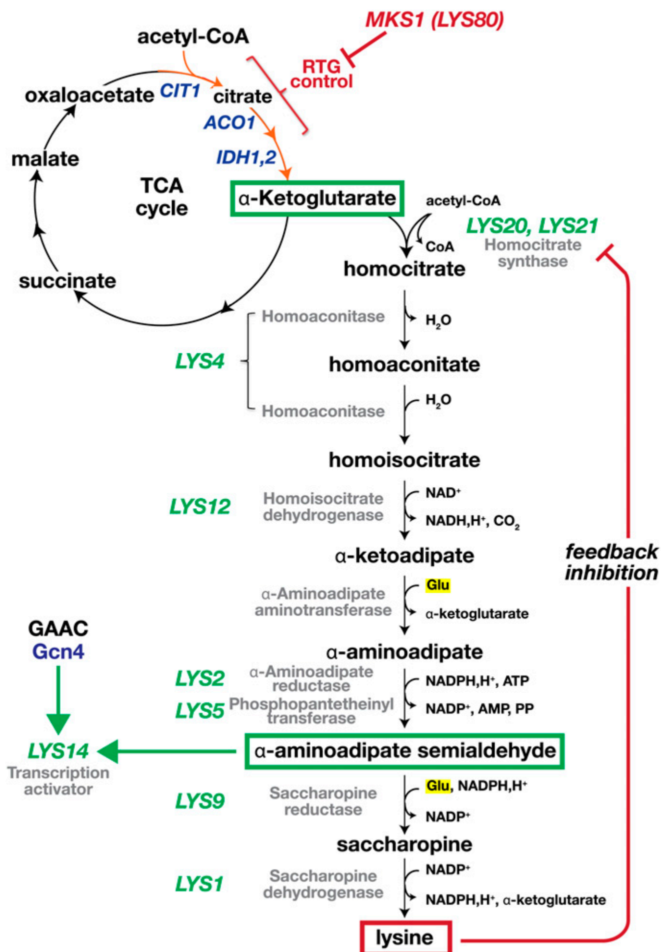

Figure 7 Lysine biosynthetic pathway. *LYS* gene expression is controlled in response to the levels of α-AAS. This pathway intermediate binds and activates the pathway-specific transcription factor Lys14. As a consequence of a pathway intermediate controlling Lys14, conditions that increase or decrease the flux through the pathway, positively or negatively, affect *LYS* gene expression, respectively. The pathway is stimulated by the precursor α-ketoglutarate and consistently activated in cells lacking *MKS1*. Conversely, due to feedback inhibition of the first step of the pathway (catalyzed by either Lys20 or Lys21), excess lysine reduces the production of α-AAS and causes apparent repression of the *LYS* genes. *LYS14* is subject to GAAC regulation, which suggests that derepression of all eight *LYS* genes under amino acid starvation conditions is mediated through Gcn4-induced *LYS14* expression (Natarajan *et al.* 2001).

new context, the ArgR/Mcm1-binding motif serves to enhance *ARG3* expression in the presence of arginine, but only in the absence of Gcn4. These findings are consistent with the more recent studies analyzing the arginine-sensitive promoters discussed above.

Carbomoyl phosphate is required during the synthesis of arginine (Figure 5). This intermediate is derived from CO_2, ATP, and the amide nitrogen of glutamine in a reaction catalyzed by the arginine-specific carbomoyl phosphate synthetase, a heterodimeric enzyme composed of a small regulatory subunit (*CPA1*) and catalytic subunit (*CPA2*). The expression of *CPA1* is regulated at the level of translation in manner that is fundamentally distinct from the mechanism controlling the translation of *GCN4* (Werner *et al.* 1987; Delbecq *et al.* 1994; Gaba *et al.* 2001). The *CPA1* mRNA has a 250-nt leader that encodes a uORF composed of 25 codons, termed the arginine attenuator peptide. In the absence of arginine, ribosomes are able to reach the downstream start codon of the Cpa1-coding sequence by scanning past the uORF. However, in the presence of high levels of cytoplasmic arginine, ribosomes synthesizing the uORF polypeptide stall at its termination codon in an arginine attenuator peptide sequence-dependent manner. As a consequence of stalled ribosomes, *CPA1* mRNA is degraded by the induction of nonsense-mediated mRNA decay (Gaba *et al.* 2005). Thus, the translational regulation of *CPA1* occurs by impairing ribosome scanning and not by affecting reinitiation, as is the case of the translational control of *GCN4* expression (Hood *et al.* 2009).

Lysine metabolism: Lysine is synthesized from α-ketoglutarate via the fungal-specific α-aminoadipate (AAA) pathway (Figure 7) (Xu *et al.* 2006). This pathway is composed of eight enzymatic steps involving nine gene products; with the exception of the gene encoding the α-aminoadipate aminotransferase, catalyzing the fourth step of the pathway, all *LYS* genes have been defined. The first step of the pathway (homocitrate synthase) is catalyzed by either Lys20 or Lys21. The homocitrate synthase activity of both Lys20 and Lys21 is feedback-inhibited by lysine (Andi *et al.* 2005). These proteins are 90% identical (Chen *et al.* 1997), and although Lys20 activity accounts for ∼70% of the flux through the pathway, both Lys20 and Lys21 can individually support wild-type growth in the absence of the other during fermentative growth on glucose (Feller *et al.* 1999; Quezada *et al.* 2008). However, during respiratory growth, *e.g.*, using ethanol as carbon source, inactivating mutations in *LYS21* but not *LYS20* impair growth. Under these conditions, the levels of Lys20 are reduced post-transcriptionally, as *LYS20* mRNA is unaffected by carbon source. Together, these findings suggest that, during respiratory growth, cells control the activity of Lys20 to avoid diverting α-ketoglutarate into lysine biosynthesis (Quezada *et al.* 2008). Interestingly, in cells grown in the absence of exogenously added lysine, under conditions where *LYS* gene expression is derepressed, Lys20 and Lys21 specifically localize to the nucleus (Chen *et al.* 1997); the functional significance of their nuclear localization remains unknown. The next two steps of the pathway from homocitrate to α-ketoadipate (*LYS4* and *LYS12*), and the final three steps from α-aminoadipate to lysine (*LYS2/LYS5*, *LYS9*, and *LYS1*) are thought to take place in the mitochondria and cytosol, respectively (Xu *et al.* 2006). Several mitochondrial carrier family members (Odc1, Odc2, and Ctp1) are implicated in the transport of AAA intermediates across the mitochondrial inner membrane (Table 5) (Breitling *et al.* 2002; Palmieri *et al.* 2006).

A fundamental feature of this pathway is that expression of the *LYS* genes is controlled in response to the levels of α-AAS. This pathway intermediate binds and activates the

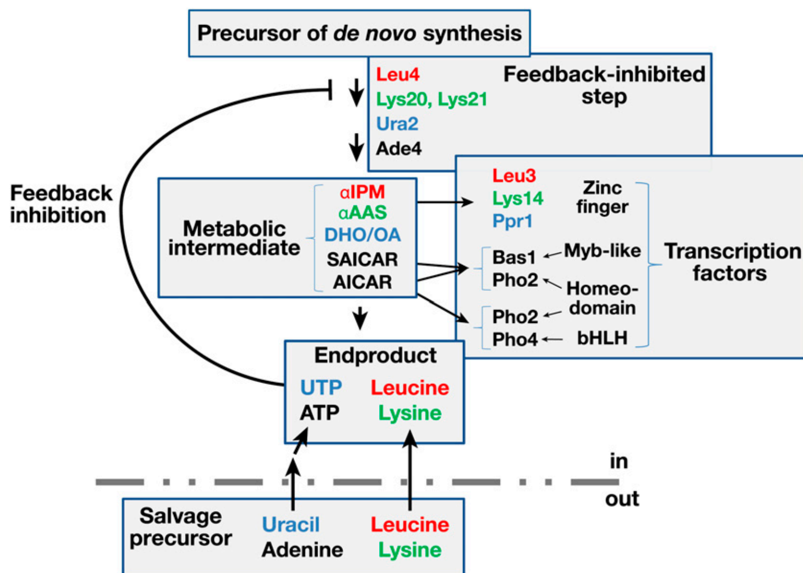

Figure 8 Transcriptional regulation of biosynthetic pathways by metabolic intermediates. The expression of genes encoding catalytic components in the lysine (green), leucine (red), pyrimidine (blue), and purine (black) is controlled by pathway-specific transcription factors that induce transcription upon binding a metabolic intermediate of the pathway. In these pathways, feedback inhibition by the end product of the first and committing step of the pathway provides the means to decrease the production of the inducer and cause the apparent repression of the pathway. This dual-sensing mechanism permits fine-tuning of biosynthetic pathways by integrating both the final end-product concentration, whether synthesized or transported into cells via salvage mechanisms, and the flux in the pathway (as sensed via the concentration of strategic metabolic intermediates).

pathway-specific transcription factor Lys14 (Becker *et al.* 1998; El Alami *et al.* 2002). Lys14 is a member of the Zn (II)$_2$Cys$_6$ binuclear cluster family of transcriptional regulators, which are constitutively nuclear and found associated with promoter sequences of the genes they regulate (reviewed in Campbell *et al.* 2008). As a consequence of a pathway intermediate controlling the capacity of Lys14 to activate gene expression, conditions that increase or decrease the flux through the pathway, positively or negatively, affect *LYS* gene expression. The pathway is stimulated by the precursor α-ketoglutarate, which accounts for the observed activation of the pathway in cells lacking *MKS1* (*LYS80*) (Feller *et al.* 1997). Inactivation of *MKS1* leads to increased α-ketoglutarate levels due to activation of the retrograde response, which induces Rgt1/Rtg3-dependent transcription of genes encoding the TCA cycle enzymes citrase synthase (*CIT1*), aconitase (*ACO1*), and isocitrate dehydrogenase (*IDH1*,*-2*) (Liu *et al.* 2003, 2005). Consequently, the increased flux in the pathway results in elevated production of α-AAS, turning on Lys14-dependent expression of all *LYS* genes (Dilova *et al.* 2002). Conversely, due to feedback inhibition of the first step of the pathway (catalyzed by either Lys20 or Lys21) (Andi *et al.* 2005), excess lysine reduces the production of α-AAS and causes apparent repression of the *LYS* genes. Similar regulatory schemes integrating both the final product concentration and the flux in the pathway (sensed via the concentration of strategic metabolic intermediates) are found in the leucine, purine, and pyrimidine synthesis pathways (Figure 8) (Flynn and Reece 1999). This dual mechanism permits fine-tuning of biosynthetic pathways.

Finally, *LYS* gene expression is coordinately induced in cells lacking functional peroxisomes, suggesting that α-AAS is normally sequestered within this organelle (Breitling *et al.* 2002). This latter finding raises the possibility that one or more steps of basal lysine biosynthesis may occur within peroxisomes, which would restrict α-AAS from entering the nucleus and preventing improper induction of Lys14-dependent gene expression.

Methionine metabolism: The synthesis of the sulfur-containing amino acids methionine and cysteine has been extensively studied (reviewed in Thomas and Surdin-Kerjan 1997). The synthetic pathways of these amino acids also provide cells with glutathione and *S*-adenosylmethionine (SAM) (Figure 9). Glutathione functions as a major redox buffer maintaining the reducing environment of the cytoplasm and is required for cell survival under cadmium and arsenic stress (Dormer *et al.* 2000; Baudouin-Cornu and Labarre 2006). SAM serves as a methyl donor and is an important precursor for the synthesis of polyamines, vitamins, phospholipids, and modified nucleotides.

The expression of the majority of genes encoding enzymes of the sulfur metabolic network requires the transcriptional activator Met4 (Thomas and Surdin-Kerjan 1997; Lee *et al.* 2010). Although the C-terminal region of Met4 contains a dimerization/DNA-binding domain of the basic-leucine zipper family, Met4 lacks DNA-binding activity. Hence the ability of Met4 to activate transcription depends on interactions with DNA-binding factors that act as dedicated adaptors for recruiting Met4 to promoters. Met4 interacts directly with either of two highly similar zinc-finger proteins, Met31 and Met32, or with the basic-helix-loop-helix protein Cbf1. In a recent transcriptome analysis, 45 core Met4-dependent promoters were identified, and each contained a Met31/Met32-binding site that consisted of a CTGTGGC motif; in 24 of these promoters, a Cbf1 motif with an invariant sequence of CACGTGA is present (Lee *et al.* 2010). Thus, the association of Cbf1 and Met31/32 with their respective DNA elements in *MET* promoters appears to provide platforms for recruiting and interacting with Met4. An additional cofactor, Met28, which also lacks DNA-binding activity, is thought to stabilize DNA-bound Met4 complexes (Kuras *et al.* 1997; Blaiseau and Thomas

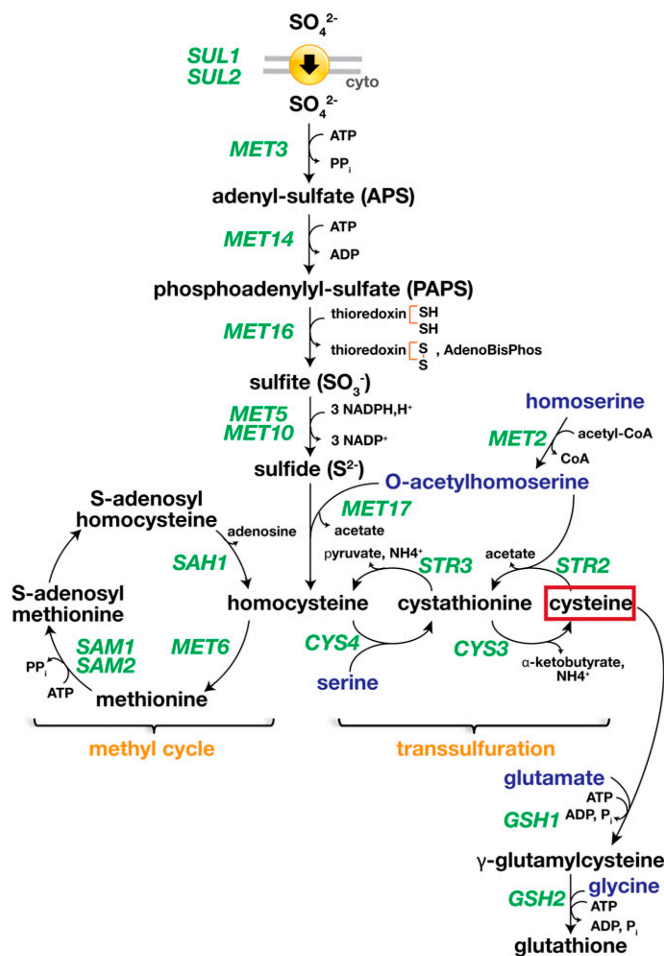

Figure 9 Sulfur metabolic network. Three major branches of the sulfur metabolic network have been defined. First, sulfate is transported into cells via the sulfate permeases (*SUL1* and *SUL2*) and is reduced to sulfide (*MET3*, *MET14*, *MET16*, *MET5*, and *MET10*). Second, sulfide is incorporated in the formation of homocysteine (*MET17*) from *O*-acetylhomoserine that is derived from homoserine (*MET2*). Third, homocysteine is converted to methionine and SAM in the methyl cycle (*MET6*, *SAM1*, *SAM2*, *SAH1*) or converted to cysteine in the two steps of the transsulfuration pathway (*CYS4* and *CYS3*). Glutathione is synthesized from cysteine (*GSH1*, *GSH2*). The sulfur-containing compounds are written in black. The levels of cysteine negatively control the activity of Met4-dependent transcription. The genes under positive control by Met4 are indicated in green.

