Mutation of a phosphorylatable residue in Put3p affects the magnitude of rapamycin-induced \textit{PUT1} activation in a Gat1p-dependent manner

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Running Title: Put3p transcriptional activity is modulated by phosphorylation of Y788.

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\textit{Saccharomyces cerevisiae} can utilise high quality (e.g., glutamine and ammonia) as well as low quality (e.g., \(\gamma\)-amino butyric acid and proline) nitrogen sources. The transcriptional activator Put3p allows yeast cells to utilise proline as a nitrogen source, through expression of the \textit{PUT1} and \textit{PUT2} genes. Put3p activates high-level transcription of these genes by binding proline directly. However, Put3p also responds to other lower quality nitrogen sources. As nitrogen quality decreases, Put3p exhibits an increase in phosphorylation concurrent with an increase in \textit{PUT} gene expression. The proline-independent activation of the \textit{PUT} genes requires both Put3p and the positively-acting GATA factors, Gln3p and Gat1p. Conversely, the phosphorylation of Put3p is not dependent on GATA factor activity. Here, we find that the mutation of Put3p at amino acid Y788 modulates the proline-independent activation of \textit{PUT1} through Gat1p. The phosphorylation of Put3p appears to influence the association of Gat1p but not Gln3p to the \textit{PUT1} promoter. Combined, our findings suggest that this may represent a mechanism through which yeast cells rapidly adapt to use proline as a nitrogen source, under nitrogen limiting conditions.

A key factor that permits \textit{Saccharomyces cerevisiae} to adapt to sub-optimal environmental conditions is its ability to utilise a wide range of nitrogen sources. Although \textit{S. cerevisiae} can metabolise many nitrogen sources, not all are preferred. Good nitrogen sources, such as glutamine and ammonia, will be preferentially consumed, while poor sources, e.g., \(\gamma\)-amino butyric acid (GABA) and proline, are scavenged only when favoured ones are no longer available. One of the least preferred nitrogen sources is proline and the genes \textit{PUT1} and \textit{PUT2} encode proteins specifically for the conversion of proline to glutamate - a more metabolically useful form of nitrogen. The expression of these genes is regulated by the
transcriptional activator, Put3p (1). Put3p is a member of the Zn(II)$_2$Cys$_6$ family of proteins, which includes Gal4p, Ppr1p and Lys14p (2). It is comprised of 979 amino acids organised, as many members of this family are, into three distinct domains (see Fig. 1c) - an N-terminal Zn(II)$_2$Cys$_6$ DNA-binding and dimerization domain, a region of unknown function loosely homologous to other family members, entitled the middle homology region (MHR), and a C-terminal activation domain (3-5).

Put3p acts as both a ligand sensor and a transcriptional activator. When proline is present at high intracellular concentrations it binds to Put3p causing a conformational change and inducing expression of the PUT genes some 20-fold (6). However, DNA footprinting has demonstrated that Put3p is bound to its target DNA sites irrespective of proline concentration (7). The constitutive DNA interaction of Put3p may allow the protein to affect the activity of the PUT genes in response to signals other than proline. For example, non-preferred nitrogen sources are capable of bringing about 2-4 fold increases in the activation of PUT1 and PUT2 from the repressed state imposed by more preferred nitrogen sources. This proline-independent activation is nitrogen source dependent and the level of activation increases as nitrogen quality decreases (8, 9). Put3p has also been observed to become increasingly phosphorylated as nitrogen quality decreases (8). While these changes in phosphorylation state are concurrent with changes in transcriptional activation, they are not dependent upon it. This indicates that the phosphorylation state of Put3p might regulate its proline-independent activation (8, 9). Thus, it would appear that Put3p responds to two separate cues to control its transcriptional activity - a proline signal which leads to conformational changes and high level transcriptional activation, and a non-preferred nitrogen source signal which results in the phosphorylation of Put3p and low level transcriptional activation.

