Stress-responsive Gln3 Localization in *Saccharomyces cerevisiae* Is Separable from and Can Overwhelm Nitrogen Source Regulation*

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Intracellular localization of *Saccharomyces cerevisiae* GATA family transcription activator, Gln3, is used as a downstream readout of rapamycin-inhibited Tor1,2 control of Tap42 and Sit4 activities. Gln3 is cytoplasmic in cells provided with repressive nitrogen sources such as glutamine and is nuclear in cells growing with a derepressive nitrogen source such as proline or those treated with rapamycin or methionine sulfoximine (Msx). Although gross Gln3-Myc° phosphorylation levels in wild type cells do not correlate with nitrogen source-determined intracellular Gln3-Myc° localization, the phosphorylation levels are markedly influenced by several environmental perturbations. Msx treatment increases Snf1-independent Gln3-Myc° phosphorylation, whereas carbon starvation increases both Snf1-dependent and -independent Gln3-Myc° phosphorylation. Here we demonstrate that a broad spectrum of environmental stresses (temperature, osmotic, and oxidative) increase Gln3-Myc° phosphorylation. In parallel, these stresses elicit rapid (<5 min for NaCl) Gln3-Myc° relocalization from the nucleus to the cytoplasm. The response of Gln3-Myc° localization to stressful conditions can completely overwhelm its response to nitrogen source quality or inhibitor-generated disruption of the Tor1,2 signal transduction pathway. Adding NaCl to cells cultured under conditions in which Gln3-Myc° is normally nuclear, i.e. proline-grown, nitrogen-starved, Msx−, caffeine−, and rapamycin-treated wild type cells, or *ure2Δ* cells, results in its prompt relocalization to the cytoplasm. Together these data identify a major new level of regulation to which Gln3 responds, and adds a new dimension to mechanistic studies of the regulation of this transcription factor.

Transcription, mediated by the GATA family transcription activators Gln3 and Gat1/Nil1, has become an important terminal readout in mechanistic studies of the clinically important, rapamycin-inhibitable Tor1,2 signal transduction pathway (1–4). From a physiological point of view, the regulation of Gln3 and Gat1 intracellular localization and nuclear function is responsible for selective utilization of nitrogen sources available to *Saccharomyces cerevisiae*; this regulatory process has been designated Nitrogen Catabolite Repression or NCR (5–9). In NCR, when readily used (i.e. repressive) nitrogen sources such as glutamine or, in some strains, ammonia are available in sufficient supply, yeast cells will not utilize poorer (i.e. derepressive) nitrogen sources in their environment. This selectivity is accomplished by repressing expression of genes encoding the permeases and catabolic enzymes required for utilization of these poorer nitrogen sources. On the other hand, when the environmental nitrogen supply becomes limiting, Gln3/Gat1-dependent transcription is derepressed. As a result, production and activation of permeases and catabolic enzymes required for utilization of poor (i.e. derepressive) nitrogen sources increase. Thus the cells are equipped to scavenge otherwise unused nitrogen sources until a better nutritional environment returns.

Phosphatidylinositol 3-kinase-related serine/threonine protein kinases, Tor1,2, are participants in NCR-sensitive transcriptional control (1–4, 10). Tor1,2 are the *S. cerevisiae* counterparts of mammalian mTor, with the human protein being a target of therapies treating multiple types of cancer and tissue rejection in transplant patients (10–17). According to the original *S. cerevisiae* model (Fig. 1, left panel), Tor1,2 received nutrient-responsive signals, which resulted in their activation (1–4). One such signal may be glutamine or a related metabolite, a conclusion derived from the observation that treating cells with the glutamine synthetase inhibitor, methionine sulfoximine (Msx), correlates with activation of the Tor1,2 pathway and its influence on downstream readouts (18, 19). Activated Tor1,2 phosphorylates and positively regulates the essential protein Tap42 (20–22). Tap42, in turn, interacts with and negatively regulates type 2A-related and type 2A serine/threonine protein phosphatases, Sit4, Pph3, and Pph21,22 (1, 3, 4, 20–26). For the sake of simplicity, we have not discussed Tip41 phosphorylation and its protein-protein interactions (22). Thus inhibited, these phosphatases were posited to be unable to dephosphorylate Gln3. Under such conditions of excess nitrogen, Gln3, was found in a complex with Ure2 (1, 4, 27), a multifunctional protein that is additionally a prion precursor (28, 29) and required participant in heavy metal ion and hydroperoxide detoxification (30, 31). The presence of the Gln3-Ure2 complex correlates with exclusion of Gln3 from the nucleus and repr...

