Mutations Dissociate Responses to Nitrogen Limitation (Nitrogen Catabolite Repression) and Rapamycin Inhibition of TorC1

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*Running title: TorC1- and NCR-dependent regulation of Gln3

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Background: Gln3 localization is hypothesized to be co-regulated by TorC1 and nitrogen limitation.

Results: gln3 mutations abrogating Gln3-Tor1 interaction abolish the Gln3 response to rapamycin without adversely affecting its response to nitrogen limitation.

Conclusions: Different Gln3 regions mediate responses to rapamycin and nitrogen limitation.

Significance: Controlled Gln3 localization occurs via two separable regulatory pathways both of which are required for overall W.T. Gln3 control.

ABSTRACT

The GATA-family transcription activator, Gln3, responds to the cell’s nitrogen requirements and environmental resources. When rapidly utilized, “good” nitrogen sources, e.g., glutamine, are plentiful, Gln3 is completely sequestered in the cytoplasm and the transcription it mediates is minimal. In contrast, during nitrogen-limiting conditions, Gln3 quickly relocates to the nucleus and activates transcription of genes required to scavenge alternative, “poor” nitrogen sources, e.g. proline. This physiological response has been designated nitrogen catabolite repression (NCR). Since rapamycin treatment also elicits nuclear Gln3 localization, TorC1 has been thought to be responsible for NCR-sensitive Gln3 regulation. However, accumulating evidence now suggests that GATA factor regulation may occur by two separate pathways, one TorC1-dependent and the other NCR-sensitive. Therefore, the present experiments were initiated to identify Gln3 amino acid substitutions capable of dissecting the individual contributions of these pathways to overall Gln3 regulation. The rationale was that different regulatory pathways might be expected to operate through distinct Gln3 sensor residues. We found that C-terminal truncations and amino acid substitutions in a 17 amino acid Gln3 peptide with a predicted propensity to fold into an α-helix partially abolished the cell’s ability to sequester Gln3 in the cytoplasm of glutamine-grown cells and eliminated the rapamycin response of Gln3 localization, but did not adversely affect its response to limiting nitrogen. However, overall wild type control of intracellular Gln3 localization requires the contributions of both individual regulatory systems. We also found that Gln3 possesses at least one Tor1-interacting site in addition to the one previously reported.

INTRODUCTION

The nitrogen catabolite repression- (NCR-) sensitive GATA transcription activator, Gln3, is one of the most often used reporters of TorC1 signaling activity in Saccharomyces cerevisiae. As such, its regulation has been investigated in substantial detail. The intracellular localization of Gln3 and another GATA-family transcription activator, Gat1, respond to both nitrogen limitation (NCR) and rapamycin treatment (Tor1-mediated regulation). The central question addressed in this work is whether NCR and rapamycin inhibitable, Tor1-mediated activity represent sequential steps of a single regulatory pathway or two independent regulatory mechanisms that work in concert to control the GATA family transcription factors.

When cells are cultured under nitrogen rich conditions, Gln3 and Gat1 are sequestered in the cytoplasm and transcription of the genes required to scavenge poor nitrogen sources is highly repressed (1-5). Ure2, a prion protein, is required for this cytoplasmic sequestration and repressed transcription (6). The additional observation that Ure2 forms a complex with Gln3 in nitrogen rich conditions led to the conclusion that formation
problem is further exacerbated by the lability and complexity of the Gln3 molecule itself (20% of its residues are serine or threonine) which have largely frustrated direct mass-spectroscopic analyses of its phosphorylation (32-35).

Given the conceptual difficulty of cleanly separating primary and secondary responses of GATA factor localization to upstream perturbations, as well as the technical problems involved in biochemical approaches, we elected to take a systematic genetic approach to the question of Gln3 regulation. The rationale is predicated on the reasoning that if Gln3 localization and function involve multiple regulatory pathways, the individual pathways might possess distinct mutable and hence potentially identifiable targets within the Gln3 molecule itself. In the present work we report serine substitutions that diminish the ability of nitrogen replete growth conditions to sequester Gln3 in the cytoplasm. These substitutions completely abolish Gln3’s response to rapamycin, but leave the NCR-sensitive response to limiting nitrogen untouched. We also show that Gln3 possesses at least one additional Tor1-interaction site beyond the one originally described.

MATERIALS AND METHODS

Strains and Culture Conditions – The Saccharomyces cerevisiae strain used as the transformation recipient in which gln3 mutant plasmids were assayed was JK9-3da (Table 1). The protein interaction assays were performed in transformants of PJ69-4a (Table 1). Growth conditions were identical to those described in Tate et al. (28). Cultures (50 ml) were grown to mid-log phase (A\textsubscript{600}nm = 0.5) in YNB (without amino acids or ammonia) minimal medium containing the indicated nitrogen source at a final concentration of 0.1%. Appropriate supplements (120 μg/ml leucine, 20 μg/ml histidine and tryptophan) were added to the medium as necessary to cover auxotrophic requirements. Where indicated, cells were treated with 200 ng/ml rapamycin for 20 mins. or 2 mM Msx for 30 mins. as described earlier.

Plasmid Construction and Protein-Protein Interaction Assays – gln3 deletion and amino acid substitution mutants were constructed using standard PCR based methods and the primer sets in Table 2. The Myc\textsuperscript{13} and ADH1 transcriptional terminator were derived from pKA62 (36). The template for all of the constructions was pRR536 which contained the wild type GLN3 gene, including its native promoter, fused in frame with Myc\textsuperscript{13} at the GLN3 translational stop codon.

PCR fragments using pRR536, pRR614 and pRR850 as template were generated and cloned into interaction vectors pACT-2 (Clontech Inc.) to yield GAL4p(AD)-gln3 fusion plasmids, pRR1065, pRR1067 and pRR1069, respectively. Two plasmids (pRR1098 and
ppRR1101), containing the 17 amino acid sequence the of Gln3_{654-670} peptide, were constructed by cloning synthetic double stranded oligonucleotides into the pACT-2 vector. All of the constructs were confirmed by DNA sequencing (University of Tennessee Health Science Center Molecular Resource Center DNA sequencing facility).

**GAL4-BD pASTOR(1-2470) and pASTOR1 (1-1760)** were generously provided by Professor Stephen Zheng (12). Positive control plasmids pTD1-1 (SV40 Large T antigen) and pLAM5 (human laminC) were obtained from Clonetech. The Gal4p-BD and AD fusion proteins were expressed together in yeast strain PJ69-4a. This strain was constructed in the W303 genetic background. We used it for our Tor1 interaction assays rather than a JK9-3da-derived strain so that Tor1-association data obtained in the present work could be directly compared with that from Carvalho et al. (12).

The protein-protein interaction assays we used were those generously provided by Carvalho et al. and measured growth of PJ69-4a transformants on synthetic complete medium (SC) lacking leucine, tryptophan, and adenine as the positive control or synthetic complete medium lacking leucine, tryptophan, and histidine plus 3 mM (final concentration) 3-amino-1,2,4-triazole (3-AT) to inhibit growth of PJ69-4a.