1998). Under sulfur-limiting conditions, these interactions enable Met4 to activate transcription through recruitment of the SAGA histone acetyltransferase and Mediator coactivator complexes (Kuras *et al.* 2002; Leroy *et al.* 2006).

The activity of Met4 is tightly controlled according to the sulfur status of the cell; the intracellular level of cysteine provides the major regulatory signals for *MET* gene expression (Hansen and Johannesen 2000). Under repressing conditions when cysteine is abundant, the activation potential of Met4 is negatively controlled by SCF^{Met30} (Kaiser *et al.* 2000; Rouillon *et al.* 2000; Kuras *et al.* 2002; Flick *et al.* 2004; Chandrasekaran *et al.* 2006; Menant *et al.* 2006; Ouni *et al.* 2010). Met30 is the substrate recognition subunit of the essential Skp1/Cdcd53/F-box protein Met30 (SCF^{Met30}) ubiquitin ligase complex. In striking contrast to most studied instances in which SCF-complex ubiquitylation targets substrates for degradation by the 26S proteasome (reviewed in Jonkers and Rep 2009), the ubiquitylation of Met4 by SCF^{Met30} is not strictly linked to its immediate degradation (Kaiser *et al.* 2000; Flick *et al.* 2004). In the context of its association with SCF^{Met30}, polyubiquitylated Met4 is stabilized by interactions with its cofactors Cbf1, Met31, and Met32 (Chandrasekaran *et al.* 2006; Chandrasekaran and Skowyra 2008). Conversely, the cofactors associated with Met4-SCF^{Met30} are polyubiquitylated and targeted for degradation (Ouni *et al.* 2010). Under cysteine-limiting conditions, due to inhibition of SCF^{Met30}, perhaps linked to the dissociation of the Met30 (Barbey *et al.* 2005), Met4 and its bound cofactors are not ubiquitylated, and *MET* gene expression is induced (Kuras *et al.* 2002; Leroy *et al.* 2006). The pools of free Cbf1, Met31, and Met32 not associated with Met4 are degraded in a constitutive manner (Ouni *et al.* 2010).

The importance of SCF^{Met30} control of Met4 activity is clearly demonstrated by the finding that the lethality resulting from the inactivation of *MET30*, leading to unbridled Met4 activation function, can be bypassed by deletion of the activation domain of Met4 or the deletion of *MET32*, but not of *CBF1*, *MET28*, or *MET31* (Patton *et al.* 2000; Su *et al.* 2005). Consistent with Met32 having an important role in *MET* gene expression, a truncated version of Met32 (Met32Δ145–192) acts as a dominant suppressor of *met30* null mutations by interfering with the recruitment of Met4 to both Cbf1 and Met31/32-dependent promoters (Su *et al.* 2008).

The transcription of *MET30* is itself regulated in response to the sulfur status of cells and is dependent upon Met4. Consequently, Met4 appears to reciprocally control its own levels through a feedback-like mechanism that regulates the amount of assembled SCF^{Met30} ubiquitin ligase (Rouillon *et al.* 2000). Finally, although GAAC is thought to have a limited role in *MET* gene expression under methionine-limiting conditions, starvation for histidine or tryptophan results in strong Gcn4-dependent induction of several *MET* genes, including *MET1*, *MET10*, *MET13*, *MET16*, *MET17*, *MET22*, *MET28*, *SUL1*, and *SUL2* (Natarajan *et al.* 2001). The fact that Gcn4 induces *MET28* suggests that GAAC may indirectly activate *MET* genes by facilitating the stability of pathway-specific activation complexes. In addition, and perhaps in a synergistic manner, *SAM1* and *SAM2*, which encode SAM synthetases, have been reported to be repressed two- to fivefold under GAAC-inducing conditions. Thus, it is possible that GAAC-dependent repression of SAM synthetase decreases the SAM pool and activates *MET* gene transcription by reducing SCF^{Met30}-mediated degradation of Met4 (Natarajan *et al.* 2001).

Serine biosynthesis: *SER3* encodes phosphoglycerate dehydrogenase, which catalyzes the first step in serine

biosynthesis from 3-phosphoglycerate (Figure 2). *SER3* expression is negatively regulated by serine availability by a newly discovered mechanism that involves the expression of *SRG1*, a small noncoding RNA (Martens *et al.* 2004). High serine levels induce transcription of *SRG1*, and its expression is associated with repositioning of nucleosomes in a region that overlaps the *SER3* promoter, which consequently represses *SER3* (Figure 10) (Hainer *et al.* 2011). Expression of *SRG1* is activated by the well-characterized transcription factor Cha4 (Martens *et al.* 2005), a member of the Zn $(II)_2Cys_6$ binuclear cluster family of transcriptional regulators (Holmberg and Schjerling 1996). In a serine-dependent manner, Cha4 recruits the SAGA and Swi/Snf coactivator complexes to the *SRG1* promoter, events also required for *SER3* repression. Importantly, Cha4 binds to UAS_{CHA} elements in the promoter of genes required for serine/threonine catabolism and, in response to serine or threonine induction, activates their expression, *e.g.*, *CHA1* encoding the catabolic serine/threonine deaminase (Holmberg and Schjerling 1996). Taken together, these findings demonstrate that serine repression of *SER3* transcription occurs by activating *SRG1* intergenic transcription. Thus, yeast uses the same transcription factor to simultaneously activate and repress opposing pathways to regulate serine biosynthesis and catabolism.

SPS-sensor signaling: extracellular amino acid-induced nitrogen source uptake

During the past 10 years, it has become clear that yeast cells possess and use plasma membrane-localized sensing systems to obtain information regarding concentrations of nutrients in the extracellular environment, including the availability of amino acids, ammonium, and glucose (reviewed in Forsberg and Ljungdahl 2001b; Zaman *et al.* 2008; Rubio-Texeira *et al.* 2010). Several of these newly discovered nutrient sensors have components that are members of protein families of well-characterized nutrient transporters. Interestingly, the ability of these transporter homologs to transduce nutrient (ligand)-induced signals across the plasma membrane appears to be independent of nutrient uptake, and thus these sensor components apparently function analogously to traditional ligand-activated receptors.

Growing yeast cells respond to the presence of micromolar amounts of extracellular amino acids by inducing the expression of genes required for their uptake. This nutrient-induced response is mediated by the SPS-sensing pathway (reviewed in Ljungdahl 2009). This pathway derives its name from the three core components of the plasma membrane-localized SPS sensor, *i.e.*, **S**sy1, **P**tr3, and **S**sy5 (Forsberg and Ljungdahl 2001a). The SPS sensor regulates gene expression by controlling the activity of two transcription factors, Stp1 and Stp2 (Figure 11A) (Andréasson and Ljungdahl 2002). These factors are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains that function as nuclear exclusion determinants (Andréasson and Ljungdahl 2004). Upon induction by extracellular

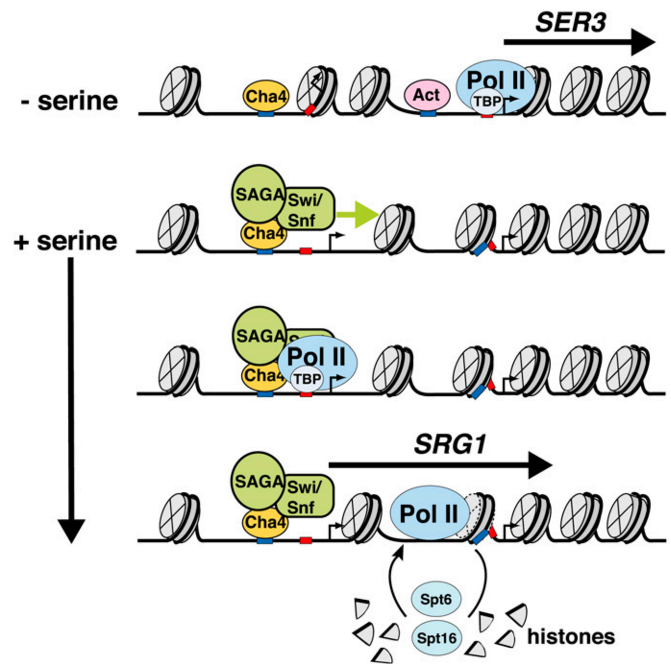

Figure 10 Model for the repression of *SER3* by *SRG1* intergenic transcription. In the absence of serine, the Cha4 activator is bound to the *SRG1* promoter but is unable to initiate transcription. The *SER3* promoter is depleted of nucleosomes allowing proteins, either an as-yet-unknown sequence-specific activator or general transcription factors, to bind and activate *SER3* transcription. In response to serine, Cha4 recruits SAGA and Swi/Snf to reposition the nucleosomes at the 5′ end of *SRG1* toward the *SER3* promoter, permitting initiation of *SRG1* transcription. These repositioned nucleosomes are then disassembled ahead of the transcribing RNA Pol II and reassembled after passage of RNA Pol II by the Spt6 and Spt16 histone chaperones. The nucleosomes being maintained by *SRG1* transcription occlude the *SER3* promoter, preventing the binding of transcription factors and *SER3* transcription. This figure and legend, orginally published in Pruneski and Martens (2011), are reproduced in accordance with Landes Bioscience policy, the publishers of *Cell Cycle*, with permission of the authors.

amino acids, the SPS sensor catalyzes an endoproteolytic processing event that cleaves the regulatory N-terminal domains. The shorter forms of Stp1 and Stp2 efficiently target to the nucleus where they bind promoters of a limited set of genes, including a subset of broad-specificity amino acid permeases (cluster 1, Table 4) and the peptide transporter Ptr2 (Didion *et al.* 1996, 1998; de Boer *et al.* 1998, 2000; Iraqui *et al.* 1999; Klasson *et al.* 1999; Wielemans *et al.* 2010; Tumusiime *et al.* 2011).

Ssy1 is a unique member of the amino acid permease family of proteins (Table 4). Ssy1 does not catalyze measurable amino acid uptake (Didion *et al.* 1998; Iraqui *et al.* 1999; Klasson *et al.* 1999), but instead functions as a receptor of extracellular amino acids (Figure 11B) (Wu *et al.* 2006). In addition to a core membrane transporter-like domain composed of 12 hydrophobic membrane-spanning segments, Ssy1 has an extended cytoplasmically oriented N-terminal domain that is not present in other amino acid permeases. Consistent with being a receptor, Ssy1 exhibits