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2 The abbreviations used are: NCR, nitrogen catabolite repression; Msx, methionine sulfoximine; DAPI, 4',6'-diamino-2-phenylindole.
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FIGURE 1. Original model (left panel) proposed for Tor1,2 regulation of Gln3 phosphorylation and localization (1, 4) as well as influence of Msx on Tor pathway regulation (18). A more recent version of the Tor regulatory pathway incorporated data from Jacinto et al. (22), Loewith et al. (51), Reinke et al. (50), Rohde et al. (25), Wang et al. (24), and Yan et al. (26). For the sake of simplicity neither model is exhaustive in detailing all of the known components and/or protein-protein interactions and protein modifications.

of Tap42 with Sit4 is required for the latter to be active (Fig. 1, right panel) (24). Furthermore, rapamycin treatment was demonstrated to release the Tap42-Sit4 complex from its association with Tor complex 1 (TORC1), also an event required for the Tap42-Sit4 complex to be active (Fig. 1, right panel) (26).

Similarly significant changes have occurred in our understanding of the relationship between Gln3-Myc\(^{13}\) phosphorylation and intracellular localization. Although rapamycin treatment and growth of cells in poor nitrogen sources bring about the same outcome, i.e. nuclear Gln3-Myc\(^{13}\) localization, the gross Gln3-Myc\(^{13}\) phosphorylation profiles do not similarly correlate (19, 32). Furthermore, Sit4 has recently been shown to be active irrespective of the nitrogen source provided to the cells, i.e. with both good and poor nitrogen sources.

When cells are treated with the Tor1,2 inhibitor, rapamycin, events opposite to those described above were envisioned. Tor1,2 no longer positively regulated Tap42, which in turn no longer bound to and inhibited Sit4 phosphatase. Thus freed from negative regulation by Tap42, Sit4 phosphatase dephosphorylated Gln3, and it was this dephosphorylated form that correlated with nuclear localization following rapamycin treatment (1, 4). However, it was subsequently found that this correlation was observed only at early times (20–30 min) following rapamycin treatment; it was not observed 60 min after treatment (32).

Although the positive correlation of short term rapamycin treatment, Gln3 dephosphorylation, and nuclear localization have been repeatedly confirmed, our understanding of the mechanistic steps from which these correlations derive are still evolving significantly (Fig. 1, right panel). An early study, which established a physiologically significant association between Tap42 and Sit4, concluded either that Tap42 positively regulated type 2A and type 2A-related phosphatase activities (i.e. the Tap42-phosphatase complex was active for a specific phosphatase function) rather than acting as a negative regulator, or alternatively that a Tap42 function was regulated by the phosphatase (20). More recently, the idea of positive Sit4 regulation by Tap42 has re-emerged in the demonstration that association (33). Finally, when \(SIT4\) is deleted, Gln3-Myc\(^{13}\) phosphorylation responds to the nature of the nitrogen source provided, i.e. an influence of the nitrogen source on Gln3-Myc\(^{13}\) phosphorylation is unmasked (33). These observations led Tate et al. (33) to suggest the nitrogen source is probably more directly connected to the regulation of one or more protein kinase activities that phosphorylate Gln3-Myc\(^{13}\) than it is to Sit4 phosphatase that continuously dephosphorylates it irrespective of the nitrogen source.

Finally, protein kinase Npr1 was reported to be a negative regulator of Gln3, because deletion of \(NPR1\) resulted in constitutive nuclear localization of Gln3 and NCR-sensitive transcription in ammonia-grown cells (34). Most recently, it has been independently shown by two groups that the \(npr1\Delta\) phenotype derives from loss of the normal ammonia permease activities in the mutant rather than direct negative regulation of Gln3 (35, 36).