Cells were classified into one of three categories: cytoplasmic (cytoplasmic Gln3-Myc fluorescence only; red bars), nuclear-cytoplastic (Gln3-Myc fluorescence appearing in the cytoplasm as well as co-localizing with DAPI-positive material; yellow bars), and nuclear (Gln3-Myc fluorescence co-localizing only with DAPI positive material; green bars). A representative collection of “standard” images demonstrating the differences in these categories is shown in Fig. 2 of ref. 28 along with a description of how the criteria were applied. Following recent recommendations (38) we assessed the precision of our scoring by analyzing the data from 10 different experiments performed over a nine month period; all appear in this work. The average values and standard deviations observed for the five conditions we assayed appear in Fig. 1.

**RESULTS**

**Use of Gln3 Structural Mutants to Dissect Gln3 Regulation** – To investigate the individual contributions of TorC1 and NCR to the regulation of Gln3 localization, we decided to use the Gln3 molecule itself not only as a reporter but also as a more direct probe to separate these regulatory pathways. However, the intractability of Gln3 to mass spectral analysis of its phosphorylation (32-35), prompted an alternative genetic approach to pursue this objective. The magnitude of the overall task – Gln3 contains 146 potentially phosphorylated serine/threonine residues – made it untenable to study the entire molecule.
at once. Therefore, we have first focused on its C-terminal region. This is the region reported to be associated with Tor1 and the one about which the least is known. It also possesses the additional asset of being distanced from known Gln3 basic functional domains, i.e., nuclear import and export, DNA binding, transcriptional activation and Ure2 interaction, situated in the N-terminal portion of the protein (8, 12, 19).

Before such genetic analyses could be confidently pursued, three important technical issues required evaluation. Taking full advantage of Gln3 amino acid substitution mutant proteins now and in the future required the capability of expeditiously introducing them into multiple wild type, TorC1 pathway and other mutant strains. This goal is most efficiently accomplished using Cent-plasmid-borne constructs. Even though Cent-based expression systems are relatively stable and the Myc\(^\text{13}\) tag has been repeatedly shown not to adversely affect Gln3 function, two pertinent questions remained: (i) would plasmid-borne mutants in which Gln3 was expressed from its own promoter, yield sufficiently reproducible data to be useful? (ii) Would the results obtained be the same as when GLN3 was situated in its native chromosomal location? To address these questions, we constructed a Cent-plasmid-borne wild type GLN3\(1\_730\) gene driven by its native promoter (pRR536) and surveyed the range of conditions that regulate Gln3 localization: (1) highly repressive, nitrogen replete conditions (glutamine-grown) where Gln3\(1\_730\)-Myc\(^\text{13}\) is completely cytoplasmic, (2) derepressive, nitrogen-limited conditions (proline-grown) where it is completely nuclear and (3) following rapamycin treatment in nitrogen-rich media where it is localized to both cellular compartments.

First, we assessed our assay’s reproducibility. Data depicted in Fig. 1A represent the averaged results of ten experiments performed over a nine month period, measuring Gln3\(1\_730\)-Myc\(^\text{13}\) localization in wild type JK9-3da transformed with wild type Gln3\(1\_730\)-Myc\(^\text{13}\) pRR536. The maximum standard deviation observed with these data was +/- ~7-8%. It is important to note that the most challenging conditions to assay are those in which Gln3\(1\_730\)-Myc\(^\text{13}\) localizes to all three scoring categories, i.e., cytoplasmic, nuclear-cytoplasmic and nuclear. These data supported the contention that our plasmid-based assays exhibited sufficient precision to be useful.

Second, we queried whether the plasmid-borne Gln3\(1\_730\)-Myc\(^\text{13}\) reporter (pRR536) yielded results similar to those obtained with a strain in which Gln3-Myc\(^\text{13}\) was situated at its native chromosomal position (TB123). Fig. 1B depicts data from four experiments performed over three years with TB123. Note that TB123 was derived from and is nearly isogenic to JK9-3da (Table 1). The only difference noted in data obtained by the two methods was a small but consistent nuclear shift of plasmid-derived Gln3\(1\_730\)-Myc\(^\text{13}\) compared to that from chromosomal Gln3\(1\_730\)-Myc\(^\text{13}\) (Fig. 1A, pRR536 and Fig. 1B, TB123).

The final technical issue was the nature of the transformation recipient. Two choices existed, wild type cells containing an unaltered chromosomal GLN3 allele in addition to the plasmid borne gln3-Myc\(^\text{13}\) mutant allele or a gln3\(\Delta\)A in which the plasmid-borne gln3-Myc\(^\text{13}\) mutant allele was the only one present in the cell. From extensive previous experience, we were well aware that diminishment or loss of Gln3 activity has far reaching secondary effects on many cellular processes. Moreover, there is no evidence that we know of demonstrating that regulation of Gln3 function is exerted through the amount of Gln3 produced unless it is ectopically over-produced at very high levels (39; Cooper et al., unpublished observations). This derives from the fact that Ure2, the titratable negative regulator of Gln3 and Gat1 (9), is present in the cell at much higher concentrations than Gln3. Given this information, we decided it was most prudent to use a wild type GLN3 strain (JK9-3da) as the transformation recipient, thereby divorcing potential secondary effects caused by Gln3 deficiency in some mutants but not others from primary effects on its localization. Comparison of data in Fig. 1A and B support the validity of this decision. Together, the results demonstrated that we could confidently make meaningful measurements of Gln3-Myc\(^\text{13}\) localization in this new genetic context.