When preferred nitrogen sources are present in the growth medium, the expression of genes required for the metabolism of non-preferred nitrogen sources, such as the PUT genes, are repressed. This phenomenon is known as Nitrogen Catabolite Repression (NCR). NCR is regulated by the TOR kinases, as part of the TORC1 complex (10-12). TORC1 is the primary signal transducer for nitrogen quality in the yeast cell and has homologues in all eukaryotic genomes examined (10-12). In high quality nitrogen environments, TORC1 is active and promotes NCR through the cytoplasmic sequestration of the positively acting GATA factors Gln3p and Gat1p. It has been shown that Gln3p is sequestered by direct physical association with Ure2p (13). However, while Gln3p is fully sequestered in the cytoplasm Gat1p remains localised to both the nuclear and cytoplasmic compartments. It is not clear how Gat1p localization is regulated (14). Upon the depletion of favored nitrogen sources, or following treatment with rapamycin, TORC1 is inactivated (12). This results in the complete nuclear localization of Gln3p and Gat1p, where they relieve NCR and activate gene expression (15, 16).

Moreover, when a good nitrogen source, such as glutamine, is available Put3p is inactive as proline transport is also inhibited by NCR. This occurs primarily through the repression of PUT4, which encodes a proline-specific transporter, and GAP1, encoding a general amino acid transporter (17, 18). Therefore, it is likely that there is a lag phase between the de-repression of proline transporters and its utilization as a nitrogen source. Proline-independent activation of PUT1 and PUT2, which are specific for proline metabolism, may represent a preparatory measure by the cell to ensure there is a sufficient level of the
enzymes present for efficient utilization of proline, when proline transport does commence. Here we show that the phosphorylation of Put3p at residue Y788 modulates proline-independent activation of the PUT genes through Gat1p, suggesting a mechanism by which PUT gene expression prepares the yeast cell for survival on proline as a nitrogen source.

**EXPERIMENTAL PROCEDURES**

**Strains and culture conditions.** FY23 yeast cells (MATa, ura3-52, trp1Δ63, leu2Δ1) carrying a C-terminally tagged PUT3 open reading frame on a MORF vector (Put3.MORF), was used for protein production and purification (19). The C-terminal tag consisted of fused 6xHis and HA tags, separated from the ZZ domain of Protein A by a 3C protease cleavage site. FY23 cells were also used to assay phosphorylation site mutants.

For chromatin immunoprecipitation, yeast strains expressing Gat1p-cAVI and Gln3p-cAVI were made by integrating a KanMX cassette bearing the cAVI tag (20) sequence into the genomic loci of GAT1 and GLN3 in FY23 put3Δ cells. The Avitag sequence was amplified from plasmid pUG6-Myc-C-Avitag (20). The resulting strains were then transformed with the plasmid pRS316-BirA-NLS, for the production of E. coli biotin ligase BirA, and either pMKL15 (wild-type PUT3) or pMKL23 (PUT3-Y788F).

**Culture methods, purification and MS/MS interrogation of Put3p.MORF by MIDAS.**

Put3p.MORF was purified from FY23 cells grown in SD media, without amino acids or ammonium sulphate (Formedium, UK), which was supplemented with 0.1% (v/v) proline, 2.0% (v/v) raffinose and essential amino acids. Cells were grown to an A$_{600}$~0.6 and protein production induced for 6 hr by the addition of 2% galactose. The induced cells were pelleted by centrifugation at 5,000 g for 5 min. Cells were initially washed, then resuspended in Buffer A (20 mM HEPES-KOH (pH 7.8), 300 mM NaCl, 10% (v/v) glycerol) with the addition of 50 mM NaF, 20 mM β-glycerophosphate, 0.1 mM Na$_3$VO$_4$ and Roche -EDTA protease inhibitor tablets. The cells were frozen in liquid nitrogen and then mechanically lysed under liquid nitrogen in a Spex 6850 Freezer Mill for 5 cycles of 2 min each, at maximum power. The lysate was thawed at room temperature, 1 mM PMSF and 1 µM okadaic acid added, and incubated with rotation at 4°C for 10 min. The lysate was then subjected to centrifugation at 5,000 g for 10 min, the supernatant removed and centrifuged again at 20,000 g for 20 min. Ni$^{2+}$-nitrioltriacetic acid agarose beads (ProBond resin; Invitrogen) (1 ml) were pre-washed with Buffer A including protease and phosphatase inhibitors, added to the cleared lysate and incubated for 1 hr with rotation at 4°C. The bead/lysate mix was loaded onto a column then washed with Buffer A including protease and phosphatase inhibitors, then Buffer A and finally Buffer A containing 500 mM NaCl, and 30 mM imidazole. Protein was eluted from the column with Buffer A containing 250 mM imidazole. The resultant fractions were analysed by SDS-PAGE and the peak fractions pooled then precipitated with 25% trichloroacetic acid (final concentration). The resulting pellet was washed once with ice cold acetone and once with ice cold acetone containing 0.5 N HCl, allowed to dry, then resuspended in lithium dodecyl sulphate (LDS) sample buffer and run on a NuPAGE Tris-glycine gel (Invitrogen). Bands corresponding to Put3p.MORF (~130 kDa) were excised from the gel and interrogated by MIDAS (21) for phosphorylation site detection.
Cloning, strain construction and mutagenesis. The PUT3 gene was deleted from FY23 by the insertion of the TRP1 gene between the SnaB1 restriction enzyme recognition sites of PUT3, resulting in an almost complete deletion of the open reading frame. GATA factor deletion strains were generated by disruption of the URE2, GLN3, or GAT1 ORFs by the kanamycin-resistance gene, as described (22).