The central role of Tap42 in the control of Sit4 and its subsequent regulation of downstream Tor1,2 targets, prompted us to investigate the effects generated by loss of Tap42 on intracellular Gln3-Myc\(^{13}\) localization and phosphorylation. The mutation we elected to analyze was the temperature-sensitive \(tap42-11\) allele because it was one of the mutations initially used to demonstrate many of the Tap42 functions in general (20) and Tap42 involvement in Gln3 regulation in particular (1). Although the \(tap42-11\) mutant is rapamycin-resistant at its permissive temperature of 24 °C, the growth inhibitory and other phenotypes it possesses require a nonpermissive temperature of 37 °C (20). Therefore, before proceeding further, we performed several control experiments, including one that assessed whether the nonpermissive temperature for \(tap42-11\) (37 °C) would affect Gln3 regulation.
These experiments led us to the surprising discovery of a new form of Gln3 regulation. (i) Gln3-Myc$^{13}$ relocates from the nucleus to the cytoplasm when proline-grown, wild type cells are shifted from 24 to 37 °C. (ii) Gln3-Myc$^{13}$ phosphorylation levels increase in parallel with its relocalization. We further showed that Gln3-Myc$^{13}$ phosphorylation and localization responded similarly to multiple stress conditions, i.e. temperature and osmotic and oxidative stress. Such a broad range of responses is characteristic of a generalized stress response, a form of regulation with which Gln3 has not previously been associated. Equally surprising, the response of Gln3-Myc$^{13}$ to environmental stress can overwhelm previously studied forms of Gln3 regulation, rapamycin, caffeine, or Msx treatment, nitrogen starvation, limiting nitrogen supply with poor nitrogen sources such as proline, and deletion of URE2. Experiments described below broaden our view of Gln3 regulation from a uni-dimensional response to nitrogen supply to a multivariant response accounting for other environmental conditions representing potentially greater and more immediate threats to the well being of a cell.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**—*S. cerevisiae* strains used in this work were TB123 (MATa, leu2-3,112, ura3-52, rme1, trp1, his4, GAL$^{+}$, HMLa, GLN3-Myc$^{13}$[KanMX]), TB136-2a (MATa, leu2-3,112, ura3-52, rme1, trp1, his4, GAL$^{+}$, HMLa, GLN3-Myc$^{13}$[KanMX], sit4::kanMX), TB138-1a (MATa, leu2-3,112, ura3-52, rme1, trp1, his4, GAL$^{+}$, HMLa, GLN3-Myc$^{13}$[KanMX], ur2::URA3), and TB123-hog1 (MATa, leu2-3,112, ura3-52, rme1, trp1, his4, GAL$^{+}$, HMLa, GLN3-Myc$^{13}$[KanMX], hog1::natMX).

Strains were grown at 30 °C (unless otherwise indicated in the figure legends) to mid-log phase (A$_{600}$ nm = 0.5) in Difco yeast nitrogen base medium (YNB) (without amino acids and ammonium sulfate), containing 2% glucose, required auxotrophic supplements (120 μg/ml leucine, 20 μg/ml uracil, 20 μg/ml histidine, 20 μg/ml tryptophan, 20 μg/ml arginine), and the nitrogen source indicated (0.1% final concentration unless otherwise indicated). Rapamycin (Sigma) (dissolved in 10% Tween 20 + 90% ethanol) was added to the cultures, where indicated, to a final concentration of 0.2 μg/ml. Caffeine (20 mM final concentration, added as a solid) or Msx (2 mM final concentration, dissolved in water) were added where indicated. The duration of treatment that occurred prior to cell harvest is indicated in the pertinent figure legends.

**Cell Collection and Protein Preparation for Western Blot Analysis**—Experiments in which yeast were transferred from one medium to another were performed as follows. An exponentially growing culture (A$_{600}$ nm = 0.4–0.5), or half of it in the case of a split culture, was quickly collected by filtration (at room temperature) and resuspended in an equal volume of the same fresh, pre-warmed (to the specified temperature), pre-aerated medium; transfer was completed in 35–45 s. Following incubation for the indicated times (see figure legends), cells were collected using the filtration method of Tate et al. (19), and protein extracts were prepared as described by Cox et al. (32). In the case of treated cultures (rapamycin, Msx, caffeine, sorbitol, sodium chloride, or hydrogen peroxide), a pretreatment (zero time) sample was collected as described above. The remainder of the culture was then treated with the indicated perturbant at the concentration and for the times indicated in the figure legends. Samples of these treated cultures were collected and processed as described above.