**Analysis of the Gln3 Region Reported To Bind Tor1** – In well performed experiments, Carvalho et al. reported that Gln3\(510\_730\)-Myc\(^{9}\) and Gln3\(600\_730\)-Myc\(^{9}\) peptides were sufficient to support association with Tor1 in a two-hybrid interaction assay, whereas a Gln3\(667\_730\)-Myc\(^{9}\) peptide was not (8, 12). Micrographic data positively correlated with the 2-hybrid results (12). Together these data led to the conclusion that Tor1 kinase interacted with the Gln3\(600\_667\)-Myc\(^{9}\) region and was responsible for nitrogen-responsive Gln3 regulation. However, we noted that the reported localization results were qualitative being derived from overall evaluation of isolated micographs in which Gln3-Myc\(^{9}\) was concluded to be either nuclear or cytoplasmic. Such qualitative evaluation would have limited or abrogated any ability to detect intermediate changes in Gln3-Myc\(^{9}\) localization if they occurred. These limitations prompted us to reevaluate the extent to which Tor1 could account for the totality of nitrogen-responsive Gln3 regulation. If inactivation of Tor1, either as a result of nitrogen limitation or rapamycin-mediated inhibition, was the sole determinant responsible for nitrogen-responsive Gln3 regulation, then elimination of or specific substitutions in Gln3 residues 600-667 should result in highly nuclear Gln3 localization similar to levels that we and others have reported in...
were the same as that seen with full length Gln3 truncations in the presence or absence of rapamycin occurred (Fig. 2A and 2B). In other words, these truncations of Gln3 were viewed from the opposite perspective. The localizations of Gln3 were insufficient to relocate Gln3-Myc quantitatively. The localizations of Gln3 were insufficient to relocate Gln3-Myc quantitatively. We initially tested the above predictions rather crudely by sequentially truncating the C-terminus of Gln3. We included in our analyses a construct, Gln3, analogous to one previously characterized by Carvalho et al.; we considered it to be a negative control (12). The data we obtained were both intriguing and provocative. From Carvalho et al.’s data, we expected Gln3 to become exclusively nuclear in wild type cells containing the truncated, glutamine-grown negative control (Gln3-Myc). This didn’t occur. Although Gln3-Myc was more nuclear than wild type Gln3, this supporting Carvalho et al.’s qualitative observation, a substantial amount of it, roughly 2/3 to 3/4 remained either cytoplasmic or nuclear-cytoplasmic when glutamine was provided as sole nitrogen source (Fig. 2A and 2B). Even when Gln3 was more drastically truncated to residues 584 (pRR622), 565 (pRR621) and 542 (pRR613), no further nuclear Gln3-Myc localization occurred (Fig. 2A and 2B). In other words, these truncations were insufficient to relocate Gln3-Myc completely into the nucleus, thus arguing that at least two regulatory mechanisms were required to account for total control of Gln3 localization. This conclusion was further supported when the data were viewed from the opposite perspective. The localizations of Gln3-Myc were much less cytoplasmic than observed for glutamine-grown, full length Gln3-Myc (Fig. 2A and 2B). This indicated that although Gln3-Myc material contributed to sequestering Gln3-Myc in the cytoplasm, these residues and their reported Tor1 interaction could account for only a portion of the cytoplasmic Gln3-Myc sequestration observed with Gln3-Myc.

We observed a second, equally important characteristic of the Gln3-Myc, Gln3-Myc and Gln3-Myc truncations. Treating them with rapamycin failed to further increase their nuclear localization. In all cases, the ratio of cytoplasmic Gln3-Myc in the presence vs. absence of rapamycin decreased from 3:1 in wild type to 1:1 in all of the mutants (Fig. 2C). We also noted that the overall intracellular distributions of the Gln3-Myc, Gln3-Myc and Gln3-Myc truncations in the presence or absence of rapamycin were the same as that seen with full length Gln3-Myc in wild type glutamine-grown, rapamycin-treated cells (Fig. 2B).

From both perspectives, the above observations suggested that high level cytoplasmic sequestration of wild type Gln3-Myc likely derived from two regulatory mechanisms. One depended on Gln3 residues 600-730, which were also responsible for the rapamycin-responsiveness of Gln3-Myc localization. The second mechanism was not rapamycin-responsive and remained unaffected even when Gln3 was truncated by as much as ~25%.

We hypothesized that if this second mechanism derived from NCR, then the cytoplasmic Gln3-Myc sequestration observed with the truncated forms of Gln3-Myc in Fig. 2 should continue to be nitrogen-responsive, i.e., when cells were provided with a derepressive nitrogen source (proline), the cytoplasmic and nuclear-cytoplasmic Gln3-Myc should relocate into the nucleus. We tested this expectation by comparing localization of the truncated versions of Gln3-Myc in repressive vs. derepressive conditions, i.e., glutamine vs. proline-grown cells (Fig. 3A and 3B). All four Gln3 truncations exhibited wild type NCR-sensitive responses. Derepressive growth conditions, using proline as sole nitrogen source, resulted in nearly complete nuclear Gln3-Myc, Gln3-Myc, Gln3-Myc and Gln3-Myc localizations. These observations supported the existence of a second regulatory mechanism and additionally demonstrated that the Gln3 target for it was N-terminal of Gln3-Myc.

**Identification of Gln3 Residues Required for the Rapamycin Response** – Although the above truncations dissected rapamycin-inhibitable, Tor1 association-dependent responses of Gln3-Myc localization away from responses that were NCR-sensitive, they represented rather blunt analytical probes. Removing up to ¼ of the Gln3 protein (130-188 residues) would not permit us to rigorously conclude that both diminished cytoplasmic sequestration of Gln3-Myc in nitrogen-replete conditions and the loss of rapamycin-responsiveness derived solely from the loss of a Tor1-association site; much higher resolution was necessary.

Using systematic amino acid substitutions, we achieved the desired degree of resolution and addressed whether substituting non-phosphorylatable or phosphomimetic alanine or aspartate residues, respectively, for serine/threonine residues in the C-terminal region of Gln3 affected its localization or the ability of TorC1 and/or rapamycin treatment to influence it. We sequentially replaced small groups of serine/threonine residues in the Gln3 region that Carvalho et al. reported to contain a Tor1-binding domain (Fig. 4A) (12). All of our substitutions were situated in full length Gln3 proteins whose production was regulated by the native GLN3 promoter. We reasoned that: (i) substituting phosphomimetic aspartate for serine/threonine residues directly participating in TorC1 control of Gln3 would
elicit constitutively cytoplasmic Gln3-Myc\textsuperscript{13} localization, (ii) substituting non-phosphorylatable alanine residues would shift Gln3-Myc\textsuperscript{13} constitutively into the nucleus, and (iii) in instances where Gln3 structure per se, rather than specific phosphorylation, was altered by the substitutions, the aspartate and alanine substitutions would generate similar results.

The first cluster of substitutions, Gln3\textsubscript{S603D, S605D, S607D, S609D-Myc}\textsuperscript{13} (pRR1014) had little demonstrable effect on Gln3-Myc\textsuperscript{13} localization either in the presence or absence of rapamycin (Fig. 4B and 4C, compare wild type Gln3\textsubscript{1-730-Myc}\textsuperscript{13} (pRR536) with Gln3\textsubscript{S603D, S605D, S607D, S609D-Myc}\textsuperscript{13} (pRR1014), Gln vs. Gln +Rap). Further, the NCR-sensitive response was similarly unaffected (Fig. 4B and 4C, compare wild type and mutant responses to glutamine (Gln) vs. proline (Pro)). We next assayed Gln3 localization in Gln3\textsubscript{S616D, S617D, S619D, S621D, S624D, S631D-Myc}\textsuperscript{13} (pRR922) and Gln3\textsubscript{S617A, S619A, S621A, S624A, S631A-Myc}\textsuperscript{13} (pRR960) (Fig. 5). Again, there was virtually no change from the wild type phenotypes (Fig. 5). Moving further towards the C-terminus, we then assayed Gln3\textsubscript{T641D, S645D, S647D, S649D, S652D-Myc}\textsuperscript{13} (pRR928) and Gln3\textsubscript{T641A, S645A, S647A, S649A, S652A-Myc}\textsuperscript{13} (pRR966) and yet again – there was no change from the wild type phenotype (Fig. 6). This was remarkable. Substituting aspartate for 25% or 16 of 53 residues, four to six at a time, and thereby substantially increasing the region's acidity yielded no demonstrable phenotype.

