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Urmylation controls Nil1p and Gln3p-dependent expression of nitrogen-catabolite repressed genes in *Saccharomyces cerevisiae*

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Abstract **Urm1 is a modifier protein that is conjugated to substrate proteins through thioester formation with the E1-like enzyme, Uba4. Here is shown that the lack of urmylation causes derepression of the *GAP1* gene (encoding a nitrogen-regulated broad-spectrum amino acid-scavenging permease) in the presence of rich nitrogen sources, and simultaneous inhibition of the expression of *CIT2*, a TCA-cycle gene involved in the production of glutamate and glutamine. This effect is dependent on the TORC1- and nutrient-regulated transcriptional factors, Nil1p and Gln3p. Evidence is provided that, in the absence of urmylation, nuclear/cytosolic shuffling of both transcriptional factors is altered, ultimately leading to inability to repress *GAP1* gene in the presence of a rich nitrogen source. Altogether, the data presented here indicate an important role of the urmylation pathway in regulating the expression of genes involved in sensing and controlling amino acids levels.**

Keywords: Urmylation; *GAP1*; *CIT2*; *GLN3*; *NIL1*

1. Introduction

Sensing of the amount and quality of nutrients available in the environment is crucial for cell survival and has been found to be largely controlled in eukaryotic cells by the molecular target of rapamycin, TOR (latest reviewed in [1,2]). Rapamycin is an antitumour and immunosuppressant macrocyclic lactone that has the ability to bind the peptidylisoprolyl isomerase FK506-binding protein (FKBP12) encoded in yeast by *FPR1* [3,4]. When bound to this protein, rapamycin causes inactivation of TOR proteins which in turn triggers a series of events within cells that closely mimic those elicited in preparation for survival and adaptation to different types of stress and nutrient starvation [1,2]. Yeasts have two separate TOR genes, *TOR1* and *TOR2*, but only *TOR2* is found to be essential [5]. Tor proteins belong to a group of kinases known as the phosphatidylinositol kinase-related kinase (PIKK) family, characterized by the presence of a carboxy-terminal serine/threonine protein kinase domain that resembles the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks) and PI4Ks [2]. In yeast, Tor proteins control different aspects of cell growth by forming part of two distinct, although partially redundant, protein complexes (TORC1 and TORC2; [1,2]). TORC1 promotes cell growth by positively regulating protein synthesis and other anabolic processes, and by negatively regulating mechanisms of stress-response and catabolic processes, such as autophagy and the expression of genes involved in the utilization of lower-quality nutrients. TORC2 seem to primarily regulate spatial aspects of cell growth such as actin/cytoskeleton organization and cell polarity [1,2]. Although sharing several components, one of the most striking differences between these two complexes is that TORC1 activity is sensitive to rapamycin whereas TORC2 is not [6].

New components of the TOR-dependent regulatory pathways are continuously being identified. In particular, recent studies have suggested a potential link between the yet poorly understood role of a newly identified ubiquitin (Ub)-like conjugation system, urmylation, and the TOR pathways [7]. URM1 (URM, for Ub-related modifier) is one of the five different Ub-like modifier proteins (Ubls) until date identified in yeast [8]. Urmylation has a unique resemblance to ATP-dependent cofactor sulfuration reactions involved in the prokaryotic synthesis of thiamin and molibdopterin, which has provided an important clue for the origin of ATP-dependent protein conjugation systems [9]. Although the nature of the putative targets for this type of regulation remains obscure, *URM1* must have an important role in eukaryotic cells since this gene has been evolutionarily conserved from yeast to mammalians, including humans [9,10]. Only one substrate for urmylation has until now been identified in yeast: Ahp1, a protein with a role in oxidative-stress response [11]. This finding potentially links urmylation to the control of stress responses. Moreover, additional work from Sprague Jr. and collaborators has shown that defects in urmylation lead to rapamycin sensitivity that can be rescued by the rapamycin-resistant allele *TOR2-1*, and has also reported interactions between this protein-modification system and invasive/pseudohyphal growth, which is usually elicited by nutrient starvation conditions such as nitrogen limitation [7].