The PUT1-lacZ reporter was created by amplifying the PUT1 promoter from -1 kb to +25 bp into the ORF from genomic DNA and ligating it to the lacZ gene, amplified from pRJR179 (3), by PCR. The resulting construct was cloned into pRJR179 as an XbaI/Kpn1 fragment. The reporter bearing plasmids were then cut with ApaI and integrated into the URA3 locus of put3Δ, ure2Δ, gal3Δ and gat1Δ cells, where it complemented the defective URA3 gene. Transformants were selected for by growth on plates lacking uracil.

The put3Δ, PUT1-lacZ strains were complemented for put3Δ by transformation with a pRS415 borne copy of PUT3, or a mutant there of. PUT3 was amplified from -639 bp to +98 bp of the ORF and cloned into pRS415 as a XhoI/XbaI fragment. The phosphorylation sites revealed by MIDAS analysis were mutated from serine to alanine or glutamic acid and from tyrosine to phenylalanine by use of the Quikchange methodology (23). Mutations were confirmed by sequencing. Oligonucleotide sequences are available on request.

Culture methods, extract preparation and β-galactosidase assays. Selected strains were grown in 3 ml cultures at 30°C to mid-log phase in SD media without amino acids or ammonium sulphate (Formedium, UK), which was supplemented with 0.1% v/v glutamine, 2% glucose and essential amino acids. Where indicated, rapamycin (final concentration of 200 ng/ml) or the drug vehicle (90% ethanol, 10% Tween-20) was added for 60 min. β-galactosidase levels in extracts were measured with the Galacto-Light Plus chemiluminescent reporter gene assay system (PE Biosystems) according to the manufactures instructions. The activity was normalised to protein concentration, which itself was calculated using a Bradford assay (24).

Chromatin immuno-precipitation. Yeast cells were grown in minimal media containing yeast nitrogen base without ammonia, and with histidine, methionine, 2% glucose and 0.5% glutamine. Cultures (100 ml) were grown to an A600 of 0.4-0.6 and then treated with 200 ng/ml rapamycin for 30 minutes. Cells were treated with 1% formaldehyde for 20 minutes at room temperature on a tilting platform in order to crosslink proteins to DNA, and then with 0.5M glycine for 5 minutes to quench the crosslinking reaction. Chromatin was extracted as described (20).

Quantitative PCR. Reactions (50 µl) were set up using 1µl ChIP sample, 10 pmol each primer and 25 µl SYBR green JumpStart mix (Sigma). All samples were set up in duplicate. Input DNA was used at 1:100 dilution to calculate IP/Input ratios. For each primer set five ten-fold serial dilutions of Input DNA were used to prepare a standard curve in order to calculate DNA concentrations of the samples.

qPCR was carried out using the Chromo4 real-time system (MJ Research) and the following program: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, 56°C for 15 sec, 72°C for 15 sec. ChIP data is expressed as a ratio of PUT1 IP:Input to HMR IP:Input. At least two
ChIP experiments were carried out for each biological sample and at least three separate yeast cultures were used to generate mean and standard deviation data.