Finally, we assayed Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) and Gln3\textsubscript{S656A, S659A, S662A-Myc}\textsuperscript{13} (pRR1038) (Fig. 7). The response was crystal clear; the phenotypes of both the aspartate (Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) and alanine (Gln3\textsubscript{S656A, S659A, S662A-Myc}\textsuperscript{13} (pRR1038) substitutions were indistinguishable. They mirrored the phenotype of the original Gln3\textsubscript{1-600-Myc}\textsuperscript{13} truncation (Compare Figs. 2 and 3 with Fig. 7). Cytoplasmic Gln3-Myc\textsuperscript{13} localization decreased from over 90% in the wild type Gln3\textsubscript{1-730-Myc}\textsuperscript{13} pRR536 to 30-40% in the mutants. Rapamycin treatment was without effect, yet the NCR response was wild type in Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) and Gln3\textsubscript{S656A, S659A, S662A-Myc}\textsuperscript{13} (pRR1038), i.e., Gln3-Myc\textsuperscript{13} shifted completely into the nuclei of proline-grown cells (Fig. 7).

In sum, only the very end of Gln3\textsubscript{600-667} was required and, within the resolution we could measure, accounted for all of the phenotypic characteristics observed in our Gln3\textsubscript{1-600-Myc}\textsuperscript{13}, Gln3\textsubscript{1-581-Myc}\textsuperscript{13}, Gln3\textsubscript{1-565-Myc}\textsuperscript{13} and Gln3\textsubscript{1-542-Myc}\textsuperscript{13} truncations as well as those reported by Carvalho \textit{et al.} (Gln3\textsubscript{1-600-Myc}\textsuperscript{8}, Gln3\textsubscript{510-730-Myc}\textsuperscript{9} and Gln3\textsubscript{600-667-Myc}\textsuperscript{9}) (12). The fact that Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) and Gln3\textsubscript{S656A, S659A, S662A-Myc}\textsuperscript{13} (pRR1038) exhibited the same intracellular distribution profiles indicated that the effects most likely derived from an alteration of the Gln3\textsubscript{S656-662} structure itself rather than from specific phosphorylation or dephosphorylation of the substituted residues acting as the direct determinant of Gln3 localization. It was additionally significant that one could dramatically increase the acidity of Gln3 over a 50 residue region without effect on its regulated localization.

\textbf{Cytoplasmic Sequestration of Gln3-Myc\textsuperscript{13} in Strains Altered in Gln3\textsubscript{600-666} Retain an Absolute ure2 Requirement} – Substitution of just three serine residues appeared to disrupt the contribution of rapamycin-inhibited, i.e., TorCl-dependent regulation of Gln3-Myc\textsuperscript{13} localization away from that imposed by NCR. If the Tor1-independent, second regulatory mechanism controlling Gln3 localization was truly NCR, then the cytoplasmic sequestration of the Gln3 truncation and Gln3\textsubscript{S656, S659, S662 substitution mutants should be ure2-dependent. As noted earlier, Ure2 had long been known to: (i) be a negative regulator of Gln3 function (1-5), (ii) form a Gln3-Ure2 complex (6-9) and (iii) be required to maintain cytoplasmic Gln3 localization in nitrogen-replete medium (6-9). To test this expectation, \\textit{CEN}-plasmids expressing Gln3\textsubscript{1-730-Myc}\textsuperscript{13} (pRR536), Gln3\textsubscript{1-600-Myc}\textsuperscript{13} (pRR614) and Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) were transformed into \textit{ure2A} RR215 and Gln3-Myc\textsuperscript{13} localization assayed. The loss of Ure2 resulted in complete nuclear localization of Gln3-Myc\textsuperscript{13} carried in all three plasmids transformed into glutamine-grown cells (Fig. 8). Thus, not only was the second Gln3 regulatory mechanism nitrogen source responsive, it also exhibited an absolute requirement for Ure2, both hallmarks of NCR-sensitive control.

\textbf{Changes in Rapamycin-Elicited Gln3\textsubscript{1-730-Myc}\textsuperscript{13} in Gln3\textsubscript{1-600-Myc}\textsuperscript{13} Gln3\textsubscript{1-581-Myc}\textsuperscript{13} and Gln3\textsubscript{1-565-Myc}\textsuperscript{13} Phosphorylation} – Treating glutamine- or YPD-grown wild type cells with rapamycin resulted not only in nuclear Gln3 localization, but also in gross Gln3 dephosphorylation thereby increasing its migration rate in SDS-PAGE gels (7, 8, 22). This was one of the foundational correlations from which the earliest model for Tor1-mediated Gln3 regulation derived (7). Therefore, the inability of Gln3\textsubscript{1-600-Myc}\textsuperscript{13} (pRR614), Gln3\textsubscript{S656D, S659D, S662D-Myc}\textsuperscript{13} (pRR850) and Gln3\textsubscript{S656A, S659A, S662A-Myc}\textsuperscript{13} (pRR1038) to relocate to the nucleus following rapamycin addition prompted us to determine whether or not rapamycin-elicited Gln3 dephosphorylation was also lost in these mutants. If gross Gln3 dephosphorylation is a determinant of its localization, then the two processes should respond in parallel to alterations in Gln3.

As expected, full length wild type Gln3\textsubscript{1-730-Myc}\textsuperscript{13} (pRR536) exhibited strong dephosphorylation following addition of rapamycin (Fig. 9). The normal slower migrating Gln3\textsubscript{1-730-Myc}\textsuperscript{13} species characteristic of hyper-phosphorylated Gln3 (upper black dot between lanes 1 and 2) was minimally detectable in the rapamycin-
treated cells, whereas the lower, hypo-phosphorylated Gln3\textsubscript{1-730}-Myc\textsuperscript{13} (pRR536) species (lower black dot between lanes 1 and 2) was prominent. In contrast, rapamycin elicited a smaller difference in Gln3\textsubscript{1-600}-Myc\textsuperscript{13} (pRR614) phosphorylation levels. In the untreated culture, the hyper-phosphorylated species (pRR614, upper black dot between lanes 1 and 2) predominated with little evidence of a clear band corresponding to the hypo-phosphorylated species (lower black dot). In Gln3\textsubscript{1-600}-Myc\textsuperscript{13} rapamycin-treated cells, both hyper- and hypo-phosphorylated forms appear. Although the hyper-phosphorylated species still predominated, the hypo-phosphorylated species was now clearly present and in greater abundance than observed in the untreated cells. Thus rapamycin-elicited Gln3 dephosphorylation had been damaged but not destroyed in the truncation mutant.