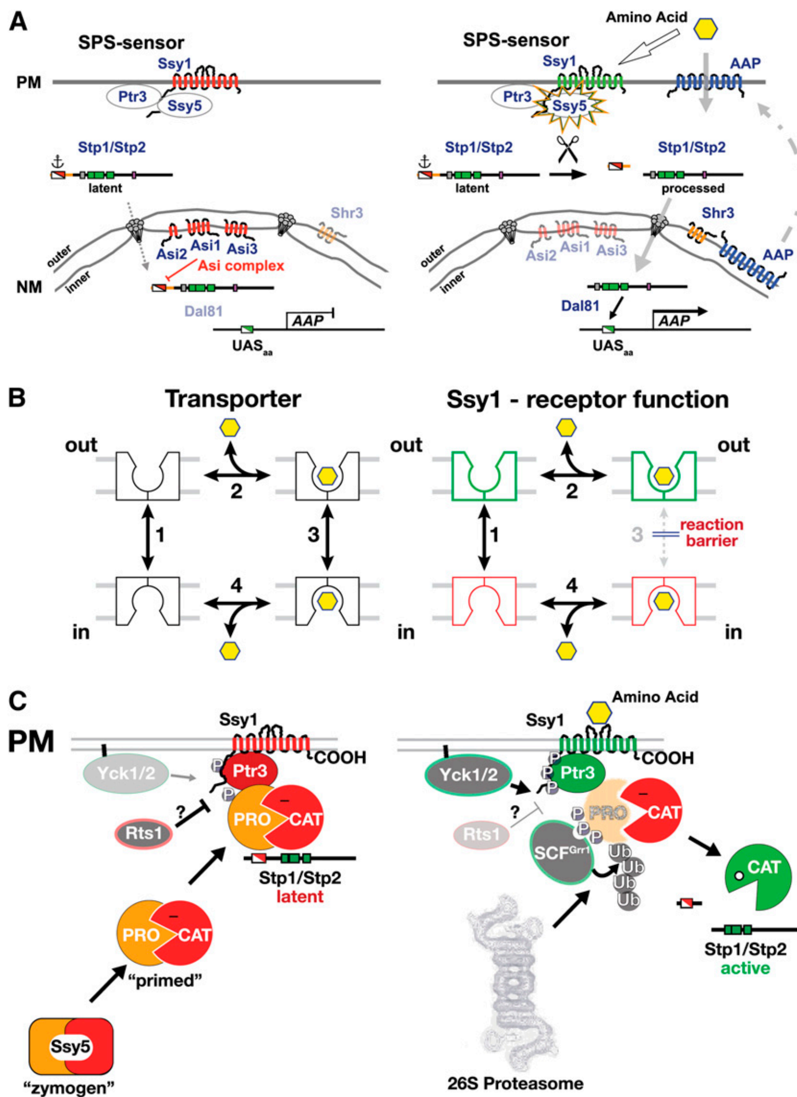

Figure 11 Schematic diagram of the SPS-sensing pathway of extracellular amino acids. (A) In cells grown in the absence of inducing amino acids (left), the SPS sensor of extracellular amino acids is present in the plasma membrane (PM) in its preactivation conformation (Forsberg and Ljungdahl 2001a), and the transcription of SPS-sensor-regulated genes, *i.e.*, amino acid permeases (AAP), occurs at basal levels, and cells exhibit low rates of amino acid uptake. The transcription factors Stp1 and Stp2 (DNA-binding motifs, green boxes) are synthesized as inactive precursors that localize to the cytosol due to the presence of their N-terminal regulatory domain (anchor) that prevents them from efficiently entering the nucleus. Low levels of full-length Stp1 and Stp2 that escape cytoplasmic retention (dashed arrow, left panel) are prevented from derepressing AAP gene expression due to activity of the Asi complex (Asi1–Asi2–Asi3) (Boban *et al.* 2006; Zargari *et al.* 2007). In the presence of extracellular amino acids (right panel), the SPS (Ssy1-Ptr3-Ssy5) sensor activates the intrinsic proteolytic activity of the Ssy5 protease, resulting in the endoproteolytic processing of Stp1 and Stp2 (scissors). The shorter activated forms of Stp1 and Stp2 lacking regulatory domains are targeted to the nucleus where, together with Dal81, they bind SPS-sensor-regulated promoters (UAS_{aa}) and induce transcription (Abdel-Sater *et al.* 2004b; Boban and Ljungdahl 2007). The increased transcription of AAP genes results in increased rates of amino uptake. AAPs are cotranslationally inserted into the ER membrane, which is contiguous with the outer nuclear membrane. Movement of AAPs to the PM (represented by the dashed arrow, right panel) requires the ER membrane-localized chaperone Shr3 (Ljungdahl *et al.* 1992; Kota and Ljungdahl 2005; Kota *et al.* 2007). (B) Transporter-based model for Ssy1 amino acid receptor function (Wu *et al.* 2006). Similar to canonical transporters, Ssy1 can attain four conformational states. However, in contrast to transporters, interconversion between the outward-facing ligand bound state and the inward-facing ligand bound state (reaction 3) is prevented by a ligand-induced reaction barrier. The outward-facing conformations of the Ssy1 sensor are thought to be signaling (green), and the inward-facing conformations are nonsignaling (red). (C) Multistep regulation of the Ssy5 endoprotease. Ssy5 is expressed as an inactive zymogen (left) composed of a prodomain that assists the folding of the Cat domain. Ssy5 is auto-processed when the Cat domain attains an active conformation. The noncovalently attached prodomain remains bound to the Cat domain, forming an inactive but catalytically competent "primed" protease complex. Primed Ssy5 is incorporated as a subcomplex of the SPS sensor via protein–protein interactions involving Ptr3, where it binds, but does not cleave, its substrates Stp1 and Stp2. In the absence of extracellular amino acids, *i.e.*, under non-inducing conditions (left), the basal level of phosphorylation of a phosphodegron in the prodomain is likely to be determined by counteracting activities of casein kinase I (Yck1 and Yck2) and the phosphatase PP2A with its regulatory subunit Rts1 (Eckert-Boulet *et al.* 2006). In the presence of extracellular amino acids (right), the primary amino acid sensor Ssy1 is stabilized in a conformation that triggers intracellular signaling. This conformation increases the level of phosphorylation of the prodomain phosphodegron (Omnus *et al.* 2011), presumably by increasing the accessibility of Yck1 or Yck2. An increased level of phosphorylation within the degron provides the requisite surface recognized by the SCF^{Grr1} complex and subsequent polyubiquitylation of lysine residues of the degron. Concomitant with being ubiquitylated, the prodomain is directly targeted for degradation by the 26S proteasome, unfettering the Stp1 and Stp2 processing activity of the Cat domain.

marked substrate (ligand) preferences; nonpolar amino acids (leucine, isoleucine, methionine, phenylalanine, and tryptophan) and polar uncharged amino acids (tyrosine, threonine) are strong inducers, whereas valine, cysteine, alanine, serine, and even citrulline induce intermediate levels, and arginine, lysine, and proline are poor inducers (Iraqui *et al.* 1999; Gaber *et al.* 2003). Ssy1 monitors the ratio of external *vs.* internal amino acids across the plasma membrane by undergoing transporter-like conformational changes between an outward-facing (signaling) and an inward-facing (nonsignaling) conformation (Wu *et al.* 2006; Poulsen *et al.* 2008). Thus, in contrast to functional transporters, but in accordance with a receptor function, amino acid binding to a single substrate-binding site appears to impose a reaction barrier that inhibits the conversion from an outward- to an inward-facing conformation. Consequently, Ssy1 signaling is sensitive to both external and internal levels of amino acids, and the SPS sensor induces gene expression only when the levels of external amino acids are higher than the levels of free amino acids in cytoplasmic pools. This

Table 4 Plasma membrane-localized transporters of nitrogenous substrates relevant to amino acid metabolism

Amino acid permease family				
Systematic name	Gene name	Functional description (substrate specificity)	Regulation	Reference
Core - Cluster I[a] – SPS sensor regulated				
YCL025c	*AGP1*	Broad substrate range, medium capacity permease (Val, Ile, Phe, Met, Ser, Leu, Thr, Cys, Asn, Tyr, Ala, Gly, Gln)[b] (Pro)	SPS-sensor, NCR, GAAC[c]	Andréasson *et al.* (2004); Iraqui *et al.* (1999); Schreve *et al.* (1998)
YBR068c	*BAP2*	Branched-chain amino acid permease (Val, Ile, Phe, Tyr, Leu, Trp, Met, Cys, Ala)[b]	SPS-sensor, GAAC[c]	Grauslund *et al.* (1995)
YDR046c	*BAP3*	Branched-chain amino acid permease (Val, Ile, Phe, Tyr, Trp, Leu, Met, Cys, Thr, Ala)[b]	SPS-sensor	Didion *et al.* (1998)
YDR508c	*GNP1*	High-affinity glutamine permease (Thr, Gln, Ser, Cys, Leu, Met, Asn)[b] (Pro)	SPS-sensor	Andréasson *et al.* (2004); Zhu *et al.* (1996)
YBR069c	*TAT1*	Tyrosine and tryptophan permease (Val, Thr)[b] (low-affinity His), (Leu)	SPS-sensor	Bajmoczi *et al.* (1998); Schmidt *et al.* (1994)
YOL020w	*TAT2*	High-affinity tryptophan permease (Phe, Tyr, Trp, Gly, Ala)[b]	SPS-sensor	Schmidt *et al.* (1994)
Core - Cluster II				
YKR039w	*GAP1*	General, high-capacity, amino acid permease (all L-amino acids, D-amino acids, GABA, peptides, polyamines)	NCR, GAAC[c]	André *et al.* (1993); Jauniaux and Grenson (1990); van Zeebroeck *et al.* (2009)
YGR191w	*HIP1*	Histidine permease		Tanaka and Fink (1985)
YLL061w	*MMP1*	High-affinity *S*-methyl methionine permease		Rouillon *et al.* (1999)
YPL274w	*SAM3*	High-affinity *S*-adenosyl methionine permease; High-affinity putrescine, spermidine, spermine (polyamine)		Rouillon *et al.* (1999); Uemura *et al.* (2007)
Core - Cluster III				
YEL063c	*CAN1*	Arginine permease (Arg)	NCR, GAAC[c]	Hoffmann (1985)
YNL270c	*ALP1*	Arginine permease (Arg)[b]		Sychrova and Chevallier (1994)
YNL268w	*LYP1*	Lysine permease (Lys, Met)[b]	GAAC[c]	Sychrova and Chevallier (1993)
Core - Unclustered				
YOR348c	*PUT4*	High-affinity proline permease (Val, Ala, Pro)[b] (GABA)	NCR	André *et al.* (1993); Jauniaux *et al.* (1987)
YPL265w	*DIP5*	Dicarboxylic amino acid permease (Ser, Ala, Asn. Asp, Gln, Gly, Gln)[b]	NCR	Regenberg *et al.* (1998)
YDR160w	*SSY1*	Receptor component of the SPS sensor		Didion *et al.* (1998); Iraqui *et al.* (1999); Klasson *et al.* (1999)
YBR132c	*AGP2*	Carnitine, spermidine, putrescine (polyamine)		Aouida *et al.* (2005); van Roermund *et al.* (1999)

(continued)

Table 4, *continued*

Systematic name	Gene name	Functional description (substrate specificity)	Regulation	Reference
		Amino acid permease family		
YFL055w	*AGP3*	Broad-substrate specificity amino acid permease (Asp, Glu, Ser)[b] (Met)		Menant *et al.* (2006); Regenberg *et al.* (1999)
Non-core - MUP Cluster				
YGR055w	*MUP1*	High-affinity methionine permease (Cys)		Isnard *et al.* (1996); Kosugi *et al.* (2001)
YHL036w	*MUP3*	Low-affinity methionine permease; inhibited by broad-substrate spectrum (Met)	GAAC[c]	Isnard *et al.* (1996)
Non-core - UGA Cluster				
YDL210w	*UGA4*	GABA permease (δ-aminolevulinic acid, putrescine) vacuole localization	NCR	André *et al.* (1993); Uemura *et al.* (2004)
YKL174c	*TPO5*	Polyamine secretion, Golgi localization		Tachihara *et al.* (2005)
YGL077c	*HNM1*	Choline permease (ethanolamine)	INO regulon	Nikawa *et al.* (1986)
YNR056c	*BIO5*	Biotin permease (7-keto 8-aminopelargonic acid)		Phalip *et al.* (1999)
		Ammonium permease family		
YGR121C	*MEP1*	Medium-affinity, high-capacity ammonium permease	NCR	Marini *et al.* (1994)
YNL142W	*MEP2*	High-affinity, low-capacity ammonium permease, ammonium sensor	NCR	Lorenz and Heitman (1998); Marini *et al.* (1997)
YPR138C	*MEP3*	Low-affinity, high-capacity ammonium permease	NCR	Marini *et al.* (1997)
		Other transporters/permeases		
DAL5 Cluster				
YJR152w	*DAL5*	Allantonate permease (ureidosuccinate/dipeptide)	NCR	Cai *et al.* (2007); Rai *et al.* (1987)
YLL055w	*YCT1*	Cysteine transporter		Kaur and Bachhawat (2007)
YGR260w	*TNA1*	Nicotinic acid transporter		Llorente and Dujon (2000)
YGR065c	*VHT1*	Biotin transporter	GAAC[c]	Stolz *et al.* (1999)
YCR028c	*FEN2*	Panthothenate transporter		Stolz and Sauer (1999)
YAL067c	*SEO1*	Not determined – sulfur compound?		Isnard *et al.* (1996)
FUR4 Cluster				
YBR021w	*FUR4*	Uracil permease		Jund *et al.* (1988)
YIR028w	*DAL4*	Allantoin permease (uracil)	NCR	Yoo *et al.* (1992)
YBL042c	*FUI1*	Uridine permease		Wagner *et al.* (1998)
YLR237w	*THI7*	Thiamine permease	Thi3	Enjo *et al.* (1997)
YOR192c	*THI72*	Thiamine permease		Enjo *et al.* (1997)
YOR071c	*NRT1*	Nicotinamide riboside (high-affinity), thiamine (low-affinity)		Belenky *et al.* (2008)
FCY2 Cluster				
YER056c	*FCY2*	Nucleobase permease (cytidine, cytosine, purine, adenine, guanine, hypoxanthine)		Weber *et al.* (1990)

(continued)

Table 4, *continued*

Other transporters/permeases				
Systematic name	Gene name	Functional description (substrate specificity)	Regulation	Reference
YGL186c	*TPN1*	Pyridoxine transporter (vitamin B6)	GAAC[c]	Stolz and Vielreicher 2003)
Unclustered				
YHL016c	*DUR3*	High-affinity polyamine (urea)	NCR	El Berry *et al.* (1993); Uemura *et al.* (2007)
YKR093w	*PTR2*	Peptide transporter (di-/tripeptides)	SPS-sensor, Cup9, NCR	Hauser *et al.* (2001); Island *et al.* (1991)
YJL212c	*OPT1*	Oligopeptide transporter (glutathione, phytochelatin)	NCR	Hauser *et al.* (2001)
YPR194c	*OPT2*	Homologous to *OPT1* (role as transporter uncertain; inactivation leads to fragmented vacuoles)	NCR	Aouida *et al.* (2009)
Transporters involved with excretion of nitrogenous compounds				
YDR384c	*ACO3*	Ammonium transport outward	SPS-sensor, GAAC	Guaragnella and Butow (2003); Palkova *et al.* (2002)
YNL065w	*AQR1*	H^+-antiporter, localized to multiple intracellular membranes/vesicles (secretion of homoserine, Thr, Ala, Asp, Glu)		Velasco *et al.* (2004)
YLL028w	*TPO1*	Polyamine transporter		Tomitori *et al.* (1999); Uemura *et al.* (2005)

[a] Clustered based onon the basis of sequence homology according to Nelissen *et al.* (1997).
[b] Substrate specificity as reported in Regenberg *et al.* (1999).
[c] GAAC-regulated expression according to Natarajan *et al.* (2001).

model elegantly accounts for the accumulated experimental data and also provides a framework for understanding the transceptor concept as applied to active nutrient carriers that combine nutrient transporter and receptor functions, including Gap1 and Pho84 (Thevelein and Voordeckers 2009; van Zeebroeck *et al.* 2009; Popova *et al.* 2010; Rubio-Texeira *et al.* 2010).

The extended N-terminal domain of Ssy1 functions as a scaffold for binding of Ptr3 and Ssy5 (Bernard and André 2001; Forsberg and Ljungdahl 2001b; Liu *et al.* 2008). Ssy5 exhibits homology to chymotrypsin-like serine proteases and is expressed as a zymogen (Abdel-Sater *et al.* 2004a; Andréasson *et al.* 2006; Poulsen *et al.* 2006). After translation, and concomitantly with folding to achieve catalytic competence, Ssy5 cleaves itself into an N-terminal prodomain and a C-terminal catalytic (Cat) domain. The prodomain remains noncovalently attached with the Cat domain, forming a primed, but inactive, protease subcomplex within the SPS sensor that associates with Stp1 and Stp2 (Andréasson *et al.* 2006). The prodomain functions as the inhibitory subunit of the SPS sensor, and its amino acid-induced degradation via the 26S proteasome correlates with the signal-propagating, endoproteolytic processing of Stp1 and Stp2 (Pfirrmann *et al.* 2010; Omnus *et al.* 2011).