**RESULTS**

*Put3p.MORF is phosphorylated at distinct domains at the N and C-terminus.* Put3p is increasingly phosphorylated under conditions of decreasing nitrogen quality, in parallel with increasing activation (8). Our previous data demonstrated that the transcriptional properties of non-phosphorylated Put3p can be activated, *in vitro*, by proline (6). In light of this, we wished to ask what the effects of these phosphorylation events were *in vivo*. This necessitated identifying the sites of phosphorylation present within Put3p. To this end, we ectopically expressed Put3p as a C-terminally tagged fusion protein (Put3p.MORF) in *Saccharomyces cerevisiae*, under the control of the GAL1 promoter (19). This was necessary as our previous studies relied on expression in insect cells, which did not result in post-translational modification of Put3p (Fig. 1a).

Once purified, Put3p.MORF was subject to multiple rounds of protease digestion and MIDAS analysis (21). It was necessary to use a combinatorial approach to protease digestion, as simply digesting Put3p.MORF with trypsin resulted in many fragments too large for MIDAS analysis. Subsequent rounds of trypsin, trypsin/AspN and trypsin/AspN/chymotrypsin digestion yielded fragments of suitable size for analysis.

Successive rounds of protease digestion and MIDAS analysis revealed the presence of six previously unidentified phosphorylation sites. As can be seen in Fig. 1b, these sites cluster at the amino terminus of the protein (S103, S129/S130 and S164), distal to the DNA binding domain, and at the carboxy-terminus (Y788, S882 and S969), proximal to and within, the activation domain. The peptide bearing S129 and S130 was shown to be phosphorylated only once. However, the juxtaposition of the two residues meant we were unable to resolve which was modified.

**Analysis of Put3p phosphorylation sites.** In order to analyse the effect of each phosphorylation event on the activity of Put3p we created a reporter system where the promoter of *PUT1* (1 kb 5' to the open reading frame) was fused to *lacZ* and integrated at the *URA3* locus. *PUT3* was then deleted by disruption of the open reading frame with the *TRP1* gene. A low copy number plasmid producing wild-Put3p maintained the same levels of activity of the integrated *PUT1-lacZ* reporter as Put3p produced from its native genomic locus (data not shown). The plasmid-borne *PUT3* gene was mutated to change the phosphorylated serine residues to alanine and the phosphorylated tyrosine to phenylalanine by site-directed mutagenesis.

The presence of poor nitrogen sources is signalled to Put3p though TORC1, *via* Gln3p and Gat1p (15, 16). Therefore, we were able to selectively activate this signal in cells growing in a rich nitrogen source by the addition of rapamycin, which inactivates TORC1, mimicking conditions of poor nitrogen quality (8, 9). Mutation of each of the phosphorylatable residues in the amino-terminal cluster of Put3p to alanine, which renders them phosphorylation-incompetent, had no effect on the ability of Put3p to activate *PUT1-lacZ* in a proline-independent manner (Fig. 2). It is possible that no single phosphorylation site had an effect on the activation of *PUT1-lacZ*, but instead acted collectively by increasing the electronegative charge of this region. We therefore...
sequentially combined the serine to alanine mutations to yield an N-terminal phosphorylation incompetent mutant (S103A, S129A, S130A, S164A). This mutant had no effect on the activation of our reporter, when compared to wild-type (Fig. 2).

For the carboxyl-terminal phosphorylation sites, the conversion of the S882 to alanine had no effect on *PUT1-lacZ* expression, while the Y788F mutant exhibited an approximately 50% reduction in activity and S969A induced an approximately 25% increase in activity. When Y788F was combined with S882A, the Y788F phenotype was dominant. However when Y788F was combined with S969A, the S969A phenotype is dominant (Fig. 3). This suggests that the phosphorylation of Y788 is an activating event, while phosphorylation of S969 is inhibitory. Although both the Y788F and S969A versions of Put3p were induced by rapamycin, both mutants exhibited their phenotype regardless of its presence (Fig. 3a).