When a more focused alteration was made in Gln3, the resulting protein, Gln3\textsubscript{s656d, s659d, s662d-Myc}\textsuperscript{13} (pRR850), exhibited a phosphorylation profile with even greater similarity to that of the wild type Gln3\textsubscript{1-740-Myc}\textsuperscript{13} (pRR536) than did Gln3\textsubscript{1-600-Myc}\textsuperscript{13} (pRR614). The more slowly migrating species dominated in untreated cells, whereas the more rapidly migrating species dominated following rapamycin addition. The same predominance of the faster migrating species was also observed with rapamycin-treated Gln3\textsubscript{s656a, s659a, s662a-Myc}\textsuperscript{13} (pRR1038). The more slowly migrating species also predominated in untreated cells. Together, these data indicated that Gln3 sequences 600-730 were substantially, though not absolutely, required for high-level, rapamycin-elicited Gln3 dephosphorylation and that within this region, Gln3 residues 656, 659, and 662 did not play a particularly important role. Therefore, Gln3\textsubscript{s656d, s659d, s662d-Myc}\textsuperscript{13} and Gln3\textsubscript{s656a, s659a, s662a-Myc}\textsuperscript{13} dephosphorylation and localization did not parallel one another in rapamycin-treated cells. These data also further demonstrated that rapamycin-elicited gross dephosphorylation of Gln3 is not sufficient to cause its relocation into the nucleus.

*Decreased cytoplasmic Gln3-Myc\textsuperscript{13} Localization Fails to Correlate with Tor1 Association* – The phenotypes of Gln3 amino acid substitutions in Gln3\textsubscript{s656d, s659d, s662d-Myc}\textsuperscript{13} (pRR850), Gln3\textsubscript{s656a, s659a, s662a-Myc}\textsuperscript{13} (pRR1038), the Gln3\textsubscript{1-600-Myc}\textsuperscript{13} truncation, and Carvalho et al.’s Gln3\textsubscript{510-731-Myc}\textsuperscript{9}, Gln3\textsubscript{600-731-Myc}\textsuperscript{9} and Gln3\textsubscript{560-667-Myc}\textsuperscript{9} truncations suggested that only Gln3 residues between 653 and 667 were required for high level cytoplasmic sequestration in nitrogen excess. If this region coincided with the Tor1 binding domain reported earlier (12), we expected the serine substitutions in Gln3\textsubscript{s656d, s659d, s662d-Myc}\textsuperscript{13} (pRR850) would eliminate the Tor1 association.

We tested this prediction with the two-hybrid assay system used and generously provided by Carvalho et al. (12). First, we confirmed, that the assay system behaved the same in our hands as in the Zheng laboratory; growth in the presence of 3-aminotriazole (3AT) signified positive *in vivo* protein-protein interaction. The positive (Fig. 10A, panels B and E, minus and plus 3AT, respectively), negative (panels A and D), and empty vector (panels C and F) controls all behaved as expected.

Confident in the assay, we transformed each of three Gln3 “prey” plasmid constructs (i) wild type Gln3\textsubscript{1-730} (pRR1065), (ii) Gln3\textsubscript{1-600} (pRR1067) or (iii) Gln3\textsubscript{s656d, s659d, s662d} (pRR1069) into a strain containing either pASTOR1-2470 (full length Tor1\textsubscript{1-2470}, panels M-O) or pASTOR1-1764 (truncated Tor1\textsubscript{1-1764}, panels G to L) as the bait plasmid (Fig. 10B) (12). The three “prey” plasmids contained wild type and mutant gln3 genes identical to those in pRR536, pRR614 and pRR850, respectively, except for elimination of the Myc\textsuperscript{13} tag. We then assayed 36 independent transformants for each pair of plasmids in the absence (panels G-I) or presence (panels J-O) of 3AT. Only pASTOR1-1764 transformants grown in the absence of 3AT, the positive control for cells containing both plasmids, are shown in the figure (panels G-I); analogous pTOR1-2470 transformants yielded similar data (data not shown).

To our great surprise, all three bait plasmids supported growth in the presence of 3AT, indicating positive associations with full length Tor1\textsubscript{1-2470} (Fig. 10B, panels M-O). Fig. 10A, panel F depicts what a lack of association with Tor1\textsubscript{1-2470} would look like. This indicated that wild type Gln3\textsubscript{1-730}, Gln3\textsubscript{1-600} and Gln3\textsubscript{s656d, s659d, s662d} all formed positive associations with full length Tor1\textsubscript{1-2470}. Based on Carvalho et al.’s earlier results, we minimally expected Gln3\textsubscript{1-600} (pRR1067) to be incapable of associating with Tor1 and hence had included it as a negative control for our two-hybrid assays. But that was not the case.

The exceptional nature of the above results prompted us to repeat the two-hybrid assays using a Tor1\textsubscript{1-1764} truncation containing the HEAT repeats reported to interact with Gln3 as the two-hybrid “bait” plasmid (pASTOR1-1764) (8). Results identical to those observed with “bait” plasmid pASTOR1-2470 were obtained, i.e., all three types of transformants grew in the presence (Fig. 10B, panels J-L) and absence (panels G-I) of 3AT.

*A Short, Potential α-helix Interacts with Tor1* – The striking incongruities between our results and those reported by Carvalho et al. required explanation. We reasoned the differences might have conceivably derived from: (i) technical defects in our assays, or (ii) a fundamental, but unrecognized difference between the previous and present experimental strategies. Indeed, the latter explanation turned out to be the source of the dispari-
ties. Based on positive interactions between Gln3_{510-730}, Gln3_{600-667} and Tor1, and lack of an interaction with the Gln3_{667-730} peptide, Carvalho et al. rightly concluded that Tor1-interacted with the Gln3_{600-667} peptide. The key feature of their strategy was the use of isolated Gln3 peptides lacking Gln3 sequences N-terminal of residues 510, 600 and 667, respectively. We, on the other hand, employed C-terminal truncations, Gln3_{600-667}, and the Gln3_{565, 569, 562} substitutions in full length Gln3 molecules. In other words, all of our constructions contained all residues N-terminal to Gln3_{600}. Therefore, if Gln3 contained more than one Tor1 interacting site N-terminal of Gln3_{600}, it would have been missed in Carvalho et al.’s experiments which accounts for the lack of congruency between our data and theirs.