Consistent with 26S proteasome involvement, amino acid-induced Stp1 and Stp2 processing requires the SCF^{Grr1} ubiquitin ligase complex (Abdel-Sater *et al.* 2004a; Andréasson and Ljungdahl 2004; Spielewoy *et al.* 2004; Liu *et al.* 2008). SCF complexes achieve specificity through association with exchangeable F-box proteins (Jonkers and Rep 2009). Grr1 is one of the best-characterized F-box proteins and is required for Ssy5 prodomain downregulation and Cat domain activation (Andréasson *et al.* 2006). Also, casein kinase I-dependent phosphorylation (either Yck1 or Yck2) is required for SPS-sensor signaling (Abdel-Sater *et al.* 2004a; Spielewoy *et al.* 2004; Liu *et al.* 2008). It has been shown that Yck1/2-dependent Ssy5 prodomain phosphorylation precedes SCF^{Grr1}-dependent ubiquitylation (Abdel-Sater *et al.* 2011). In other signaling pathways, Yck1/2-catalyzed phosphorylation has been shown to lead to Grr1-dependent polyubiquitylation and subsequent degradation of the modified substrates by the 26S proteasome (Moriya and Johnston 2004; Spielewoy *et al.* 2004). Hence, the SPS sensor coordinates the activity of general signaling components to achieve a highly specific output (Omnus *et al.* 2011) (Figure 11C).

The constitutive nuclear factor Dal81 has an important and synergistic role in amplifying the induced expression of SPS-sensor genes (Boban and Ljungdahl 2007). Dal81 is also required for several other well-characterized nitrogen source utilization pathways, including urea, allantoin, and GABA. Dal81 functions together with an inducer-specific transcription factor, *i.e.*, Uga3 (Jacobs *et al.* 1981; Turoscy

and Cooper 1982; Coornaert *et al.* 1991), Dal82 (Vissers *et al.* 1990), or Stp1 and Stp2 (Iraqui *et al.* 1999; Abdel-Sater *et al.* 2004b; Boban and Ljungdahl 2007) to activate target genes. The fact that these pathways share a common coactivator enables cells to integrate the signals derived from these different nitrogen sources due to competitive binding of the specific factors to Dal81 (Abdel-Sater *et al.* 2004b). A distinct hierarchy exists, and the induced targeting of activated forms of Stp1 and Stp2 overrides allantoate- and GABA-induced signals, enabling cells to take advantage of the availability of amino acids as preferred nitrogen sources. Integration appears to be achieved by the preferential recruitment of Dal81 to SPS-sensor-regulated promoters. Accordingly, in an SPS-sensor-dependent manner, the presence of extracellular amino acids impair Dal82-dependent expression of *DAL5*, encoding allantoate/ureidosuccinate permease (Cai *et al.* 2007), and Uga3-dependent gene expression of *UGA4*, the GABA permease (Cardillo *et al.* 2010).

Overlapping transcriptional regulatory networks contribute to the expression of several SPS-sensor-controlled genes. The promoter region of *AGP1* encoding a broad-specificity amino acid permease is under NCR control; however, its expression is strictly dependent on inducing signals mediated by the SPS sensor (Iraqui *et al.* 1999; Abdel-Sater *et al.* 2004b; Wielemans *et al.* 2010). Stp1 binding to UAS_{aa} motifs in the promoters of SPS-sensor-regulated genes is insensitive to the nitrogen status of the cell and to factors controlling NCR, *i.e.*, Gln3, Ure2, and Gzf3 (Godard *et al.* 2007). In cells grown with the preferred nitrogen source ammonium, the addition of amino acids leads to the rapid induction of *AGP1* expression. In cells lacking a functional SPS-sensing pathway, *AGP1* expression is not induced, and the P_{AGP1} promoter is unresponsive to mutations that inactivate Ure2. Conversely, amino acid-induced expression of *AGP1* is greatly augmented in cells grown in the presence of nonpreferred nitrogen sources (Abdel-Sater *et al.* 2004b; Godard *et al.* 2007). Thus, in contrast to other permease genes responsive to NCR control (*e.g.*, *GAP1*, *DAL5*, and *MEP2*), Gln3 is not able to activate *AGP1* expression in the absence of synergistic signals initiated by the presence of extracellular amino acids that are transduced via the SPS-sensing pathway.

The analysis of dipeptide uptake in yeast also provides a clear demonstration of how SPS-sensor-induced signals are integrated with other nutritionally regulated activities. Amino acid-induced and SPS-sensor-mediated activation of dipeptide transporter *PTR2* expression is a requisite for dipeptide uptake (Island *et al.* 1987; Barnes *et al.* 1998; Hauser *et al.* 2001); however, full induction of peptide uptake requires an additional activation step (Byrd *et al.* 1998; Turner *et al.* 2000; Hwang and Varshavsky 2008; Xia *et al.* 2008). Peptides entering the cell that contain N-terminal amino acids recognized according to the N-end rule as destabilizing (Dohmen *et al.* 1994; Varshavsky 2008), allosterically activate Ubr1, an E3 ubiquitin ligase, which in turn accelerates degradation of the transcriptional repressor Cup9. A decreased level of Cup9 gives rise to fully derepressed *PTR2* expression and dipeptide transport. Although Ubr1 and Cup9 are absolutely required for full induction of *PTR2* transcription, they do not affect P_{AGP1}-*lacZ* (Bernard and André 2001) or *BAP2* (Alagramam *et al.* 1995) expression.

Membrane transporter systems and compartmentalization

The compartmentalized eukaryotic cell architecture is a complicating factor in evaluating the flow of metabolites associated with amino acid and nucleotide biosynthesis. To fully understand metabolic regulation, the dynamic distribution of compartmentalized metabolites will eventually have to be taken into account. Although this was already pointed out by Cooper (1982b) and Jones and Fink (1982), it remains a substantial challenge. Great progress has been made in understanding how metabolites are transported into cells and into or out of organelles. Here full-genome sequence data have contributed greatly to the identification of families of transport proteins (Saier 2000; Brohee *et al.* 2010). Subsequent cell biological analyses have established the intracellular localization of these transporters, and in many cases purification and reconstitution experiments have provided precise mechanistic understanding of their function.

Membrane transport systems of nitrogenous compounds relevant to amino acid metabolism are schematically depicted in Figure 12. Transporters and permeases that function at the plasma membrane and primarily facilitate metabolite uptake into cells are listed in Table 4. Transport across the plasma membrane is facilitated via H^+-symport energized by the plasma membrane H^+-ATPase Pma1 (Serrano *et al.* 1986; Horák 1997). A fundamental theme that has emerged from the functional analysis of plasma membrane transport is that, in many instances, individual substrates are transported by several different systems, which, although displaying different kinetic specificities, function redundantly (Regenberg *et al.* 1999). The expression of redundant systems with differing catalytic properties allows yeast cells to extract necessary nutrients from a great variety of environments.

Transport across the inner mitochondrial membrane is catalyzed by the mitochondrial carrier protein (MCP) family; a selected subset of MCPs that are intimately linked to amino acid metabolism is listed in Table 5 (Palmieri *et al.* 2006). The MCPs exhibit a variety of transport mechanisms, including uniport, symport, and antiport, and the transport can occur in an electroneutral, proton-mediated, or electrophoretic manner. In the case of bidirectional antiport exchange, the direction of transport is determined by the relative concentrations of substrates in the cytoplasmic and mitochondrial substrate.

The vacuole is an organelle with a well-established role in amino acid homeostasis (Matile and Wiemken 1967; Cooper 1982a; Kitamoto *et al.* 1988; Klionsky *et al.* 1990; Jacquemin-Faure *et al.* 1994; Sekito *et al.* 2008). The vacuolar transport systems are listed in Table 6 (Sekito *et al.* 2008). Metabolite

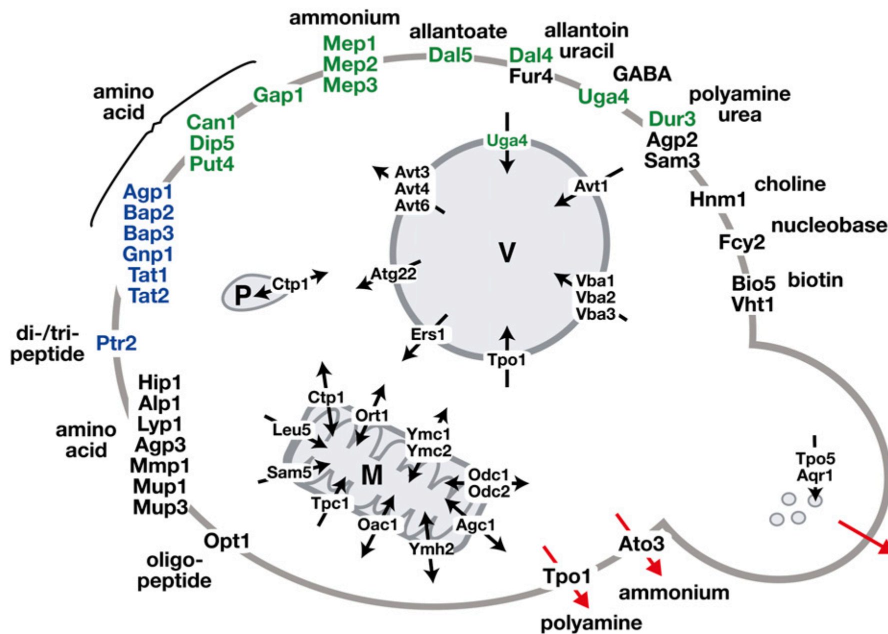

Figure 12 Membrane transport systems of nitrogenous compounds relevant to amino acid metabolism. Plasma membrane-localized permeases/transporters are shown with their corresponding substrates. The expression of transport proteins in green text is under nitrogen regulation (NCR). The expression of transport proteins in blue text is transcriptionally controlled by the SPS sensor of extracellular amino acids. Transporters thought to be involved in the excretion of amino acids, either functioning in the late secretory pathway or at the plasma membrane, are shown with red outwardly pointing arrows. Transporters localized to intracellular organelle membranes, *i.e.*, mitochondria (M), peroxisome (P), and vacuole (V), are depicted; the arrows indicate the direction of the transport catalyzed.

transport across the vacuolar membrane is energized by the oligomeric vacuolar V_0V_1 H^+-ATPase (Uchida *et al.* 1985; Anraku *et al.* 1992), and movement of substrates across the membrane occurs by either H^+-antiport (In) or H^+-symport (Out). Importantly, the vacuole is a major storage compartment for amino acids, and cells have discrete pools of amino acids; *i.e.*, the basic amino acids (His, Arg, and Lys) are sequestered in the vacuole, whereas the acid amino acids (Asp and Glu) are selectively excluded.

Interestingly, efforts to understand TORC1 signaling appear to be narrowing in on intracellular trafficking of membrane proteins, including the general amino acid permease (*GAP1*). TORC1 localizes to the vacuolar/endosomal membrane (Wedaman *et al.* 2003), which in essence is a major crossroad for protein sorting (Nickerson *et al.* 2009). The membrane-anchored EGO–GSE complex, found associated with the late endosome and vacuolar membranes, is required for TORC1 localization and activation (Dubouloz *et al.* 2005; Binda *et al.* 2009) and for proper Gap1 trafficking from the endosome to the plasma membrane during amino acid limitation (Gao and Kaiser 2006). Consistent with a link to vacuole function, null alleles of genes encoding class C-VPS components, *e.g.*, *PEP3*, exhibit synthetic lethality with a *tor1*Δ null allele (Zurita-Martinez *et al.* 2007). The presence of glutamate or glutamine suppresses the synthetic lethality of a *pep3 tor1* mutant, indicating that nitrogen metabolism and vacuolar function are intimately intertwined. Class C-VPS mutants do not have identifiable vacuolar structures (Bowers and Stevens 2005), have low levels of intracellular pools of amino acids, and are unable to survive nitrogen starvation (Zurita-Martinez *et al.* 2007). Proper TOR signaling apparently requires a vacuole/endosomal membrane platform The central role of the vacuole in nitrogen metabolism may explain the nitrogen-related phenotypes associated with rapamycin treatment.

Nucleotides

Nucleotides are critical players in a multitude of very different cellular processes. Purines and pyrimidines are the basic components of nucleic acids, and ATP is the central cellular energy supply. In addition, GTP and modified nucleotides such as cyclic AMP are signaling molecules. Finally, nucleotides are incorporated in cofactors (*e.g.*, NAD and coenzyme A) and serve as precursors (*e.g.*, UDP-glucose and GDP-mannose). Purine and pyrimidine synthesis occurs through distinct metabolic pathways highly conserved among both prokaryotic and eukaryotic species. These pathways (Figure 13 and Figure 15; see also Table 7 and Table 8) combine *de novo* synthesis from amino acids and sugar with nucleotide recycling from precursors available in the growth medium or provided via degradation of macromolecules. The purine and pyrimidine pathways ensure net synthesis of nucleotides but also allow interconversion of nucleobases, nucleosides, and nucleotides, thus permitting a proper balance of the final products to be achieved. Intracellular concentrations of purine and pyrimidine nucleotides are presented in Table 9.

Regulation of pyrimidine metabolism

The *de novo* pyrimidine pathway is regulated at the enzymatic level through feedback inhibition of the first enzyme, Ura2, by the final product UTP (reviewed in Jones and Fink 1982). Moreover, several genes of the pathway are also upregulated at the transcriptional level in response to pyrimidine starvation (Jones and Fink 1982). The elegant genetic work of Lacroute and coworkers in the early eighties allowed identification of Ppr1 as a zinc-finger transcription factor required for expression of the *URA1* and *URA3* genes (Loison *et al.* 1980; Losson and Lacroute 1981; Kammerer *et al.* 1984). Ppr1 is also likely to positively regulate *URA4*

Table 5 Mitochondrial carrier family: transporters directly coupled to amino acid metabolism

Systematic name	Gene name	Description (substrate specificity)	Transport in/out organelle	Reference
YPR021c	*AGC1*[a]	Aspartate-glutamate transport (Asp, Glu)	aspartate → out, in ← glutamate	Cavero *et al.* (2003)
YPL134c	*ODC1*	Lysine and glutamate biosynthesis/lysine catabolism/nitrogen assimilation (α-ketoglutarate, α-ketoadipate)	α-ketoadipate → out, in ← α-ketoglutarate	Palmieri *et al.* (2001)
YOR222w	*ODC2*[a]	Lysine and glutamate biosynthesis/lysine catabolism/nitrogen assimilation (α-ketoglutarate, α-ketoadipate)	α-ketoadipate → out, in ← α-ketoglutarate	Palmieri *et al.* (2001)
YOR130c	*ORT1*[a] (*ARG11*)	Arginine biosynthesis (ornithine)	ornithine → out, in ← H^+	Crabeel *et al.* (1996); Palmieri *et al.* (1997); Soetens *et al.* (1998)
YBR291c	*CTP1*	Lysine biosynthesis - Lys14 regulated; suggested role in peroxisome membrane (citrate, malate)	citrate → out, in ← malate	Breitling *et al.* (2002); Kaplan *et al.* (1995)
YKL120w	*OAC1*[a]	Leucine biosynthesis (oxalacetate, α-isopropyl malate)	α-isopropylmalate → out, in ← oxalacetate	Marobbio *et al.* (2008)
YBL089w	*YMH2*	Arginine biosynthesis (citrate, α-ketoglutarate)	citrate → out, in → α-ketoglutarate	Castegna *et al.* (2010)
YHR002w	*LEU5*	Leucine biosynthesis - CoA transport (cofactor A)	in	Prohl *et al.* (2001)
YPR058w	*YMC1*[a]	Lysine and glutamate biosynthesis (α-ketoglutarate)	n.d.	Trotter *et al.* (2005)
YBR104w	*YMC2*[a]	Lysine and glutamate biosynthesis (α-ketoglutarate)	n.d.	Trotter *et al.* (2005)
YNL003c	*SAM5* (*PET8*)	Methionine biosynthesis (*S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAHC)) ThPP = Thiamine pyrophosphate TMP = Thiamine monophosphate	In ← SAM, SAHC → out	Marobbio *et al.* (2003)

[a] GAAC-regulated expression (Natarajan *et al.* 2001)

and *URA10* on the basis of data from *in vitro* binding to promoter sequences (Roy *et al.* 1990; Roy 1992). However, the role of Ppr1 in transcription has not been evaluated genome-wide, which could reveal interesting cross-pathway regulation.