**Indirect Immunofluorescence Microscopy**—Cell collection and fixation for indirect immunofluorescence were performed using the method of Cox et al. (37) as modified by Tate et al. (33, 35). Gln3-Myc$^{13}$ localization was visualized using 9E10(c-Myc) monoclonal antibody (Covance MMS-150P, at a dilution of 1:1000) as the primary antibody and Alexa Fluor 594 goat antimouse IgG antibody (Molecular Probes, at a dilution of 1:200) as secondary antibody. DNA was visualized using 4′,6′-diamino-2-phenylindole (DAPI) contained in the mounting medium (Sigma) (37).

Cells were imaged using a Zeiss Axioplan 2 imaging microscope with a 100× Plan-Apochromat 1.40 oil objective at room temperature. Images were acquired using a Zeiss Axiocam and AxioVision 3.0 software (Zeiss). Images were processed with Adobe Photoshop and Illustrator programs. Gamma settings were altered where necessary to avoid any change in cellular detail during processing; changes were applied uniformly to the image.

**Determination of Intracellular Gln3-Myc$^{13}$ Distribution**—To provide a more representative and complete view of Gln3-Myc$^{13}$ behavior than can be obtained from a single image of four to eight cells, we scored Gln3-Myc$^{13}$ localization in 200 or more cells that appeared in multiple, randomly chosen microscopic fields of each experimental sample, including the ones from which the images we present were taken. These cells were placed in one of three categories as follows: cytoplasmic (cytoplasmic fluorescence only), nuclear-cytoplasmic (fluorescence appeared in the cytoplasm as well as co-localizing with DAPI-positive material), and nuclear (co-localizing only with DAPI-positive material). The limitations and reproducibility of the scoring method are described in Tate et al. (33, 35). It is important to emphasize again here, as described in detail earlier, that the nuclear-cytoplasmic category is, of necessity, arbitrary. Placing cells in that category is based on subjective visual observation by the individual scoring the cells; it is not an objective instrument-based measurement. When Gln3-Myc$^{13}$ is not restricted to a single cellular compartment, scoring depends upon repeated decisions that conclude whether Gln3-Myc$^{13}$ in a given cell is localized in the center nuclear-cytoplasmic category or a category flanking it. Although intracellular Gln3-Myc$^{13}$ distribution was scored as carefully and consistently as possible, our interpretations and conclusions are based on straightforwardly detected changes in overall distribution patterns that are apparent in the microscopic images. Histograms of cell scoring results that accompany the images provide supplemental descriptions of our observations. They will undoubtedly differ in detail, however, from those of another observer, who sets their localization category dividing lines differently. Experiments similar to those described were repeated two or more times with similar results. Experiment to experiment variation can be ascertained by comparing similar experimental conditions.
within the present work (e.g. the 30 °C proline value in Fig. 2B, with the 0 min or control proline values in Fig. 2, D–F, Fig. 3, B and D, Fig. 7F, and Fig. 11B, or similar time points for the ure2 mutant experiments in Fig. 11, B and D), as well as between similar experimental conditions in present and previous work (33, 35). Finally, although we have used the appearance of Gln3-Myc13 fluorescence in both cellular compartments as a descriptor of our observations, we do not a priori assume this appearance represents a separate and unique physiological state.

In Vitro Alkaline Phosphatase Treatment of Cell Extracts—Alkaline phosphatase treatment of crude cell extracts was performed as described earlier (19).

RESULTS

Intracellular Localization of Gln3-Myc13 Responds to Increased Temperature—This investigation was originally initiated to study the effects of altering Tap42 activity on intracellular Gln3-Myc13 localization and phosphorylation. The mutant we elected to analyze contained the temperature-sensitive tap42-11 allele. Our study began with several control experiments, the most important of which tested the prerequisite condition that increased temperature, required to alter some Tap42 activities, did not otherwise affect Gln3-Myc13 behavior in wild type cells. As expected, Gln3-Myc13 was cytoplasmic in ammonia-grown cells whether cultured at 24 and 30 °C or shifted from 24 to 37 °C for 45 min (Fig. 2A, Am). To provide a more complete picture of what we observed microscopically, we categorized Gln3-Myc13 localization (see “Materials and Methods”) in multiple fields of the same slide from which the images in Fig. 2A were taken (Fig. 2B, left histogram). In proline-grown cells, Gln3-Myc13 was, as also expected, nuclear in most cells cultured at 24 or 30 °C (Fig. 2, A, Pro, and B, right histogram). Unexpectedly, however, Gln3-Myc13 was cytoplasmic in
nearly all proline-grown cells transferred from 24 to 37 °C (Figs. 2, A, Pro, and B, right histogram).