Given these findings, we generated mock-phosphorylated mutants to verify the phosphorylation-incompetent phenotypes. Substitution of serine for glutamic acid can, in some instances, mimic phosphorylation because of the presence of a bulky negatively charged functional group. However as there are no naturally occurring aromatic amino acids with a carboxylic acid group, in place of a hydroxyl group, it is not possible to create a mock phosphorylated tyrosine residue. When we assayed the S969E strain we discovered that it had a very similar phenotype to the phosphorylation-incompetent strain, S969A. This observation may occur through the perturbation of the activation domain, which Put3p is known to be sensitive to (3). The fact that both the S969A and S969E phenotypes were dominant to Y788F supports the idea that this phenotype arises from perturbation of the activation domain (Fig. 3b).

In order to dismiss the possibility that the Y788F phenotype resulted from non-specific effects of the mutation, we challenged both glutamine and proline grown cells with rapamycin or the drug vehicle (Fig. 4). Neither the Y788F mutation, nor treatment with rapamycin had any effect on the proline-dependent activation of Put3p, while the proline-independent activation again exhibited a ~50% reduction. This suggests that the Y788F phenotype does not result from non-specific effects of the mutation on Put3p function.

**Effect of phosphorylation at Y788 on GATA factor-mediated proline-independent activation of PUT1-lacZ.** Proline-independent activation of Put3p is mediated through the GATA factors Gln3p and Gat1p, which are, in turn, regulated wholly, or in part, by Ure2p (11, 14). Given this, we wanted to determine if phosphorylation of Y788 had an effect on these factors. We disrupted the ORF encoding each GATA factor with the kanamycin-resistance gene (22) in the *put3Δ, PUT1-lacZ* background. These strains were then assayed for the activity of Y788F relative to wild-type Put3p.

Ure2p negatively regulates of Gln3p by direct physical association. Gat1p, however, does not associate with Ure2p and is only indirectly regulated by it (14, 15). Deletion of Ure2p results in constitutive nuclear localization of Gln3p and the constitutive dual nuclear-cytoplasmic localisation of Gat1p (14). It also has been shown to hyperactivate Put3p (25). As this activation is most likely a result of constitutive Gln3p recruitment, we would therefore expect the Y788F phenotype to be preserved in this
mutant if it operates through Gln3p. This, however, is not the case. In all the \textit{ure2}\Delta strains, regulation of proline-independent activation is lost and cells show similar levels of activation whether or not rapamycin is present. In addition, under these conditions there is no discernible phenotype associated with the Y788F mutation in comparison to the wild-type protein (Fig. 5a).

When the positive regulators Gln3p and Gat1p are deleted individually a difference becomes apparent. While \textit{gat1}\Delta cells remain responsive to rapamycin, Y788F and wild-type Put3p maintain similar levels of activity in each condition; the Y788F phenotype is again lost (Fig. 5b). However, the \textit{gln3}\Delta strains not only retain their responsiveness to rapamycin, but also retain the Y788F phenotype (Fig. 5c). These data suggest that phosphorylation of Y788 exerts its modulating effect through Gat1p. In addition, the dependence of the Y788F mutation on Gat1p provides evidence that the mutation does not simply cause an inhibitory effect on activation by Put3p by, for example, altering the activation domain of the protein.

\textit{Gat1p and Gln3p binding at the PUT1 promoter.} To determine the effect of both wild-type Put3p and the Y788F mutant on the ability to recruit Gat1p or Gln3p to the \textit{PUT1} promoter, chromatin immunoprecipitation analysis was performed (Fig. 6). The protein being tested was tagged, at its genomic locus, using a biotin tag (20). The efficacy of this system was tested by initially tagging Rpb3p, the largest subunit of RNA polymerase II. In the presence of rapamycin, two-fold more Rpb3p could be identified at the transcriptional start site of \textit{PUT1}. In comparison, this rapamycin-dependent increase in RNA polymerase II association was not observed in a region of the \textit{PUT1} promoter that encompasses the Put3p-binding site and the GATA binding sites (Fig. 6a).

Increased binding of both Gat1p and Gln3p in response to rapamycin treatment could be observed at the \textit{DAL5} promoter (data not shown). In cells bearing wild-type Put3p, the presence of rapamycin induced a greater level of Gat1p binding at the \textit{PUT1} promoter (Fig. 6b). Conversely, the presence of rapamycin had little effect on the binding of Gln3p to the same promoter region (Fig. 6c). The Y788F version of Put3p elicited a higher level of association of Gat1p to the \textit{PUT1} promoter that was insensitive to the presence of rapamycin (Fig. 6b) and, again, the binding of Gln3p to this promoter was unaffected by this mutation.