The early observation that yeast mutants accumulating orotic acid (OA) and dihydro-orotate (DHO) show increased DHO dehydrogenase (Ura1) and orotidine 5′-monophosphate decarboxylase (Ura3) activity (Lacroute 1968) suggested that these metabolic intermediates could play a direct role in the pyrimidine pathway regulation. In this model, a specific pathway intermediate functions as a cofactor for the transcriptional activator, and the abundance of the metabolite is downregulated by feedback inhibition (Figure 8). This assumption was tested directly using purified Ppr1 *in vitro* (Flynn and Reece 1999), where addition of either DHO or OA to the reaction stimulated Ppr1-dependent transcription (Flynn and Reece 1999). This reconstitution experiment established that Ppr1 directly senses the levels of pyrimidine biosynthesis intermediates and modulates pyrimidine enzyme synthesis in response to the flux in the pathway. Interestingly, it was also found that efficient binding of Ppr1 to DNA required a yet-unidentified small molecule (Flynn and Reece 1999). Identification of this molecule in the future could uncover new aspects of pyrimidine biosynthesis regulation.

Transcription of *URA2*, encoding the first committed step of UTP synthesis, is also upregulated in response to pyrimidine depletion (Exinger and Lacroute 1992; Kwapisz *et al.* 2008). However, expression of *URA2* is not Ppr1 dependent (Losson and Lacroute 1981; Kwapisz *et al.* 2008), but is regulated by a novel mechanism recently described. This mechanism involves alternative transcription start sites that lead to the synthesis of small "cryptic unstable transcripts" (CUTs) (Thiebaut *et al.* 2008) but also to a downstream T-rich region (Kwapisz *et al.* 2008). "Nonproductive" transcription from the upstream sites downregulates expression of the *URA2* mRNA while uracil deprivation activates selection of the functional *URA2* mRNA start site by a mechanism that does not involve lower expression of the CUTs (Thiebaut *et al.* 2008) (Figure 14). Interestingly, mechanisms involving noncoding RNAs appear to regulate other nucleotide genes such as *URA8*, *IMD2*, and *ADE12* (Davis and Ares 2006; Steinmetz *et al.* 2006; Kuehner and Brow 2008; Kwapisz *et al.* 2008; Thiebaut *et al.* 2008). Strikingly, these four genes encode the first committed steps of UTP, CTP, GTP, and ATP synthesis (see Figures 13 and 15). Although not fully understood at the molecular level, these regulatory processes apparently involve direct sensing of nucleotide concentration by the transcription machinery and thus efficiently connect individual nucleotide synthesis to its actual availability.

Table 6 Vacuole-localized amino acid transport proteins

Systematic name	Gene name	Substrate specificity	Transport in/out of organelle	Reference
AVT subfamily				
YJR001w	*AVT1*[a]	Ile, Leu, Asn, Gln, Tyr	In	Russnak *et al.* (2001)
YEL064c	*AVT2*	Not known, may localize to ER	—	Russnak *et al.* (2001)
YKL146w	*AVT3*[b]	Ile, Leu, Asn, Gln, Tyr	Out	Russnak *et al.* (2001); Yang *et al.* (2006)
YNL101w	*AVT4*[a,b]	Ile, Leu, Asn, Gln, Tyr	Out	Russnak *et al.* (2001); Yang *et al.* (2006)
YBL089w	*AVT5*	Not known	—	Russnak *et al.* (2001)
YER119c	*AVT6*[b]	Asp, Glu	Out	Chahomchuen *et al.* (2009); Russnak *et al.* (2001)
YIL088c	*AVT7*[a]	Not known	—	Russnak *et al.* (2001)
VBA subfamily				
YMR088c	*VBA1*[a]	His, Lys	In	Shimazu *et al.* (2005)
YBR293w	*VBA2*	Arg, His, Lys, Tyr	In	Shimazu *et al.* (2005)
YCL069w	*VBA3*	His, Lys	In	Shimazu *et al.* (2005)
YDR119w	*VBA4*	Not known	—	Shimazu *et al.* (2005)
YKR105c	*VBA5*	Not known	—	Shimazu *et al.* (2005)
Other transport proteins				
YCL038c	*ATG22*	Ile, Leu, Tyr	Out	Sychrova and Chevallier (1994); Yang *et al.* (2006)
YEL063c	*UGA4*	GABA, putrescine	In	Uemura *et al.* (2004)
YNL268w	*ERS1*	Cys	Out	Gao *et al.* (2005)

[a] NCR-sensitive expression.
[b] Required for viability upon nitrogen starvation.

Regulation of the purine de novo synthesis pathway

The transcription factors responsible for activation of purine *de novo* pathway genes were identified while studying the histidine pathway regulation. The pioneering work by Fink and coworkers on the transcriptional regulation of the *HIS4* gene revealed the important role of the Myb-related Bas1 and homeodomain Bas2 transcription factors (Arndt *et al.* 1987; Tice-Baldwin *et al.* 1989). Knockout of these genes was found to result in adenine bradytrophy, suggesting that they could also regulate purine biosynthesis genes. This hypothesis was correct, and Bas1 and Bas2 were found to be required for expression of several *ADE* genes *in vivo* and to bind to their promoter *in vitro* (Daignan-Fornier and Fink 1992). On the basis of proteome, transcriptome, and gene-by-gene analysis, it was found that Bas1 and Bas2 activate the expression of all 10 AMP-biosynthesis genes except *ADE16* (Daignan-Fornier and Fink 1992; Denis *et al.* 1998) and bind to a specific promoter region of these genes (Daignan-Fornier and Fink 1992; Rolfes *et al.* 1997; Pinson *et al.* 1998). Furthermore, Bas1/Bas2 also mediate adenine-repressible transcriptional activation of genes of other pathways metabolically connected to the purine pathway, such as histidine (*HIS1*, *HIS4*, and *HIS7*), glutamine (*GLN1*), or one-carbon-unit (*SHM2*, *MTD1*) metabolism genes (Figure 15) (Arndt *et al.* 1987; Springer *et al.* 1996; Denis and Daignan-Fornier 1998). Bas1 binding to the promoter region of most of these genes has been confirmed *in vivo* by ChIP-CHIP analysis (Mieczkowski *et al.* 2006). Importantly, Bas2 is also known as Pho2, a major regulator of phosphate utilization in yeast (see *Purine phosphate connection: more signal molecules*) (Arndt *et al.* 1987). In general, both Bas1 and Pho2 are required for transcriptional activation although one-carbon-unit metabolism genes are much more dependent on Bas1 than on Bas2/Pho2 (Denis and Daignan-Fornier 1998; Subramanian *et al.* 2005).

All the genes activated by Bas1 and Bas2/Pho2 also respond to extracellular adenine, their expression being low when adenine is abundant in the growth medium (Tice-Baldwin *et al.* 1989; Daignan-Fornier and Fink 1992; Springer *et al.* 1996; Denis *et al.* 1998; Denis and Daignan-Fornier 1998). To get an insight into the molecular mechanisms linking adenine availability to transcriptional activation, Rolfes *et al.* (1997) used chimeras between LexA and either Bas1 or Pho2. They found that lexA-Pho2 could activate transcription independently of Bas1 and in an adenine-independent way, while lexA-Bas1 activation was both Pho2 dependent and adenine responsive (Zhang *et al.* 1997). They proposed that adenine limitation favors formation of a complex between Bas1 and Pho2, thus unmasking the Bas1 activation domain (Zhang *et al.* 1997). Consistently, a Bas1–Pho2 fusion chimera activated expression of the *ADE* genes in an adenine-independent way (Pinson *et al.* 2000). Further analyses using Bas1 and Pho2 fused to the transcription activation domain of VP16 suggested that Bas1 binds to the *ADE* gene promoters and recruits Pho2 (Pinson *et al.* 2000). This was further demonstrated by chromatin immunoprecipitation (ChIP) analysis (Som *et al.* 2005).

How does adenine limitation stimulate Bas1–Pho2 interaction? A genetic analysis of mutants unresponsive to adenine has shown that adenine needs to be taken up and metabolized to ADP to exert its regulatory role (Guetsova *et al.* 1997; Rébora *et al.* 2001). This is consistent with the observation that extracellular hypoxanthine downregulates expression of an *ADE1-lacZ* fusion while guanine has little

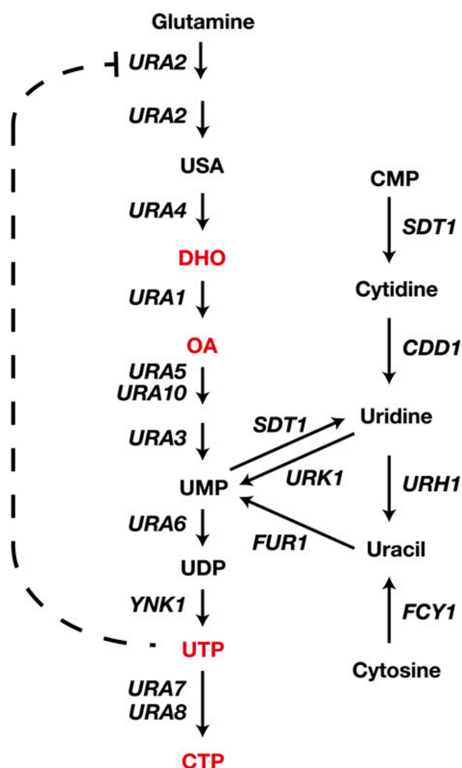

Figure 13 Pyrimidine synthesis and salvage pathways. DHO, dihydroorotate; OA, orotic acid; USA, ureidosuccinic acid. Gene names are italicized. Regulatory molecules are shown in red.

effect (Guetsova *et al.* 1997) because hypoxanthine can be converted to adenylic nucleotides while guanine cannot (Figure 15). Strikingly, most of the deregulated mutants affected the *ADE13* gene encoding adenylosuccinate lyase, suggesting a key role for this step of the pathway (Figure 15) (Rébora *et al.* 2001). Further genetic analyses revealed that AICAR (5′-phosphoribosyl-5-amino-4-imidazole carboxamide) and SAICAR (succinyl-AICAR), the metabolic intermediates just downstream and upstream of Ade13, respectively, play a pivotal role by promoting interactions between Bas1 and Pho2 (Rébora *et al.* 2001, 2005). Several lines of evidence supported this idea. First, a direct correlation was found between intracellular AICAR concentrations, measured by HPLC, and *ADE* gene expression (Pinson *et al.* 2009). Second, a Bas1–Pho2 fusion makes expression of the *ADE* genes (S)AICAR independent (Rébora *et al.* 2001; Pinson *et al.* 2009). Finally, two-hybrid studies revealed that the Bas1–Pho2 interaction is stimulated under conditions where (S)AICAR accumulates (Rébora *et al.* 2001; Pinson *et al.* 2009), and consistently, these conditions stimulate Bas1-dependent recruitment of Pho2 to the *ADE5*, *7* and *ADE17* promoters, as determined by ChIP (Pinson *et al.* 2009). Affinity chromatography revealed that AICAR binds Pho2 but not Bas1 *in vitro* strongly, suggesting that Pho2 could be an AICAR sensor through direct interaction with the small molecule (Pinson *et al.* 2009). Importantly AICAR, but not SAICAR, also affects interaction of Pho2 with another transcription factor, Pho4, and thus modulates expression of phosphate utilization genes (see *Purine phosphate connection: more signal molecules* and Figure 8) (Pinson *et al.* 2009). Together, these results suggest that somehow AICAR binding to Pho2 potentiates this transcription factor by stimulating the interaction of Pho2 with its partners.

The link between exogenous adenine and (S)AICAR synthesis is thought to occur through enzymatic regulation of the first step of the pathway, catalyzed by Ade4, which is downregulated by ADP and ATP *in vitro* (Rébora *et al.* 2001). Indeed, the intracellular concentration of ADP and ATP is clearly higher in adenine-replete conditions (Gauthier *et al.* 2008), while the (S)AICAR concentration decreases (Hurlimann *et al.* 2011). Thus, both ATP, the final product of the pathway, and a metabolic intermediate, (S)AICAR, are required for proper transcriptional regulation of adenylic nucleotides. Although the mechanisms are different, the general scheme is very close to that of the pyrimidine pathway, where UTP feedback inhibits Ura2, the first enzyme of the pathway, thus modulating the synthesis of orotate and dihydroorotate, two metabolic intermediates that directly stimulate the transcription factor Ppr1 (see *Regulation of pyrimidine metabolism*). Dual-sensing mechanisms are well suited for regulation of nonlinear pathways, such as the purine *de novo* pathway, which can be fed on the side by the histidine pathway and which branches to allow ATP and GTP synthesis (Figure 15).