Our functional assays had already demonstrated that minimally, Gln3 residues 656-662 were required for full cytoplasmic sequestration of Gln3-Myc and that those in Gln3_{600-653} were not (Figs. 5-8). Therefore, we focused our attention on a 17 amino acid Gln3_{654-670} peptide that contained Gln3_{656-666}, a sequence predicted by the Psipred secondary structure prediction method to potentially fold into an α-helix (Fig. 4A and 11B) (40, 41). Further, the substituted serine residues in Gln3_{565, 569, 562}-Myc (pRR880) were all situated on the same face of this putative Gln3_{654-670} α-helix peptide, if it existed in this form (Fig. 11, substituted serine residues appear as white letters). We reasoned, based on these results, that the Gln3_{654-670} peptide potentially contained the Tor1 interacting site situated in the C-terminal region of Gln3. This in turn predicted that the wild type Gln3_{654-670} peptide (pRR1098) would interact with Tor1, whereas this peptide containing aspartate or alanine substitutions [Gln3_{565, 569, 562}-Myc (pRR1101); Gln3_{565A, 569A, 562A} (pRR1160)] would not. These predictions were validated experimentally. The wild type Gln3_{654-670} peptide interacted with Tor1_{1,2470} (Fig. 12B, images M and Q), whereas the mutant peptides did not (Fig. 12B, images N and R). Note that wild type Gln3_{1,730} and Gln3_{565D, 569D, 562D} (pRR1065 and pRR1069, respectively) again both yielded positive interactions with Tor1_{1,2470} (Fig. 12B, images G and H in the absence and panels K and L and O and P in the presence of 3AT). Fig. 12A, panels A-F depict the 2-hybrid assay controls, all of which yielded the expected results. Finally, we noted that when the entire set of independent replicate transformants, containing either wild type or mutant forms of the Gln3_{654-670} peptide, were viewed as a whole, it appeared that the alanine substitutions exhibited a slightly greater interaction with Tor1_{1,2470} (growth on +3AT) than the aspartate substitutions. Together, these data indicated that Gln3 possessed at least two sites that were able to associate, directly or indirectly, with Tor1 and that one of them was situated between Gln3 residues 654 and 670.

**DISCUSSION**

**Parsing Tor1- and NCR-Associated Regulation of Gln3 Localization** – Experiments interrogating the C-terminal region of Gln3 have genetically separated Tor1- and NCR-associated regulation of Gln3 localization. Specifically, elimination of / or amino acid substitutions in a 11 amino acid peptide with a theoretical potential to fold into an α-helix, Gln3_{656-666}, abolished the ability of: (i) Gln3 to associate with Tor1 in a two-hybrid assay, (ii) nitrogen rich conditions to completely sequester Gln3 in the cytoplasm, and (iii) rapamycin to elicit increased nuclear Gln3 localization in glutamine-grown cells. The Tor1-Gln3 association requirement for complete cytoplasmic sequestration is consistent with a requirement of the Tor1 kinase activity to achieve this end. This interpretation is consistent with Carvalho et al.’s *in vitro* demonstration of Tor1 being able to phosphorylate Gln3 (12). On the other hand, the ability of Gln3 localization to exhibit a normal response to the nitrogen source provided, a hallmark of NCR-sensitive regulation, remained intact and responsive to the negative regulation of Ure2 in the absence of this Tor1 association and inability to respond to rapamycin addition. These observations argue that Tor1 association-dependent, rapamycin-elicted Gln3 regulation is a distinct and genetically separable pathway from nitrogen source-responsive, NCR-sensitive Gln3 regulation.

**Additivity of TorC1-Dependent and NCR-Sensitive Regulation** – Beyond genetically dissecting the contributions of Tor1- and NCR-associated Gln3 regulation, the data also demonstrate the required collaboration of the two regulatory mechanisms to achieve the overall nitrogen-responsive control of Gln3 localization. NCR-mediated regulation alone in cells cultured in nitrogen-replete glutamine medium was insufficient to completely sequester Gln3 in the cytoplasm; a Tor1 association-dependent contribution was also needed. On the other hand, loss of the Tor1 contribution resulted in a nuclear shift in Gln3 localization, but again one that was far from complete. Even under the best conditions, high concentrations of rapamycin were incapable of achieving more than ~30% nuclear Gln3-Myc localization with similar amounts remaining completely cytoplasmic and nuclear-cytoplasmic, respectively (Fig. 1). Complete nuclear Gln3 localization could only be achieved when cells, devoid of the Gln3_{566-666}-Tor1 association, were cultured under derepressive conditions, i.e., in nitrogen-limiting proline medium, or in an *ure2Δ*. This is the first time that the combined action of Tor1- and NCR-associated participation have been demonstrated to be additively required for overall wild type regulation.

It may be mere coincidence that the Gln3-Myc intracellular distribution profiles observed in mutants...
where the Tor1-Gln3$_{656-666}$ association was eliminated are remarkably similar to those observed with rapamycin-treated, wild type, glutamine-grown cells [Compare Fig. 1 wild type Gln3$_{1-730}$-Myc$^{13}$ (pR5536) +Rap with Fig. 2 truncations +/- Rap or Fig. 8, Gln3$_{656D}$, S659D, S662D-Myc$^{13}$ (pR850) and Gln3$_{656A}$, S659A, S662A-Myc$^{13}$ (pR1038) +/- Rap]. However, this is a lot of positive correlation to be mere coincidence. Rapamycin treatment could mimic the loss of the Tor1-Gln3 interaction, but could go no further. In other words, rapamycin effects were restricted to reversing the outcome of Tor1 association-dependent actions. To maintain proper perspective, however, it is important to point out that the data neither indicate that residues putatively phosphorylated by Tor1 are the same as those putatively dephosphorylated by rapamycin-elicited phosphatase action, nor that rapamycin treatment is preventing Tor1-dependent phosphorylation. Putative Tor1 association-dependent phosphorylation and cytoplasmic sequestration may well be a completely independent event from rapamycin-elicited phosphatase action and the partial shift of Gln3 into the nucleus.

Although previous data strongly suggested that two separate regulatory systems were responsible for regulating Gln3 localization (20-23, 25-31), they suffered from the unavoidable caveats that observed results could potentially have derived from indirect effects of the conditions employed. This caveat derived from the fact that inhibitors functioned at remote sites from the reporter protein whose localization was being influenced, Gln3-Myc$^{13}$, and mutant studies involved molecules other than Gln3 itself. In the present work these caveats have been greatly minimized or eliminated because separation of TorC1- and NCR-associated components of Gln3 regulation derived from direct alteration of its target protein, the Gln3-Myc$^{13}$ molecule itself.

With What Does Gln3 Interact? – Data in this work and the earlier study of Carvalho et al. clearly demonstrate a positive Tor1-Gln3 interaction using the standard two-hybrid interaction assay. However, this assay has the caveat of not specifying whether the physiologically significant, in vivo interaction(s) visualized are direct or indirect. One need look no further than Carvalho et al.’s data for such a precedent. Their two-hybrid results visualized Tor1 interactions with Gln3, Ure2, Dal81, Dal82, Gat1, and Gzf3 (8). Gln3 is known to interact with Ure2 and Dal82 (6-8, 42-44). However, Dal81 interacts with Gln3 only in a Dal82-dependent manner (44). Therefore, it may be premature to conclude that Gln3 binds directly to Tor1. Although in vitro Tor1 clearly mediates Gln3 phosphorylation (8), it is worth noting that Gln3 contains 146 serine/threonine residues providing a wide in vitro selection of possible phosphorylation candidates that may or may not be physiologically significant. However, the present study provides an excellent highly defined probe to search for other molecules with which Gln3$_{656-666}$ interacts. This information, which we are attempting to acquire, may contribute to a more accurate and detailed view of the mechanism through which TorC1 regulates Gln3.