\section*{DISCUSSION}

The expression of \textit{PUT} genes for proline utilisation (\textit{PUT1} and \textit{PUT2}) is regulated by two pathways – direct sensing of proline by Put3p and general nitrogen catabolite repression (NCR). While the interaction of Put3p with proline is well characterised (6), the interaction with NCR regulators is not. It is, however, known that Put3p becomes increasingly phosphorylated as nitrogen source quality decreases and that this coincides with increased \textit{PUT} gene expression (8, 9). This suggests that this Put3p phosphorylation is linked with NCR regulation of \textit{PUT} gene expression and prompted us to ask what role phosphorylation plays in the proline-independent regulation of the \textit{PUT} genes.

It is known that other members of the Zn(II)\textsubscript{2}Cys\textsubscript{6} family are phosphorylated concurrently with activation, it has been shown that a number of these events occur not as a cause of transcriptional activation, but rather as a consequence of it (26). For instance, Gal4p is phosphorylated at four
sites in a manner coincident with transcriptional activation. However, only one of these phosphorylation sites has an appreciable effect on the activation process itself (27, 28). The kinase responsible for phosphorylating Gal4p is Srb10p, a component of the RNA PolII holoenzyme (27). It therefore appears that, while phosphorylation affects activation, it only occurs when Gal4p is activating transcription and is not part of a separate signalling pathway impinging on Gal4p function.

Put3p becomes phosphorylated in a nitrogen source dependent, but activation independent manner. This suggests that there are distinct mechanisms regulating these events. In this work, we identified several phosphorylation sites in Put3p. Mutational analysis suggests that the majority of these sites have no effect on modulating the activity of Put3p activity. Our data has, however, revealed that mutation of tyrosine 788 to phenylalanine (Y788F) effects the NCR-dependent activation of the PUT1 promoter.

Previous studies involving promoter manipulation and deletion analysis have revealed that GATA factors are required for proline-independent activation of PUT1 (29). In addition, it has been proposed that Gat1p has a stronger influence on PUT1 gene expression than Gln3p (8, 9). These data, coupled with the finding that Put3p phosphorylation is not dependent on GATA factors (9), suggest that phosphorylation of Put3p might affect the ability of GATA factors to regulate proline-independent PUT1 expression. Our work confirms this, as a ~50% reduction in PUT1 activity was observed in the Put3p (Y788F) background. Furthermore, this phenotype was preserved in gln3Δ, but not gat1Δ strains, suggesting that it is Gat1p that interacts with the Y788 phosphorylation signal.

The ChIP data (Fig. 6) suggest that the Y788F mutation results in constitutive binding of Gat1p to the PUT1 promoter. There is some evidence to suggest that not all Gat1p is excluded from the nucleus under NCR (unlike Gln3p) (14). It is possible, therefore, that the Y788 phosphorylation event serves a permissive function, allowing Gat1p to bind and activate PUT1 only when NCR is relieved, and that the Y788F mutation causes aberrant binding of Gat1p, which consequently interferes with normal activation function. These results agree with our gene expression data, which show that PUT1 activation by rapamycin is indistinguishable from the wild-type when Gat1p is deleted in the Y788F background (Fig. 5). In the absence of Gat1p, another factor may be able to bind the PUT1 promoter which is not influenced by Y788 phosphorylation. It is possible that Gln3p may represent this factor, but results presented here and elsewhere (9) would suggest that Gln3p has only a minor role in PUT gene activation and that the authentic factor is, as of yet, unidentified.

In summary, we demonstrate that mutation of only one phosphorylatable residue within Put3p (Y788) affects the magnitude of PUT1 activation in response to rapamycin in a manner that depends on Gat1p. We suggest that phosphorylation of this residue is an important determinant in this process. However, it is possible that this mutation may affect Put3p in a manner not dependent on phosphorylation. For example, the mutation may by directly affect the interactions between Put3p and Gat1p or the way in which the complex interacts with other factors to modulate its ability to regulate transcriptional activation.