Regulation of GTP synthesis

Expression of genes required for GTP synthesis is not coregulated with that of genes for AMP synthesis. The *IMD* genes and *GUA1* do not respond to adenine as *ADE* genes do (Escobar-Henriques and Daignan-Fornier 2001). In fact, *IMD2/3/4* genes are strongly downregulated when guanine is added to the growth medium, while *GUA1* expression is unaffected (Escobar-Henriques and Daignan-Fornier 2001). This effect of guanine is abolished in the *fcy2*, *hpt1*, and *guk1* guanine utilization mutants, indicating that guanine metabolism is required for GTP to exert its regulatory role (Escobar-Henriques and Daignan-Fornier 2001). In contrast, inhibitors of IMPDH activity, such as mycophenolic acid (MPA) or 6-azauracil (6AU) that lower GTP pools (Exinger and Lacroute 1992), strongly induce *IMDs* genes (Shaw and Reines 2000; Escobar-Henriques and Daignan-Fornier 2001; Saint-Marc *et al.* 2009).

An analysis of the *IMD2* promoter, aimed at deciphering the molecular mechanisms leading to transcriptional regulation, revealed several unusual features. First, a regulatory element lying >200 bp upstream of the transcription start, carries a typical TATA box motif and is required for binding of the TATA-box-binding protein on the *IMD2* promoter (Escobar-Henriques and Daignan-Fornier 2001; Escobar-Henriques *et al.* 2003a). Deletion of this TATA box motif resulted in constitutive expression of *IMD2* mRNA at a level that was intermediary between fully repressed and fully induced levels (Shaw *et al.* 2001; Escobar-Henriques *et al.*

Table 7 Pyrimidine metabolism genes

Gene	ORF	Activity in UTP synthesis	References
URA2	*YJL130C*	Bifunctional: carbamoylphosphate synthetase - aspartate transcarbamylase	Denis-Duphil (1989); Nagy *et al.* (1989); Souciet *et al.* (1987)
URA4	*YLR420W*	Dihydroorotase	Guyonvarch *et al.* (1988)
URA1	*YKL216W*	Dihydroorotate dehydrogenase	Roy (1992)
URA5	*YML106W*	Major orotate phosphoribosyltransferase isozyme	de Montigny *et al.* (1989)
URA10	*YMR271C*	Minor orotate phosphoribosyltransferase isozyme	de Montigny *et al.* (1990)
URA3	*YEL021W*	Orotidine-5′-phosphate decarboxylase	Storms *et al.* (1979)
URA6	*YKL024C*	Uridylate kinase	Liljelund and Lacroute (1986)
YNK1	*YKL067W*	Nucleoside diphosphate kinase	Fukuchi *et al.* (1993)
		Activity in CTP synthesis	
URA7	*YBL039C*	Major CTP synthase isozyme	Ozier-Kalogeropoulos *et al.* (1991)
URA8	*YJR103W*	Minor CTP synthase isozyme	Ozier-Kalogeropoulos *et al.* (1994)
		Activity in salvage	
FCY1	*YPR062W*	Cytosine deaminase	Erbs *et al.* (1997)
FUR1	*YHR128W*	Uracil phosphoribosyltransferase	de Montigny *et al.* (1990)
SDT1	*YGL224C*	Pyrimidine nucleotidase	Nakanishi and Sekimizu (2002)
URK1	*YNR012W*	Uridine kinase	Kern (1990)
URH1	*YDR400W*	Uridine-cytidine *N*-ribohydrolase	Kurtz *et al.* (2002)
CDD1	*YLR245C*	Cytidine deaminase	Kurtz *et al.* (1999)

2003b; Kuehner and Brow 2008). A repressive element was also identified between the TATA box and the transcriptional start site (Shaw *et al.* 2001) and was shown to have transcription terminator properties (Jenks *et al.* 2008).

Recently, two groups studying RNA stability and transcription termination at the genome-wide scale observed unusual features at the *IMD2* locus. These studies revealed multiple unstable transcripts (CUTs) produced from the *IMD2* promoter region (Figure 14) (Davis and Ares 2006; Steinmetz *et al.* 2006). Importantly, abundance of the CUTs appears inversely correlated to that of *IMD2* mRNA (Davis and Ares 2006; Steinmetz *et al.* 2006). Since the CUTs always start with a G while *IMD2* mRNA starts with an A, Brow and coworkers proposed that somehow the transcription machinery senses GTP concentration and responds to it via differential start-site utilization (Steinmetz *et al.* 2006). This model was globally supported by an analysis of various mutations in the *IMD2* promoter (Kuehner and Brow 2008). The precise mechanism is not yet understood, and it could involve transcription elongation factors, since the corresponding mutants poorly induce *IMD2* expression in the presence of MPA or 6AU and are hypersensitive to these drugs (Shaw and Reines 2000; Desmoucelles *et al.* 2002; Riles *et al.* 2004).

Nucleotide balance

Proper nucleotide balance appears to result from individual nucleotide sensing and adjustment. However, since CTP is synthesized from UTP, and since both GTP and ATP are made from IMP, the synthesis of individual nucleotides does not occur independently. Yet, synthesis of each nucleotide appears to be finely regulated by nucleotide-dependent transcriptional regulations involving noncoding RNAs and specific responses to limitation in each given nucleotide. Enzymatic mechanisms are also involved in ensuring the nucleotide balance; for example, GMP synthesis requires ATP at the Gua1-catalyzed step, while AMP synthesis requires GTP for Ade12-dependent activity (Figure 15). What happens if the nucleotide balance is disturbed? To tackle this question, specific mutations disrupting the purine nucleotide balance that result in lower ATP or GTP concentration (Gauthier *et al.* 2008; Saint-Marc *et al.* 2009; Iglesias-Gato *et al.* 2011) or that strongly increase the GTP pool (Breton *et al.* 2008) were constructed. While these mutations had a strong impact on yeast growth and resulted in general transcriptional and translational responses, no evidence for a common specific cellular response to defective nucleotide balance emerged. It thus seems that yeast cells do not have an integrated response to nucleotide imbalance, most probably because regulatory mechanisms ensuring proper nucleotide balance are highly robust.

Regulation in response to growth phase

As building blocks, nucleotides are required mainly during growth and division. Their synthesis should therefore be much lower in quiescent cells, in which efficient recycling should fulfill most requirements. Indeed, most purine biosynthesis genes are downregulated at the transcriptional level upon entry into stationary phase. Interestingly, in *Bacillus subtilis*, diminution of intracellular GTP concentration is required for proper entry into stationary phase (Ratnayake-Lecamwasam *et al.* 2001). In yeast, expression of IMPDH-coding genes (*IMD*s) is strongly affected by growth phase (Shaw *et al.* 2001; Escobar-Henriques *et al.* 2003a). A mutagenesis of the *IMD2* gene revealed that the sequence involved in growth phase regulation lies in the coding region of the gene (Escobar-Henriques *et al.* 2003a). Somehow this sequence affects recruitment of the TATA-box-binding protein to the upstream *IMD2* promoter

Table 8 Purine metabolism genes

Gene	ORF	Activity in IMP synthesis	References
ADE4	*YMR300C*	Phosphoribosyl-pyrophosphate amidotransferase	Mantsala and Zalkin (1984)
ADE5,-7	*YGL234W*	glycinamide Glycinamide ribotide synthetase	Henikoff (1986)
ADE8	*YDR408C*	Phosphoribosyl-glycinamide transformylase	White *et al.* (1988)
ADE6	*YGR061C*	Formylglycinamidine-ribonucleotide -synthetase	Giani *et al.* (1991)
ADE5,-7	*YGL234W*	Amino-imidazole ribotide synthetase	Henikoff (1986)
ADE2	*YOR128C*	Phosphoribosyl-amino-imidazole carboxylase	Stotz and Linder (1990)
ADE1	*YAR015W*	*N*-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase	Crowley and Kaback (1984)
ADE13	*YLR359W*	Adenylosuccinate lyase	Guetsova *et al.* (1997)
ADE16	*YLR028C*	Isoform of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase	Tibbetts and Appling (1997)
ADE17	*YMR120C*	Isoform of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase	Tibbetts and Appling (1997)
		Activity in ADP synthesis	
ADE12	*YNL220W*	Adenylosuccinate synthase	Andreichuk Iu *et al.* (1995)
ADE13	*YLR359W*	Adenylosuccinate lyase	Guetsova *et al.* (1997)
ADK1	*YDR226W*	Adenylate kinase	Konrad (1988); Magdolen *et al.* (1987)
		Activity in GDP synthesis	
IMD1	*YAR073W*	IMP dehydrogenase isoform, probable pseudogene	Escobar-Henriques and Daignan-Fornier (2001); Hyle *et al.* (2003)
IMD2	*YHR216W*	IMP dehydrogenase isoform	Escobar-Henriques and Daignan-Fornier (2001); Hyle *et al.* (2003)
IMD3	*YLR432W*	IMP dehydrogenase isoform	Escobar-Henriques and Daignan-Fornier (2001); Hyle *et al.* (2003)
IMD4	*YML056C*	IMP dehydrogenase isoform	Escobar-Henriques and Daignan-Fornier (2001); Hyle *et al.* (2003)
GUA1	*YMR217W*	GMP synthase	Dujardin *et al.* (1994)
GUK1	*YDR454C*	Guanylate kinase	Konrad (1992)
		Activity in purine salvage	
AAH1	*YNL141W*	Adenine deaminase	Ribard *et al.* (2003)
AMD1	*YML035C*	AMP deaminase	Meyer *et al.* (1989)
APT1	*YML022W*	Adenine phosphoribosyltransferase	Alfonzo *et al.* (1999)
HPT1	*YDR399W*	Hypoxanthine-guanine phosphoribosyltransferase	Guetsova *et al.* (1997)
XPT1	*YJR133W*	Xanthine phosphoribosyl transferase	Alfonzo *et al.* (1999)
ADO1	*YJR105W*	Adenosine kinase	Lecoq *et al.* (2001a)
ISN1	*YOR155C*	Inosine 5′-monophosphate (IMP)-5′-nucleotidase	Itoh *et al.* (2003)
PNP1	*YLR209C*	Inosine and guanosine phosphorylase	Lecoq *et al.* (2001b)
GUD1	*YDL238C*	Guanine deaminase	Saint-Marc and Daignan-Fornier (2004)

regions (Escobar-Henriques *et al.* 2003a), but the precise mechanisms have not yet been elucidated.

For adenine deaminase (Aah1), regulation upon entry into stationary phase occurs both at the transcriptional and the post-transcriptional levels. A genetic screen for mutants abolishing this regulation revealed that transcriptional regulation involves the kinase Ssn3 and its cyclin Ssn8 (Escusa *et al.* 2006), which together are involved in phosphorylation of the RNA polymerase II C-terminal domain. The post-transcriptional regulation occurs through degradation of Aah1 by the proteasome and is mediated by a SCF complex involving the F-box protein Saf1, which is itself upregulated in stationary phase (Escusa *et al.* 2006, 2007). However, a *saf1* mutant can enter and exit stationary phase normally, and the role of nucleotide synthesis in stationary-phase establishment is still unclear.

Several purine biosynthesis enzymes (Ade4, Ade5,7, Ade17, Ade12) were found to form punctate cytoplasmic foci in cells grown to stationary phase (Narayanaswamy *et al.* 2009). For Ade4-GFP, these foci could be reversed by addition of adenine or hypoxanthine to the growth medium (Narayanaswamy *et al.* 2009). This intriguing property is reminiscent of that of purine biosynthesis enzymes in HeLa cells that tend to cluster upon purine limitation (An *et al.* 2008). The existence of such complexes in yeast remains to be established.

Phosphate

Identification of phosphate-responsive genes

Phosphate is an abundant molecule that is incorporated into ATP and transferred from ATP to a large number of small

Table 9 Intracellular nucleotide concentrations

Nucleotide	Concentration (mM)
ATP	4.6 ± 0.6
GTP	1.3 ± 0.2
UTP	1.6 ± 0.1
CTP	0.73 ± 0.03
ADP	0.82 ± 0.081
GDP	0.39 ± 0.036
UDP	0.093 ± 0.007
AMP	0.049 ± 0.012
GMP	0.12 ± 0.011
UMP	0.394 ± 0.061
CMP	0.017 ± 0.006
IMP	0.178 ± 0.019
dATP	0.031 ± 0.008
dGTP	0.016 ± 0.005
dCTP	0.023 ± 0.004
dTTP	0.022 ± 0.007

FY4 prototrophic strain was grown in SD CASA (0.5% ammonium sulfate, 0.17% yeast nitrogen base, and 2% glucose, supplemented with 0.2% casamino acids) (Benoît Pinson, personal communication).

biomolecules including nucleotides, sugars, and lipids, but also macromolecules such as proteins. In yeast, the total phosphate concentration is in the hundred-millimolar range. Phosphate, like carbon, nitrogen, or sulfur, is required for progression through the cell cycle, and cells starved for phosphate arrest in G1 (Saldanha *et al.* 2004). Yeast cells have developed complex responses to adapt to phosphate scarcity, and the physiological response to inorganic phosphate availability has been studied for a long time. Most of the work in the past was based on the expression of excreted phosphatases that were detected using a chromogenic substrate (reviewed in Johnston and Carlson 1992). More recent transcriptomic approaches have provided an overview of the phosphate response in yeast.

In pioneering work, Brown and coworkers used microarray analysis to identify phosphate-responsive genes that are induced when cells are shifted from high to low inorganic phosphate (Pi) medium (Ogawa *et al.* 2000). Subsequently, Piper and coworkers used chemostat cultures to analyze phosphate-responsive genes and identified a set of genes specifically up- or downregulated in cells grown under low-phosphate conditions (Boer *et al.* 2003). These studies gave very consistent results, but also revealed sets of genes induced only upon starvation or only when cells are adapted to growth on low Pi (Table 10). Some of these differences could be due to differences in Pi concentration in the two experiments. Indeed, it is known that intermediate phosphate concentration (low Pi *vs.* starvation) may differentially affect expression of the PHO genes (Lam *et al.* 2008).

Phosphate-responsive genes belong to several functional categories: organic phosphate utilization genes (phosphatases and transporters), polyphosphate metabolism, regulatory factors (*PHO81*, *SPL2*), and other metabolic pathways (histidine, trehalose, glycogen, inositol phosphate) (Table 10). While upregulation of organic phosphate utilization genes in response to phosphate limitation is not surprising, induction of polyphosphate synthesis genes or cross-pathway regulations is less intuitive and will be discussed individually in the following sections.