Gln3 Contains More Than One Tor1-Interacting Site and Possible Implications – The data in this work also demonstrate that, based on two-hybrid assays, Gln3 contains one or more Tor1-interacting sites beyond the one situated at Gln3$_{656-666}$. The most compelling evidence is that Gln3$_{1-600}$, lacking the demonstrated site at Gln3$_{656-666}$, exhibits a positive interaction with both Tor1$_{1-2470}$ and Tor1$_{1-1760}$. Further, reasoning described in the Results section provides a consistent explanation of how such a putative additional Tor1-interacting site(s) may have escaped earlier detection. If additional Tor1-interacting site(s) do, in fact, exist in Gln3, they open an important question, what is their function with respect to Gln3 regulation? Our future investigations will be directed toward locating additional putative Tor1-association site(s) in Gln3 and addressing this question.

Finally, two additional items merit emphasis: (i) the observations from which our conclusions derive would not have been possible without the ability to reliably quantify Gln3-Myc$^{13}$ localization and (ii) it is quite remarkable that systematic introduction of extensive alterations throughout the Gln3$_{600-655}$ region (25% of the residues) had no significant effect on rapamycin-influenced or NCR-sensitive regulation of Gln3 localization; all but one of the multiple substitutions failed to generate a mutant phenotype. This suggests that beyond direct or indirect interaction with Tor1, the bulk of this region either plays only a limited or minimally direct role in Gln3’s regulated localization or that the role it plays tolerates significant alteration.

Acknowledgements – The authors would like to thank Professors Elizabeth Craig and Steven Zheng for plasmids and strains, Dr. Thomas Cunningham of the University of Tennessee Health Science Center Molecular Resource Center DNA Sequencing Facility for performing all of the DNA sequencing needed to confirm the structures of the plasmids we constructed, Drs. Martha Howe and Lorraine Albritton for help with protein secondary structure prediction programs. We much appreciated the helpful comments of Drs. Evelyne Dubois, Isabelle Georis, and Michael A. Whitt for their helpful comments This work was supported by NIH grant GM-35642-22-23.

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FIGURE LEGENDS

FIG. 1. Evaluation of new assays used to as-
assess the intracellular distribution of wild type Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} expressed from CEN-based plasmids. The histograms for each of the conditions assayed in these experiments are the averages of 10 experiments performed with CEN-based pRR536 over a 9 month period (Panel A), or four experiments performed in wild type TB123 in which Gln3-Myc\textsuperscript{13} is situated at its native chromosomal position over a three year period (Panel B). The error bars represent plus or minus one standard deviation. pRR536 transformants and TB123 were grown in YNB medium with glutamine (Gln) or proline (Pro) as sole nitrogen source. Where indicated (+Rap) the cultures were treated with rapamycin. Red bars indicate Gln3-Myc\textsuperscript{13} staining in the cytoplasm only, yellow bars indicate both cytoplasmic and nuclear Gln3-Myc\textsuperscript{13} staining, and green bars indicate Gln3-Myc\textsuperscript{13} staining in the nucleus only. Panels of standard images illustrating the characteristics of each scoring category appear in Fig. 2 of ref. 28. Strain JK9-3da was the transformation recipient.

FIG. 2. Localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} or Gln3\textsuperscript{1-600-Myc\textsuperscript{13}}, Gln3\textsuperscript{1-584-Myc\textsuperscript{13}}, Gln3\textsuperscript{1-565-Myc\textsuperscript{13}} and Gln3\textsuperscript{1-642-Myc\textsuperscript{13}} truncations in untreated and rapamycin-treated, glutamine-grown transformants. Panel A depicts representative images from which the corresponding histograms in Panel B were generated. Panel C depicts the ratio of cytoplasmic staining untreated vs. rapamycin-treated, glutamine-grown cells for each of the truncation proteins analyzed in Panel B. Red bars indicate Gln3-Myc\textsuperscript{13} staining in the cytoplasm only, yellow bars indicate both cytoplasmic and nuclear staining, and green bars indicate staining in the nucleus only. Strain JK9-3da was the transformation recipient.

FIG. 3. Localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} or Gln3\textsuperscript{1-600-Myc\textsuperscript{13}}, Gln3\textsuperscript{1-584-Myc\textsuperscript{13}}, Gln3\textsuperscript{1-565-Myc\textsuperscript{13}} and Gln3\textsuperscript{1-642-Myc\textsuperscript{13}} truncations in glutamine- (Gln), or proline- (Pro) grown transformants. The experimental format was the same as in Fig. 2 except for the addition of a third growth condition, proline provided as sole nitrogen source.

FIG. 4. Intracellular localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} (pRR536) and Gln3\textsuperscript{563D,600D,601D,605D,606D-Myc\textsuperscript{13}} (pRR1014) in untreated glutamine- (Gln), or proline- (Pro) grown transformants or in rapamycin-treated, (+Rap) glutamine-grown cells. Panel A. Gln3 amino acid sequence 601-670 showing the locations of serine/threonine substitutions in the plasmids we used in this study as well as the location of sequences with the potential to form an \(\alpha\)-helix. Panels B and C. The experimental format was the same as in Fig. 2 except for the addition of a third growth condition, proline provided as sole nitrogen source.

FIG. 5. Localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} (pRR536), Gln3\textsuperscript{S616D, S617D, S619D, S621D, S631D-Myc\textsuperscript{13}} (pRR922), and Gln3\textsuperscript{S616A, S617A, S619A, S621A, S624A, S631A-Myc\textsuperscript{13}} (pRR960) in untreated glutamine- (Gln in Panels A-D), or proline- (Panels C and D) grown transformants or in glutamine-grown, rapamycin-treated (+Rap in Panels A and B) transformants. The experimental format was the same as in Fig. 2 except for the addition of a third growth condition, proline provided as sole nitrogen source.

FIG. 6. Localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} (pRR536), Gln3\textsuperscript{S641D, S645D, S647D, S649D, S652D, S653D-Myc\textsuperscript{13}} (pRR928) and Gln3\textsuperscript{S641A, S645A, S647A, S649A, S652A, S653A-Myc\textsuperscript{13}} (pRR966) in untreated glutamine- (Gln in Panels A-D), or proline- (Pro in Panels C and D) grown transformants or in rapamycin-treated (+Rap in Panels A and B). The experimental format was the same as in Fig. 2 except for the addition of a third growth condition, proline provided as sole nitrogen source.

FIG. 7. Localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} (pRR536), Gln3\textsuperscript{S656D, S659D, S662D-Myc\textsuperscript{13}} (pRR850) and Gln3\textsuperscript{S656A, S659A, S662A-Myc\textsuperscript{13}} (pRR1038) in untreated glutamine- (Gln in Panels A-D), proline- (Pro in Panels C and D) grown transformants or in rapamycin-treated (+Rap in Panels A and B). The experimental format was the same as in Fig. 2 except for the addition of a third growth condition, proline provided as sole nitrogen source.