Although Gln3-Myc13 localization appeared to respond markedly to increased temperature, it was possible the effect did not derive from increasing the temperature to 37 °C but from transferring the cells to fresh pre-aerated 37 °C medium, i.e. it was the fresh medium rather than increased temperature that caused Gln3-Myc13 relocation. Testing this possibility, we observed that transferring proline-grown cells cultured at 30 °C to fresh 30 °C proline medium resulted in transient relocation of Gln3-Myc13 from the nucleus to the cytoplasm, coinciding with an earlier observation (Fig. 2, C and D, left histogram) (38). Relocation derived from the fresh medium rather than the transfer process itself, because it did not occur when cells were harvested by filtration and returned to the same spent medium (Fig. 2, C, spent medium, and D, right histogram). Although we have not identified the source of this perturbation, it does not derive from changes in pH that occur during growth. The same results occurred whether or not the pH of the fresh medium was adjusted to that of the spent medium prior to cell transfer (data not shown). To eliminate the medium shift variable all together, we increased the temperature of a culture (50 ml) grown at 24 to 37 °C by transferring it to a 1-liter flask that was adjusted to that of the spent medium prior to cell transfer (data not shown). To determine whether decreased Gln3-Myc13 mobility at the higher temperature was associated with proline as the nitrogen source, or was a general characteristic of temperature stress per se, we performed a further experiment using ammonia-grown cells. Gln3-Myc13 mobility detectably decreased within 15 min of transferring ammonia-grown cells from 24 to 37 °C (Fig. 4, B, lane A, and B), reaching minimal levels by 30 min post-transfer (Fig. 4B, lane C). The fastest migrating species in

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**Intracellular Gln3-Myc13 Localization Is Sensitive to Osmotic Stress**—The third environmental condition we assayed was oxidative stress by adding a low concentration of hydrogen peroxide (0.3 mM) to a proline-grown culture. Gln3-Myc13 was localized to the nuclei of the majority of untreated cells (Fig. 3, A and B, None). In contrast, Gln3-Myc13 was cytoplasmic or nuclear-cytoplasmic in the majority of cells following the addition of hydrogen peroxide (Figs. 3A, \(H_2O_2\) and B, \(H_2O_2\)). The response to hydrogen peroxide addition was less drastic than observed with NaCl or sorbitol, i.e. more nuclear staining remained following treatment with hydrogen peroxide than with NaCl or sorbitol (Fig. 3, A, \(H_2O_2\), NaCl, and Sorb., and B, \(H_2O_2\), NaCl, and Sorb.).

Together, the above data suggested intracellular Gln3-Myc13 localization responded to multiple environmental stresses. It is important to note that experiments measuring intracellular Gln3-Myc13 localization could not be performed with ammonia or glutamine as nitrogen sources, because Gln3-Myc13 is already localized to the cytoplasm of nearly all cells under these growth conditions (Fig. 2, A, Amn., and B, Fig. 7, C and D, and Fig. 7A in Ref. 33). Finally, deletion of SIT4 in proline-grown cells had no demonstrable effect on stress-induced Gln3-Myc13 intracellular localization (data not shown); Gln3-Myc13 was cytoplasmic in un stressed, proline-grown sit4Δ in this strain background.

**Gln3-Myc13 Phosphorylation Increases in Response to Temperature-induced Stress**—Dramatic intracellular relocation of Gln3-Myc13 observed in response to environmental stresses raised the possibility that its phosphorylation state might be affected in parallel. Therefore, proline-grown wild type cells were maintained at 24 °C or transferred from 24 to 37 °C, using the 1-liter heat-sink flask method described above. Crude cell extracts were then prepared, and Gln3-Myc13 phosphorylation was assayed by Western blot analyses. Gln3-Myc13 mobility decreased in extracts derived from cells shifted to 37 °C relative to those at 24 °C (Fig. 4A, lane A, and C versus B and D), behavior characteristic of increased phosphorylation (Fig. 4 of Ref. 32). To determine whether decreased Gln3-Myc13 mobility at the higher temperature was associated with proline as the nitrogen source, or was a general characteristic of temperature stress per se, we performed a further experiment using ammonia-grown cells. Gln3-Myc13 mobility detectably decreased within 15 min of transferring ammonia-grown cells from 24 to 37 °C (Fig. 4, B, lane A, and B), reaching minimal levels by 30 min post-transfer (Fig. 4B, lane C). The fastest migrating species in