Phosphorylation of Pho4 and subcellular localization in response to phosphate availability

On the basis of thorough genetic analysis of the phosphate response using acid phosphatase activity as a readout, three positive regulators (*PHO2*, *PHO4*, *PHO81*) and two negative regulators (*PHO80* and *PHO85*) have been identified. Epistasis studies carried out in the early 1970s by the Oshima group indicated that these genes function within a linear regulatory cascade (Figure 16) (Johnston and Carlson 1992). The most downstream effectors of the cascade, Pho2 and Pho4, are transcription factors (Johnston and Carlson 1992) that are modulated by the proteins in the upper part of the cascade through complex molecular mechanisms that were only recently uncovered. The elegant work in the mid-1990s by the O'Shea laboratory provided a clear breakthrough in our understanding of how yeast cells regulate phosphate utilization. Pho85 and Pho80 were shown to be a protein kinase and a cyclin, respectively, that coimmunoprecipitated as a complex (Kaffman *et al.* 1994). Furthermore, the Pho80–Pho85 complex was found to coimmunoprecipitate with Pho4, could phosphorylate Pho4 *in vitro* (Kaffman *et al.* 1994), and is required for Pho4

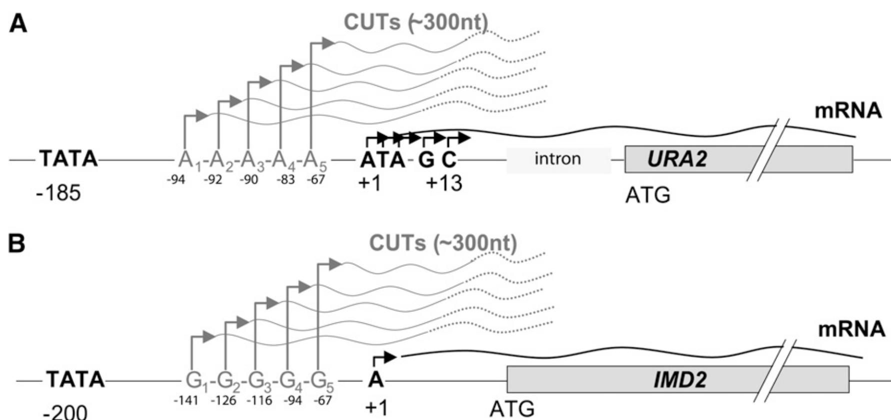

Figure 14 Organization of the *URA2* and *IMD2* promoter regions. The transcription start sites are shown, and their respective distances from the mRNA start site are indicated. Unstable transcripts are shown in gray.

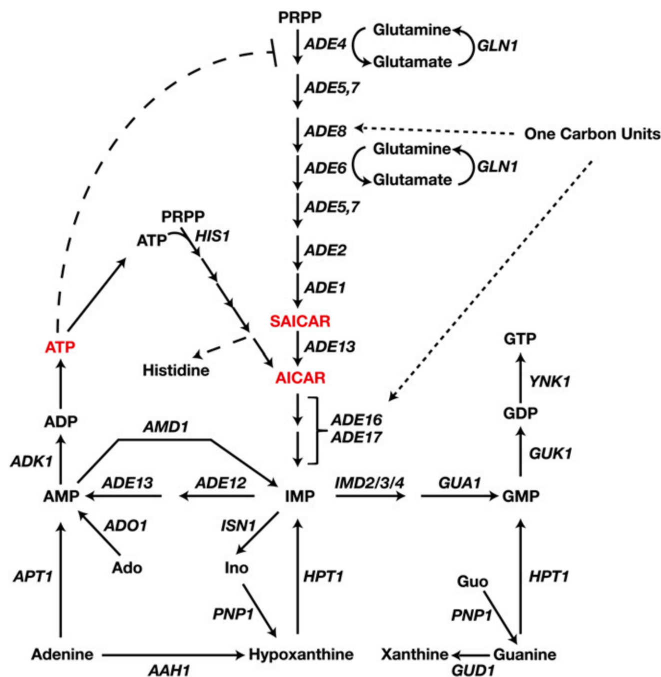

Figure 15 Purine and histidine pathways in yeast. Ado, adenosine; AICAR, 5′-phosphoribosyl-5-amino-4-imidazole carboxamide; Ino, inosine; guo, guanosine; IMP, inosine 5′-monophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; SAICAR, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-amino-imidazole. Gene names are italicized. Regulatory molecules are shown in red.

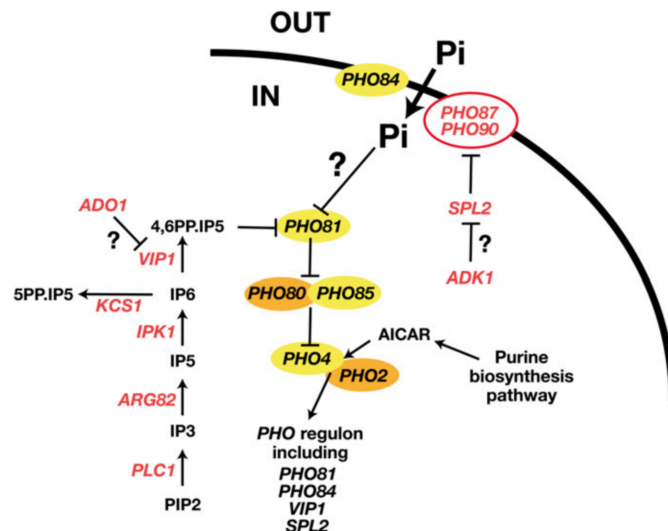

Figure 16 Regulation of phosphate utilization. The phosphate regulatory cascade is shown. New genes recently identified as important for regulation of phosphate utilization are shown in red. Question marks designate the steps for which no molecular mechanism has been documented yet.

hyperphosphorylation *in vivo* (Kaffman *et al.* 1994). Five phosphorylated peptides dependent on the Pho80–Pho85 complex were identified (Kaffman *et al.* 1994), and the cognate-phosphorylated serine residues were identified by mutagenesis and phosphopeptide mapping (O'Neill *et al.* 1996). Importantly, phosphorylation of Pho4 by Pho80–Pho85 impedes nuclear localization; Pho4 is localized in the nucleus under low phosphate conditions or when the phospho-acceptor serines are mutated, and conversely, Pho4 is cytosolic in the presence of high phosphate when Pho80–Pho85 is active (Figure 6) (O'Neill *et al*. 1996). Phosphorylation of two serine residues is specifically required for proper export of Pho4 to the cytosol in response to high phosphate via interaction of Pho4 with the exportin Msn5 (Komeili and O'Shea 1999). Phosphorylation of a third serine residue inhibited interaction of Pho4 with the Pse1 importin and Pho4 nuclear import (Komeili and O'Shea 1999). Finally, a fourth serine residue was found to modulate interaction of Pho4 with Pho2 (Komeili and O'Shea 1999). Conversely, a mutation in a serine residue in Pho2, which can be phosphorylated *in vitro* by Cdc28, affects its interaction with Pho4 (Liu *et al.* 2000). However, there is no evidence that this potential modification of Pho2 could be responsive to the phosphate switch *in vivo*. Since both Pho2 and Pho4 are required for expression of the phosphate-responsive genes because their interaction is reduced by phosphate as revealed by two-hybrid experiments (Hirst *et al.* 1994) and since the two proteins bind cooperatively *in vitro* to the promoters of the target genes (Barbaric *et al.* 1996), their ability to interact probably limits their transcriptional activation capacity. Importantly, a Pho4 mutant lacking all the Pho80–Pho85 phosphorylation sites is fully derepressed for Pho5 expression under high-phosphate conditions, indicating that phosphorylation of Pho4 is the main way to regulate *PHO5* expression (Komeili and O'Shea 1999). It should be stressed that the regulation of Pho4 activity by phosphorylation is not an on/off mechanism and that intermediary situations leading to partial phosphorylation of Pho4 and specific enrichment of the transcription factor at specific promoters as revealed by ChIP exist (Springer *et al.* 2003). A study of Pho4 binding, utilizing genomic nucleotide arrays to analyze DNA sequences that coprecipitate (ChIP on CHIP), revealed that Pho4 is associated with promoters under high-phosphate conditions, indicating that phosphorylation of Pho4 does not fully exclude it from the nucleus (Nishizawa *et al.* 2008).

Role of an intermediate metabolite (IP7) in the regulation of Pho81

While it became clear that subcellular localization of Pho4 was critical for the phosphate response, two major questions remained to be answered: what is the nature of the molecular signal generated in response to inorganic phosphate availability? And how is it transmitted to the Pho80–Pho85 complex? On the basis of epistasis studies, Pho81, the regulator just upstream of the Pho80–Pho85 complex, is considered to be the best candidate as a phosphate sensor.

Pho81 is a positive regulator of the PHO pathway that acts through negative regulation of Pho80–Pho85 (Ueda *et al.* 1975) (Figure 16). Indeed, the kinase–cyclin complex could be co-immunoprecipitated with Pho81 and

Table 10 Phosphate-responsive genes

	Starvation[a]	Chemostat[b]	Both experiments
Phosphate utilization and regulation	*PHO5 PHO12 PHO8*	*PHO3*	*SPL2 PHO89 PHO84 PHO11, PHO81 PHO86*
Polyphosphate metabolism	*PPN1 VTC2*		*VTC1 VTC3 VTC4*
Phospholipid metabolism	*GPD1 PLB1*	*PLB3 INM1 GIT1 KCS1 DDP1 TAX4*	*HOR2 GDE1 PHM8 VIP1 SUR1*
Sugar metabolism	*GLK1 PGM2 TSL1 TPS2 GSY1GLC3 CIT2 GRE3*		*PYK2 NTH2*
Amino acid and nucleotide metabolism	*HIS1 MET6 CHA1*	*PRS4 PPR1 SDT1 IMD4*	
Cell wall	*DAN1 DAN4 TIR1 TIR3 KRE2 RCR1*		
Other	*KRE29 PLM2 CTF19 RTC3 MGA1 PTK2 MAF1 DDR48 EMI2 ERG28 LAS1 TMA10 RTS3 AMS1 PMC1 YCR007C RCN1 CMK2 SPC110 HSP42 SSA4 YPK2 ZRT1 CTT1 DIA1 YNL208W MSC1 PHM7 AIM17 YJR061W YOR385WYLR149C YOR289W YMR291W YMR007W YBR051W YJL107C*	*ALR1 TRK2 ERC1 AST1 UFO1 ZAP1 APG2 AUT4 BUD23 FLO9 YLH47 PMU1 SHE9 YDL109C QDR1 DML1 SQS1 ZPS1 COS10 GFD2 YHR210C YGR079W YER186C YLR346C YNR014W YNL046W YBL070C YOR343C YMR279C*	*ICY1 PHM6 YNL217W YAR069C YJL119C*

[a] Ogawa *et al.* (2000)
[b] Boer *et al.* (2003)

immunoprecipitated Pho80–Pho85 activity was higher in a strain lacking *PHO81* while it was inhibited *in vitro* by addition of purified Pho81 (Schneider *et al.* 1994). A small region of Pho81 has similarity to CDK inhibitors, and this region is sufficient to inhibit kinase activity although with a much higher IC_{50} than the entire Pho81 protein (Schneider *et al.* 1994). Several studies aimed at identifying functional domains in the large Pho81 protein have allowed narrowing down the minimal regulatory domain (Ogawa *et al.* 1995; Huang *et al.* 2001); however, the roles of the other parts of the protein are not clearly established. Still, a regulatory role of the Pho81 amino-terminus is expected, as there are four different point mutations in this region, leading to its constitutive activation (Creasy *et al.* 1993; Ogawa *et al.* 1995). Pho81 is found mainly in the nucleus although it is also detected in the cytosol and at the plasma membrane (Huang *et al.* 2001). It is clear that nuclear localization of Pho81 is not regulated by inorganic phosphate availability (Huang *et al.* 2001). Interestingly, *PHO81* itself is a phosphate-responsive gene, thus resulting in a positive feedback loop.

The molecular nature of the signal received by Pho81 was recently identified using an *in vitro* assay based on the formation of the Pho81–Pho80–Pho85 complex (Lee *et al.* 2007). These authors observed that a cellular extract from cells grown in low phosphate was sufficient to inhibit the kinase activity. Further fractionation revealed that the inhibitor is a small molecule that was identified as α-myo-d-inositol heptakisphosphate (IP7) by NMR and mass spectrometry and was further confirmed using the synthetic compound (Lee *et al.* 2007). Consistently, IP7 was more abundant in cells under low-phosphate conditions, and Pho4 localization was reduced or elevated, respectively, by mutations impairing IP7 synthesis (*vip1*) or degradation (*ddp1*) (Figure 6) (Lee *et al.* 2007). Since Pho81 constitutively interacts with the Pho80–Pho85 kinase complex, IP7 is thought to reversibly change Pho81 conformation and affect accessibility of the kinase substrate (Lee *et al.* 2008). Although several earlier reports, including transcriptome analyses, had linked insositol phosphate metabolism with the phosphate response (Flick and Thorner 1998; El Alami *et al*. 2003; Steger *et al*. 2003; Auesukaree *et al*. 2005), the results were not entirely consistent. Mutations in genes of the inositol polyphosphate pathway—*PLC1*, *ARG82*, or *KCS1* (Figure 6)—constitutively express several genes of the *PHO* regulon (El Alami *et al.* 2003), suggesting that their regulatory role is not limited to synthesis of IP7 under low-phosphate conditions. In another report, Plc1 and Arg82 were found necessary for chromatin remodeling of the *PHO5* promoter upon induction in a *pho80ts* mutant (Steger *et al.* 2003). These studies have incontestably uncovered a complex interplay between inositol polyphosphate metabolism and regulation of phosphate-responsive genes.

Thus the current model of phosphate response is as follows: under low-phosphate conditions, IP7 becomes more abundant and binding of IP7 to Pho81 results in inhibition of the protein kinase activity of Pho80–Pho85. The resulting lower phosphorylation of Pho4 favors its nuclear localization and its interaction with Pho2 and attendant transcriptional activation of the phosphate-responsive genes. At this point, the molecular mechanism leading to increased IP7 concentration in response to phosphate starvation is not elucidated. In this perspective, the regulation of the inositol phosphate pathway genes in response to phosphate limitation appears interesting (Table 10). Kcs1 and Ddp1 that contribute to diminish IP7 concentration (by degradation or competition for the IP7 synthesis substrate) are induced only in the chemostat experiment (Boer *et al.* 2003) while Vip1, which synthesizes IP7, is induced immediately after Pi limitation (Ogawa *et al.* 2000) and maintained high in the chemostat experiment (Boer *et al.* 2003). It is tempting to speculate that transcriptional regulation of these genes could contribute to finely tune IP7 concentration upon Pi limitation.