FIG. 8. Effect of deleting URE2 on the localization of full length Gln3\textsuperscript{1-730-Myc\textsuperscript{13}} (pRR536), Gln3\textsuperscript{1-600-Myc\textsuperscript{13}} (pRR614) and Gln3\textsuperscript{S656D, S659D, S662D-Myc\textsuperscript{13}} (pRR850) in untreated and rapamycin-treated, glutamine-grown transformants. The experimental format was the same as in Fig. 2. Strain RR215 was the transformation recipient.

FIG. 9. Gln3-Myc\textsuperscript{13} phosphorylation profiles observed with wild type (pRR536), Gln3\textsuperscript{1-600-Myc\textsuperscript{13}} truncation (pRR614), Gln3\textsuperscript{S656D, S659D, S662D-Myc\textsuperscript{13}}
Myc$^{13}$ (pRR850) and Gln3$^{S656A, S659A, S662A-Myca^{13}}$ (pRR1038) amino acid substitution proteins. The conditions in which transformants containing these plasmids were grown appear above the lanes. Plasmids used in the experiments are shown at the right of each panel. Growth conditions were as in Fig3.

**FIG. 10.** Two-hybrid assessment of Tor1 association with full length Gln3$^{1-730}$ (pRR1065), Gln3$^{S656D, S659D, S662D}$ (pRR1069). The assays were performed as described in Methods. The transformation recipient in all cases was strain PJ69-4a. The positive control was constructed by sequentially transforming PJ69-4a with pAV3-1 and pTD1-1. The negative control was a transformant containing pASTOR1-2470 (Gal4 DNA binding domain-Tor1 fusion) and pACT2 (corresponding empty vector). pRR1065, pRR1067, and pRR1069 consisted of the Gal4 activation domain fused N-terminal of the respective Gln3 genes. Viability and the test for a two-hybrid association of the transformants was determined by streaking them on the same medium in the presence and absence of 3 mM 3'-aminotriazole (3AT). The procedures, strain, fusion vectors and control plasmids were those used by Carvalho et al. (12).

**Fig. 11.** Wild type Gln3 residues 656-666 depicted as situated on an $\alpha$-helical wheel. The serine residues substituted in Gln3$^{S656D, S659D, S662D-Myca^{13}}$ (pRR850) and Gln3$^{S656A, S659A, S662A-Myca^{13}}$ (pRR1038) are indicated in white letters.

**Fig. 12.** Two-hybrid assessment of Tor1 association with full length Gln3$^{1-730}$ (pRR1065), Gln3$^{1-730, S656D, S659D, S662D}$ (pRR1069), Gln3$^{654-670}$ wild type peptide (pRR1098) and Gln3$^{654-670, S656D, S659D, S662D}$ peptide (pRR1101). The format and strategy of this experiment was the same as that in Fig. 10.
Table 1. Saccharomyces cerevisiae strains used in this work.

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1JK9-3d was constructed by Jeanette Kunz (Michael Hall’s laboratory). Joseph Heitman isolated *MATα* and *MATα* strains isogenic to JK9-3d by mating type switching. JK9-3da is a hybrid strain containing contributions from the following strains: S288c, a strain from the Oshima lab, and an unidentified strain from the Herskowitz lab. It was chosen because of its robust growth, sporulation efficiency, and good growth on galactose (GAL+). It may have a SUP mutation that allows translation through premature STOP codons and therefore produces functional alleles with many point mutations.

2DGY63::171 is in the W303 strain background.
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<td>pRR1160 Gln3&lt;sub&gt;654-670&lt;/sub&gt; S656A, S659A, S662A</td>
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*Sequence Gln3<sub>1947-1952</sub>, 5'-AGACCT-3' was changed to 5'-CGGCCG-3' to generate a unique EagI restriction site for cloning purposes. These changes did not alter the amino acid sequence of the protein.*
(A) Plasmid borne GLN3 (pRR536)

(B) Chromosomally GLN3 (TB123)
Fig. 5

(A) pRR536, W.T.  pRR922  pRR960
- Gln  +Rap  Gln  +Rap  Gln  +Rap

Gln3-Myc:  
- Gln  +Rap  Gln  +Rap  Gln  +Rap

DAPI:  
- Gln  +Rap  Gln  +Rap  Gln  +Rap

(B) Cytoplasmic  Nucl.-Cyto.  Nuclear
- Gln  +Rap  Gln  +Rap  Gln  +Rap

Percentage of Cells

(C) pRR536, W.T.  pRR922  pRR960
- Gln  Pro  Gln  Pro  Gln  Pro

Gln3-Myc:  
- Gln  Pro  Gln  Pro  Gln  Pro

DAPI:  
- Gln  Pro  Gln  Pro  Gln  Pro

(D) Cytoplasmic  Nucl.-Cyto.  Nuclear
- Gln  Pro  Gln  Pro  Gln  Pro

Percentage of Cells
**CONTROL PLASMIDS**

(A)

**Media Composition**

- **SC-Leu-Trp**
  - Negative Control: pAS2-1 & pACT2
  - Positive Control: pAV3-1 & pTD1-1
  - Vector Control: pASTOR1-1764 & pACT2

- **SC-His-Leu-Trp + 3AT**
  - Negative Control: pAS2-1 & pACT2
  - Positive Control: pAV3-1 & pTD1-1
  - Vector Control: pASTOR1-1764 & pACT2

(B)

**Gln3 TEST PLASMIDS**

**Media Composition**

- **SC-Leu-Trp**
  - Gln3<sub>1-730</sub> Wild Type: pASTOR1-1764 & pRR1065
  - Gln3<sub>1-600</sub> Truncation: pASTOR1-1764 & pRR1067
  - Gln3<sub>S656D,S659D,S662D</sub>: pASTOR1-1764 & pRR1069

- **SC-His-Leu-Trp + 3AT**
  - Gln3<sub>1-730</sub> Wild Type: pASTOR1-1764 & pRR1065
  - Gln3<sub>1-600</sub> Truncation: pASTOR1-1764 & pRR1067
  - Gln3<sub>S656D,S659D,S662D</sub>: pASTOR1-1764 & pRR1069

- **SC-His-Leu-Trp + 3AT**
  - Gln3<sub>1-730</sub> Wild Type: pASTOR1-2470 & pRR1065
  - Gln3<sub>1-600</sub> Truncation: pASTOR1-2470 & pRR1067
  - Gln3<sub>S656D,S659D,S662D</sub>: pASTOR1-2470 & pRR1069
### CONTROL PLASMIDS

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### Gln3 TEST PLASMIDS

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gln3 Mutations Dissociate Responses to Nitrogen Limitation (Nitrogen Catabolite Repression) and Rapamycin Inhibition of TorC1
Rajendra Rai, Jennifer J. Tate, David R. Nelson and Terrance G. Cooper

J. Biol. Chem. published online December 5, 2012

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