Our current model of PUT gene activation is described as follows. In rich media, TORC1 mediates the inhibition of PUT1 by two
pathways: first by inhibition of proline transport (\textit{PUT4}, \textit{GAP1}), and second by inhibition of GATA factor activity (Gat1p, Gln3p, possibly other proteins). The lack of phosphorylation on Y788 of Put3p inhibits low-level \textit{PUT1} activation by any Gat1p in the nucleus. In poor nitrogen conditions, repression by TORC1 is relieved. GATA factors are able to enter the nucleus. Phosphorylation of Put3p, by an unidentified kinase, allows activation of \textit{PUT1} by Gat1p - concurrent with the movement of Gat1p localisation from nuclear-cytoplasmic, to strictly nuclear. This permits the expression of the \textit{PUT1} while proline transport is initiated. Once proline transport commences, the presence of excess proline in the cell then results in full Put3p activation and high-level \textit{PUT1} expression. The events resulting from the switch from rich to poor nitrogen sources thus represents a 'priming' event whereby the cell becomes prepared to utilise proline as a nitrogen source.

The fact that Y788 is the only phosphorylation site for which we were able to detect an effect by blocking phosphorylation, narrows the possible factors regulating this event. In \textit{S. cerevisiae} there are no true protein tyrosine kinases, only dual specificity serine/threonine-tyrosine protein kinases. Moreover, there are only eleven proteins in the yeast kinome possessing this activity - four of these proteins are involved in MAP kinase cascades (Bck1p, Mkk1p, Mkk2p, Pbs2), six in cell cycle (Rad53, Swe1, Bud32, Mck1p, Mps1p and Yak1p) and one of unknown function related to Cdc2p (Kns1p) (30-34). Of these kinases, Yak1p may be the most likely candidate to act on Put3p. Yak1p responds to nutrients, is nuclear and is responsible for cell cycle arrest upon glucose limitation (35). The response of Yak1p to glucose limitation does not render it incompatible with nitrogen sensing pathways, as it is well established that both carbon and nitrogen sensing pathways converge on Gat1p and Gln3p. Not only is the expression of invertase, the product of the \textit{SUC2} gene, influenced by both Gat1p and Gln3p, it has also been shown that glucose, as well as nitrogen limitation, results in increased expression of \textit{GAPI}, \textit{GDH1} and \textit{PUT1} (36-38). Finally, it has been demonstrated that the cross-talk between GATA factors and glucose limitation is executed through the phosphorylation of Gln3p by Snf1p - the primary mediator of glucose repression in yeast. Given this, it is possible that Yak1p, a glucose responsive kinase, may phosphorylate Put3p in response limitation of other nutrients.

The positive identification of the kinase responsible for Y788 phosphorylation, and the further determination of its interactions with Gat1p will allow dissection of the mechanism by which the NCR pathway interacts with a metabolite-specific pathway to increase the adaptability of the cell.

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FIGURE LEGENDS

Fig. 1. Detection of the phosphorylation state of Put3p. a, Put3p purified from insect and S. cerevisiae cells. Protein samples were incubated at 37°C for 1 hr with either CIP or buffer. The difference in the apparent molecular weight of the two protein preparations is due to the size of the tags used, His (4.3 kDa) or MORF (19 kDa). The asterisk indicates the location of the lower mobility phosphorylated version of Put3p. b, Identified phosphopeptides from the proteolytic digestion of Put3p purified from yeast. The phosphorylated amino acid is each peptide is highlighted in red. Peptides were generated using either trypsin (cleavage sites indicated by the backslash), chymotrypsin or AspN, or a combination of these proteases. c, A schematic representation of Put3p illustrating the location of phosphorylation sites and sub-domains.

Fig. 2. Effects of N-terminal phosphorylation sites on PUT1: lacZ activation. Relative β-galactosidase activity of put3Δ, PUT1-lacZ cells carrying pRS415, a pRS415 plasmid expressing wild-type Put3p, or a version of Put3p bearing the annotated mutation(s). Cells were grown in media containing glutamate to an A600 ≈ 0.6 and treated with either rapamycin (grey bars) or the drug vehicle (open bars) for 1 hr. Values represent the mean of three separate cultures subjected the same treatment and error bars indicate standard deviation.