The quality and quantity of nitrogen sources are carefully assessed by yeast cells through monitoring of the relative levels of intracellular glutamine (Gln) and glutamate (Glu) [12,13]. Gln and Glu are central precursors for the synthesis of tricarboxylic acids (TCA) cycle intermediates, their relative levels playing a key role in the generation of energy as well as in anabolic reactions, such as the biosynthesis of amino acids and other nitrogen-containing metabolites [12]. Hence, Gln and Glu are the preferred nitrogen environmental sources for cells. External absence of these two nitrogen sources and/or reduced levels of amino acids in the environment trigger degradation of most of the highly-specific amino acid permeases, and promote the synthesis and sorting to the plasma membrane of lower-quality nitrogen-scavenging permeases such as the general

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amino acid permease, Gap1p, a broad-spectrum amino acid permease able to internalize all naturally occurring amino acids [14–18]. Lower-quality nitrogen sources internalized by Gap1p are then used to synthesize Glu and Gln.

Genes directly and indirectly involved in the synthesis of Glu/Gln are tightly regulated transcriptionally and post-transcriptionally. In a simple picture, enzymatic activities acting in the earlier steps of the TCA cycle are mainly responsible for the generation of glutamate and glutamine. *CIT2* encodes a citrate synthase involved in the first catalytic step for the generation of α-ketoglutarate from acetyl-CoA [12]. *GDH1* encodes a $NADP^+$-linked glutamate dehydrogenase that uses ammonia derived from the internalized nitrogen sources to convert α-ketoglutarate into glutamate, and *GLN1* encodes a glutamine synthetase, which also uses ammonia to further convert glutamate into glutamine [12]. *GAP1* is positively coregulated with *GDH1* and *GLN1* to couple the internalization of low-quality nitrogen sources through Gap1p with their use in the production of Glu and Gln. Increasing levels of Glu and Gln ultimately lead to downregulation of the expression of these genes and activity of their products through a negative feedback loop [12,13,19].

Genes involved in the earliest steps of the TCA cycle, such as *CIT2*, are positively regulated by the Rtg 1/3 transcription factors, and negatively regulated by Mks 1p (in combination with Bmh1, and Bmh2), and the TOR-associated protein Lst8, in response to the intracellular levels of Glu/Gln (retrograde (RTG) pathway; reviewed in [20]). The transcription of *GAP1*, *GDH1*, and *GLN1*, is controlled by the two GATA transcriptional factors, Nil1p and Gln3p [19,21–23]. Gln3p is repressed on glutamine or ammonia medium, whereas Nil1p is repressed on glutamate or glutamine medium [12]. Gln3p-dependent transcription is repressed by a physical interaction with the negative regulator Ure2p, which sequesters Gln3p in the cytosol when glutamine is present [24,25]. A similar regulation exerted on Nil1p has not yet been demonstrated. Recent evidence has shown that although the sets of genes responsive to Nil1p and Gln3p regulation overlap, the activation of Nil1p is mainly aimed at the generation of energy via production of TCA cycle intermediates from the precursor glutamate, whereas the activation of Gln3p is involved in the collection of alternative nitrogen sources to synthesize glutamine [26]. Although the main role of Nil1p seems to be the maintenance of proper levels of glutamate in the presence of poor carbon sources, the upregulation of certain genes regulated by Nil1p triggered by an intermediate-quality nitrogen source such as ammonia, also favor the production of glutamine from glutamate.