Phosphate uptake and sensing

A lot of recent work has been devoted to understanding how yeast cells sense phosphate. Do yeast cells sense external and/or internal phosphate concentration? How is it converted into a transduction signal? As for other nutrients such as glucose or amino acids (Forsberg and Ljungdahl 2001b), phosphate uptake and sensing appear intimately connected.

Inorganic phosphate uptake in *Saccharomyces cerevisiae* involves several transporters able to ensure phosphate uptake over a wide range of Pi concentrations. Two high-affinity transporters were identified as Pho84 and Pho89 (Bun-Ya *et al.* 1991; Martínez and Persson 1998). A Pho84–GFP fusion was found at the plasma membrane (Petersson *et al.* 1999). Both *PHO84* and *PHO89* were among the most highly inducible genes upon phosphate limitation (Ogawa *et al.* 2000). While Pho84 cotransports phosphate with H^+, Pho89 is a phosphate/Na^+ symporter and works most efficiently under basic conditions, which are not usual physiological conditions for yeast cells (Zvyagilskaya *et al.* 2008). Indeed, on the basis of knockout experiments, Pho84 appears to be the major high-affinity phosphate transporter (Pattison-Granberg and Persson 2000).

Importantly, mutations in the *PHO84* gene result in constitutive expression of *PHO5*, and epistasis studies have placed *PHO84* upstream of all the regulatory components of the PHO pathway (Lenburg and O'Shea 1996). Phosphate uptake was significantly reduced in the *pho84* mutant even at high-orthophosphate concentrations, thus suggesting that constitutive expression of *PHO5* in the *pho84* mutant could be due to decreased internal phosphate concentration (Wykoff and O'Shea 2001); This assumption was further supported by direct measurement of internal phosphate concentration by ^{31}P NMR (Auesukaree *et al.* 2004; Pinson *et al.* 2004). This regulatory defect of *PHO5* expression in the *pho84* mutant could be compensated by overexpressing low-affinity phosphate transporters, and thus it is unlikely that Pho84 acts as a critical phosphate sensor in the phosphate regulatory pathway (Wykoff and O'Shea 2001). However, in work based on mutant analysis and use of agonists such as glycerol-3-phosphate, Pho84 was found to be involved in phosphate signaling to the protein kinase A pathway (Popova *et al.* 2010). Importantly, a good correlation was observed between internal phosphate concentration and *PHO5* expression in several mutants (Auesukaree *et al.* 2004), thus suggesting that there is an internal phosphate-concentration-sensing mechanism. How it is connected to Pho81 regulation of the Pho80-85 kinase via an IP7-dependent and/or -independent mechanism remains to be determined.

Three low-affinity phosphate transporters—Pho87, Pho90 and Pho91—were characterized and found to be strictly required in the absence of high-affinity transporters (Wykoff and O'Shea 2001). However, more recently, Pho91 was shown to be a vacuolar phosphate transporter (Hurlimann *et al.* 2007). Mutations in the low-affinity phosphate transporter genes led to upregulation of phosphate-regulated genes (Auesukaree *et al.* 2003; Pinson *et al.* 2004). Importantly, this transcriptional response was not associated with a lower intracellular phosphate concentration, suggesting that these transporters contribute to phosphate sensing independently of internal phosphate concentration (Pinson *et al.* 2004). This result suggests the existence of an extracellular phosphate-sensing mechanism that could be mediated by the low-affinity transporters. Strikingly, the low-affinity phosphate transporters carry an hydrophilic amino-terminal extension that is reminiscent of the carboxy-terminus extension found in the well-described glucose sensors Snf3 and Rgt2 (Özcan *et al.* 1996). More recently, the amino-terminal regions of the Pho87 and Pho90 transporters (named SPX domains) and the small Spl2 protein were found to negatively regulate phosphate uptake by these transporters (Wykoff *et al.* 2007; Hurlimann *et al.* 2009). The effect of Spl2 overexpression on phosphate uptake was dependent on the presence of the SPX domain, and consistently Spl2 was found to interact with the SPX domain in split ubiquitin and co-immunoprecipitation assays (Hurlimann *et al.* 2009). Spl2 is a phosphate-responsive gene (Ogawa *et al.* 2000) initially isolated as a suppressor of a phospholipase C mutant (Flick and Thorner 1998). Under low-phosphate conditions, induction of Spl2 expression leads to downregulation of low-affinity transport, which is thought to result in lower intracellular phosphate and attendant activation of the *PHO* regulon (Wykoff *et al.* 2007) although this aspect has not yet been addressed experimentally. This feedback loop is required for the bistable properties of the system that result in heterogeneous (either low or high) expression of the *PHO84* in individual cells of a clonal population, as revealed by flow cytometry using a GFP construct driven by the *PHO84* promoter (Wykoff *et al.* 2007).

It should be stressed that SPX domains are found in 10 yeast proteins (*PHO81*, *PHO87*, *PHO90*, *PHO91*, *VTC2*, *VTC3*, *VTC4*, *GDE1*, *SYG1*, and *YDR089W*), of which 8 are closely linked to phosphate metabolism; whether all the SPX domains are regulated by Spl2 is not known. Importantly, in plants, the SPX domain occurs as well in proteins involved in maintaining phosphate homeostasis (Duan *et al.* 2008), suggesting that it could be directly involved in phosphate sensing.

Purine phosphate connection: more signal molecules

Systematic high-throughput screens for mutants constitutively expressing *PHO5* revealed, among many mutants, two purine metabolism mutants: *ado1* and *adk1* (Auesukaree *et al.* 2005; Huang and O'Shea 2005). *ADO1* and *ADK1*, respectively, encode adenosine and adenylate kinase, which successively phosphorylate adenosine to AMP and then ADP (Figure 15). The corresponding mutants affect the phosphate response through different pathways. The effect of *ado1* is abolished in a *vip1* but not in a *spl2* background, whereas the *adk1* effect is *vip1* independent and *spl2* dependent (Gauthier *et al.* 2008). While the role of Ado1 in

the phosphate response is unclear, Adk1 effects could be due to lower ATP concentrations in the mutant strain. Indeed, other conditions resulting in lower ATP, such as growth in the absence of adenine, lead to upregulation of *PHO84* expression (Gauthier *et al.* 2008). However, it should be stressed that this presumed regulatory role for ATP is based on coincidental observations and that no molecular mechanism linking ATP concentration to Spl2-dependent regulation has been identified yet. Whether direct or not, such a central role for ATP could reflect the fact that incorporation of phosphate into biomolecules always requires ATP at some point.

More surprisingly, an important role for the purine pathway metabolic intermediate AICAR in the regulation of *PHO* genes was revealed by transcriptome analysis of mutants accumulating various amounts of AICAR (Pinson *et al.* 2009). AICAR, but not SAICAR, was found to promote interaction between Pho2 and Pho4 *in vivo* in a two-hybrid assay, and a Pho2-Pho4 chimera was not responsive to AICAR (Figure 16) (Pinson *et al.* 2009). Both Pho2 and Pho4 bound an AICAR column *in vitro*, indicating that the effect of AICAR on these transcription factors could be direct (Pinson *et al.* 2009). Because AICAR is a major regulator of purine biosynthesis genes, it is possible that coregulation of the two pathways reflects the fact that purine synthesis significantly contributes to phosphate consumption. It is noteworthy that *HIS1*, which encodes the first committed step in the histidine pathway, is upregulated at the transcriptional level under low Pi conditions (Ogawa *et al.* 2000). The induction of *HIS1* was not observed in the chemostat experiment (Table 10), suggesting that this response is transient and that AICAR could be used to boost the transcriptional response.

Importantly, the above mentioned upregulation of *PHO84* in the *adk1* mutant is independent of the AICAR response. Indeed, *PHO84* is still upregulated in an *adk1 ade8 his1* mutant unable to synthesize AICAR as testified by the inability of this triple mutant to upregulate *ADE17* expression (Gauthier *et al.* 2008). Thus, upregulation of the *PHO* genes in the AICAR-accumulating mutant *ade16 ade17* or in the *adk1* mutant most likely occurs through different means, thereby illustrating the complex regulatory network between purine and phosphate metabolism.

Polyphosphates as a means to save and buffer intracellular phosphate

While it is still unclear whether intracellular orthophosphate is directly sensed and used as a signal, the situation is even more complicated due to compartmentalization and storage of inorganic polyphosphate (polyP). PolyP is composed of linear phosphate polymers, which can represent >30 mM equivalent Pi while free cytosolic orthophosphate concentrations are ∼20 mM (Pinson *et al.* 2004). Most polyP is stored in the vacuole, although some is found in other cellular compartments (*e.g.*, nucleus and mitochondria) (Urech *et al.* 1978; Saito *et al.* 2005).

Genome-wide analysis of phosphate-responsive genes has allowed the identification of several genes involved in polyP metabolism (Ogawa *et al.* 2000). Importantly, measurement of phosphate uptake in the polyP synthesis mutants revealed that polyP accumulation acts as a phosphate sink required for sustained Pi uptake (Ogawa *et al.* 2000). This led to the hypothesis that polyP acts as a buffer that can be mobilized when extracellular phosphate is transiently limiting (Thomas and O'Shea 2005) or when intracellular consumption fluctuates along the cell cycle (Neef and Kladde 2003).

A systematic search for knockout mutants affecting polyP content revealed 255 genes connected to this process (Freimoser *et al.* 2006), although the reasons why most of these mutants affect polyP concentration are largely unknown.

Regulation by noncoding RNAs

While the idea of pervasive transcription is emerging, it is striking that transcription of noncoding RNAs has been reported at several yeast loci connected to phosphate metabolism where they were suspected to play important regulatory roles. Antisense transcripts have been detected at the *PHO5* and *PHO84* loci (Camblong *et al.* 2007; Uhler *et al.* 2007). These transcripts, which are degraded by the nuclear exosome, are clearly more abundant in exosome mutants (Camblong *et al.* 2007; Uhler *et al.* 2007). In the case of *PHO5*, antisense transcription appears to affect the speed of chromatin remodeling during transcription activation (Uhler *et al.* 2007). For *PHO84*, antisense transcription was observed during chronological aging (Camblong *et al.* 2007). In this case, antisense transcription was found to result in the recruitment of the histone deacetylase Hda1 and thereby downregulate the authentic *PHO84* promoter (Camblong *et al.* 2007). Importantly, antisense at *PHO84* was found to act both in *cis* and in *trans*, as shown by ectopic expression of the antisense and use of a ribozyme inserted in the antisense sequence to cleave the antisense after its synthesis (Camblong *et al.* 2009). In a third study carried out on the *KCS1* locus, both antisense and intragenic transcripts were detected (Nishizawa *et al.* 2008). Importantly, expression of the noncoding transcripts was dependent on the transcription factor Pho4 and responded to phosphate availability (Nishizawa *et al.* 2008). However, since the abundance of these transcripts did not significantly affect *KCS1* expression (Nishizawa *et al.* 2008), it is not yet clear whether these transcripts play a regulatory role such as the one established in the case of *PHO84* (Camblong *et al.* 2007). Further roles for noncodingRNAs in regulation of phosphate utilization are waiting to be discovered.

Future Directions

The cellular components required for amino acid, nucleotide, and phosphate metabolism in yeast can be subdivided in four interrelated rudimental categories: (1) the permeases in the plasma membrane that facilitate uptake and secretion of metabolites; (2) the metabolic sensors that

directly control enzymatic activity or indirectly regulate metabolic pathways by altering patterns of gene expression; (3) the enzymes catalyzing the synthesis, catabolism, and interconversion of metabolites; and (4) the intracellular organelles, including the vacuole, mitochondria, and peroxisome that compartmentalize metabolic processes and also serve as storage compartments. Although great progress has been made in understanding these rudiments, major holes in understanding remain, in particular how individual components are coordinated to function in synchrony.

The compartmentalized structure of eukaryotic cells requires intracellular targeting mechanisms to ensure correct localization of transport systems. Cell biological approaches continue to provide insight into the intracellular traffic of nutrient permeases/transporters in the early (ER) and late (Golgi and post-Golgi) stages of the secretory pathway. In particular, an increased understanding of the routing mechanisms may provide answers as to how cells sense their overall metabolic state. For example, it is intriguing to consider the general amino acid permease (Gap1) as an example. Gap1 is known to function as a transceptor in amino acid-starved cells that responds to the reintroduction of amino acids and initiates signals activating the cAMP-protein kinase pathway (Thevelein and Voordeckers 2009; van Zeebroeck *et al.* 2009; Popova *et al.* 2010; Rubio-Texeira *et al.* 2010). Proper routing of Gap1 from endosomal compartments to the plasma membrane depends on it being functionally active (Gao and Kaiser 2006; Risinger *et al.* 2006); *i.e.*, to be sorted correctly, Gap1 must be able to switch its conformation between an outward facing (lumenal) and an inward facing (cytoplasm) conformation (Cain and Kaiser 2011). The combination of these findings raises the possibility that, as Gap1 progresses through the secretory pathway, it may have the capacity to sample the amino acid levels within cells. Hence, in similarity to the amino acid receptor of the external amino acid Ssy1 (Wu *et al.* 2006; Poulsen *et al.* 2008), transceptors may be the ultimate source of internal nutrient-based signals affecting important signaling components, *e.g.*, TORC1.

Finally, there is a growing appreciation of the importance of metabolites in regulating many biological processes. In the future, we expect that the ability to correlate data obtained from metabolomic and classical gene expression analyses will be highly informative and critical for making progress toward a more complete understanding. For example, in the case of the cellular response to phosphate limitation, it is clear that transcriptome data obtained from batch (phosphate starvation) and from chemostat cultures (adapted to phosphate limitation) reveal different, albeit overlapping, sets of genes. The differences in gene expression are likely due to differences in the metabolic state of cells; consequently, metabolomic analysis should provide crucial information to help decipher the molecular nature of the phosphate limitation signal(s). Also, the great expansion of whole-genome information on many different fungi enables sophisticated comparisons to be carried out, which will likely yield interesting insights regarding metabolic regulation and may reveal precise information of general features and specific adaptations.

Acknowledgments

We thank members of the Ljungdahl and Daignan-Fornier laboratories for fruitful discussions and patience. J. A. Martens (University of Pittsburgh) is gratefully acknowledged for granting permission to reproduce the schematic diagram depicted in Figure 10. The vastness of the subject matter and space limitations have precluded referencing all relevant papers, and undoubtedly we have failed to cite some papers of equal or greater value than the ones cited; we apologize for the inadvertent omission of uncited work. Original research in our laboratories is supported by the Swedish Research Council (P.O.L.) and Conseil Régional d'Aquitaine and Agence Nationale de la Recherche grant no. BLAN06-1_136912 (B.D.-F.).

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Communicating editor: A. Hinnebusch