Fig. 3. Effects of C-terminal phosphorylation sites on PUT1: lacZ activation. a, Relative β-galactosidase activity of C-terminal phosphorylation incompetent mutants. b, Relative β-galactosidase activity of C-terminal mock-phosphorylation mutants. put3Δ, PUT1-lacZ cells carrying pRS415 (39), the pRS415 plasmid expressing wild-type Put3p, or a version of Put3p bearing the annotated mutation(s) were grown in media containing glutamate to an A600 ≈ 0.6 and treated with either rapamycin (grey bars) or the drug vehicle (open bars) for 1 hr. Values represent the mean of three separate cultures subjected the same treatment and error bars indicate standard deviation.

Fig. 4. Relative β-galactosidase activity of Put3p.Y788F grown in either glutamine or proline +/- rapamycin. put3Δ cells carrying either pRS415 (open bars), the pRS415 plasmid expressing wild-type Put3p (grey bars), or a version of Put3p bearing Y788F mutation (black bars) were grown in media containing either glutamate or proline to an A600 ≈ 0.6 and treated with either rapamycin or the drug vehicle for 1 hr. Values represent the mean of three separate cultures subjected the same treatment and error bars indicate standard deviation.

Fig. 5. Relative β-galactosidase activity of Put3p.Y788F in yeast cells GATA factor delete backgrounds. a, put3Δ, PUT1-lacZ and put3Δ, ure2Δ, PUT1-lacZ cells carrying either pRS415, the pRS415 plasmid expressing wild-type Put3p, or a version of Put3p bearing Y788F mutation in the presence and absence of rapamycin. b, put3Δ, PUT1-lacZ and put3Δ, gat1Δ, PUT1-lacZ cells carrying either pRS415, the pRS415 plasmid expressing wild-type Put3p, or a version of Put3p bearing Y788F mutation in the presence and absence of rapamycin. c, put3Δ, PUT1-lacZ and put3Δ, gln3Δ PUT1-lacZ cells carrying
either pRS415, the pRS415 plasmid expressing wild-type Put3p, or a version of Put3p bearing Y788F mutation in the presence and absence of rapamycin. Cells were grown in media containing glutamine to an $A_{600} \approx 0.6$ and treated with either rapamycin (grey bars) or the drug vehicle (open bars) for 1 hr. Values represent the mean of three separate cultures subjected the same treatment and error bars indicate standard deviation.

Fig. 6. The interaction of Gat1p and Gln3p with the PUT1 promoter in the presence of wild-type and Y788F versions of Put3p. a, Cells expressing Rpb3p-cAVI were treated with rapamycin and ChIP-qPCR was performed for either the PUT1 start-site (-98 to +52) or the PUT1 upstream region (-303 to -178, as indicated). ChIP-qPCR data is expressed as IP:IN ratio of PUT1 to HMR. Data was normalised to the wild-type rapamycin control. Error bars represent standard deviations for at least 3 biological replicates. An * indicated a $p$ value of less than 0.05 in comparison to the wild-type control. b, As in a, using cells expressing Gat1p-cAVI and either the wild-type or Y788F versions of Put3p were treated with rapamycin and ChIP-qPCR was performed for the PUT1 upstream region (-303 to -178). c, As in b, using cells expressing Gln3p-cAVI.
(a) Leverentz et al., Fig. 1

| Insect cells | S. cerevisiae | Phosphatase |
|--------------|--------------|-------------|
| -            | -            | -           |
| +            | +            | *           |

(b) Site Peptide
S103: K/ALLENGPVSVR/
S129/130: DTLVSSAPAAPIF
S164: R/SYDHSLEK/
Y788: K/YNDILK/
S882: DIVNVNNSSEPSTF
S969: DPTAAAGSETDFT

(c) Leverentz et al., Fig. 1
Leverentz et al., Fig. 2
Leverentz et al., Fig. 3
Leverentz et al., Fig. 4
Leverentz et al., Fig. 6
Mutation of a phosphorylatable residue in Put3p affects the magnitude of rapamycin-induced PUT1 activation in a Gat1p-dependent manner
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