TORC1 is inactivated in the presence of rapamycin because glutamine production, which posits the signal for nitrogen excess, is inhibited so that such signal no longer reaches Tor [23]. Inactivation of TORC1 by rapamycin or in response to nutrient starvation causes Gln3p dissociation from Ure2p, which in turn allows Gln3p entry to the nucleus, where it activates the expression of nitrogen-catabolite repressed genes [27]. Interestingly, the lack of Ure2p alters the pattern of urmylated substrates in a Gln3p-dependent manner, suggesting that urmylation may be used as an additional modification system to regulate proteins related to the Gln3p–Ure2p pathway [7]. In the present work, evidence is presented that urmylation affects nuclear-cytosolic localization of the transcriptional factors Nil1p and Gln3p, thereby constituting an additional level of regulation in the expression of genes involved in the synthesis of Glu and Gln. This finding provides further evidence showing that urmylation has an important role in the control of nutrient sensing in TOR-related pathways.

2. Materials and methods

2.1. Strains, plasmids, and media

All the yeast strains used in this study (listed in Table 1) are in the S288C background, which displays high Gap1p expression and activity when ammonia is the nitrogen source [28]. All complete gene deletions described here were obtained by replacement of the functional ORF of the corresponding gene by homologous recombination with either a *kanMX4/6* or a *natMX4* cassette [29,30] in the wild type strain CKY835 [18] or in strains derived from the same genetic background.

Plasmids used in this study are pMS29, a P_{GAP1}-*LacZ* fusion at codon 53 of *GAP1* in the *URA3-CEN* vector pBL101 [31]; pEC261, a P_{CIT2}-*LacZ* fusion after codon 3 of *CIT2* in pRS316 [32]; and pMRT14, which contains the constitutive promoter P_{ADH1} followed by 3×FLAG sequence, *URM1* ORF fused in frame to 3×FLAG, and *kanMX6* marker cloned in *Xba*I/*Eco*RI sites present in the multi cloning site of pRS316.

Minimal medium was prepared using yeast nitrogen base without amino acids and without ammonium sulfate (Difco, Detroit, MI), 2% glucose, and a nitrogen source: 0.1% glutamine, 0.1% glutamate, 0.5% ammonium sulfate, or urea 0.2%. All growth experiments were carried out at 24 °C except when indicated. For the rapamycin treatment, YPD standard plates were prepared containing 10 or 50 nM of rapamycin (Sigma-Aldrich, St. Louis, MO).

Table 1
Yeast strains

Strain	Genotype	Reference
CKY835	MATα *ura3-52*	[18]
MRTY305	MATα *urm1*Δ::*kanMX4 ura3-52*	This study
MRTY306	MATα *uba4*Δ::*kanMX4 ura3-52*	This study
CKY834	MATα *GAP1-GFP::kanMX6*	[18]
MRTY382	MATα *urm1*Δ::*kanMX4 GAP1-GFP::kanMX6 ura3-52*	This study
MRTY383	MATα *uba4*Δ::*kanMX4 GAP1-GFP::kanMX6 ura3-52*	This study
CKY777	MATα *ure2*Δ::*kanMX6 ura3-52*	[32]
CKY778	MATα *gln3*Δ::*kanMX6*	[32]
CKY888	MATα *nil1*Δ::*kanMX6 ura3-52*	C.A. Kaiser
MRTY378	MATα *urm1*Δ::*kanMX4 nil1*Δ::*kanMX6 ura3-52*	This study
MRTY380	MATα *urm1*Δ::*kanMX4 ure2*Δ::*kanMX6 ura3-52*	This study
MRTY384	MATα *urm1*Δ::*kanMX4 gln3*Δ::*kanMX6 ura3-52*	This study
MRTY404	MATα *NIL1-HA*::*kanMX6 ura3-52*	This study
MRTY405	MATα *GLN3-myc::kanMX6 ura3-52*	This study
MRTY406	MATα *urm1*Δ::*kanMX4 NIL1-HA*::*kanMX6 ura3-52*	This study
MRTY407	MATα *urm1*Δ::*kanMX4 GLN3-myc::kanMX6 ura3-52*	This study