**FIGURE 2.** Temperature stress or transfer of cells from one medium to another elicits Gln3-Myc13 relocation from the nucleus to cytoplasm of proline-grown cells. Panels A and B, TB123 cells were cultured at 24 °C (24h) or 30 °C (30h) in YNB-ammonia (N/A) or-proline (Pro) medium. Alternatively, they were cultured at 24 °C and then transferred to fresh pre-aerated medium at 37 °C for 45 min. Cells were prepared, and microscopic images obtained, processed, and intracellular Gln3-Myc13 distribution scored as described under “Materials and Methods”; cytoplasmic (red bars), nuclear-cytoplasmic (nucl.-cyto., yellow bars), or nuclear (green bars). Panels C and D, TB123 was cultured to mid-log phase at 30 °C in YNB-proline. At zero time a sample was collected for assay (0’ Control). The remaining culture was divided into two portions. One portion was harvested by filtration and transferred to an equal volume of pre-warmed, pre-aerated medium (Fresh Medium) identical to that in which they were cultured. The second portion was similarly harvested and then resuspended in the same medium from which they had just been harvested (Spent Medium). Samples were then taken for assay at the times (in minutes) indicated. Panels E and F, three cultures of TB123 were grown in YNB-proline medium, two at 24 °C and a third at 30 °C. The temperature of one 24 °C culture (50 ml in a 125-ml flask) was increased by transferring it to a 1-liter flask pre-warmed to 37 °C. After 10 min in that flask, the cells were returned to a 125-ml flask pre-warmed to 37 °C and incubated for an additional 50 min (60 total min at 37 °C). The 30 °C culture (30h) was used as an untreated control. As an additional control, the second 24 °C culture was transferred to a 1-liter flask equilibrated at 24 °C. After 10 min in the 1-liter flask, the culture was returned to a 125-ml flask equilibrated at 24 °C and incubated for 10 min. After the incubations, samples of each culture were collected and processed for assay. In panels A, C, and E, images of DAPI-positive material (blue) are presented below corresponding Gln3-Myc13 images (red).
lane A had disappeared in lane C and a slower mobility species appeared (Fig. 4B, lower and upper black dots, respectively).

Decreased Gln3-Myc13 mobility following temperature shift was similar to that which occurred after Msx treatment, although the relative distribution among the various resolved species was not identical (Fig. 4C, compare lanes A, C, and D). Following Msx treatment at 37 °C, the slowest mobility species of Gln3-Myc13 was predominant (Fig. 4C, lane D), whereas a more even distribution among the three principal Gln3-Myc13 species was observed in extracts of cells subjected to temperature shift in the absence of Msx (Fig. 4C, lane C).

If the decreased Gln3-Myc13 mobility observed above derived from increased phosphorylation, it should be sensitive to in vitro treatment with alkaline phosphatase (1, 4, 19, 32). Therefore, extracts were prepared from cells incubated at 37 °C for 60 min and treated with calf intestine alkaline phosphatase (CIP) with and without sodium pyrophosphate (Na4P2O7).

FIGURE 4. Temperature stress increases Gln3-Myc13 phosphorylation. Panel A, wild type (TB123) cells were cultured in YNB-proline medium at 24 °C and then shifted to 37 °C using the 1-liter heat-sink flask method of temperature shift as described in Fig. 2, E and F. After 60 min of incubation at 37 °C, cells were harvested for Western blot analyses as described under “Materials and Methods.” Panel B, cells were cultured in YNB-ammonia medium at 24 °C, transferred to fresh pre-warmed, pre-aerated ammonia medium at 37 °C, and samples harvested and processed for Western blot assay at the indicated times (in minutes) thereafter. Panel C, cells were cultured to mid-log phase in YNB-ammonia medium at 24 or 30 °C (lanes A, B, E, and F). At that time they were harvested (lanes A and B) or treated for 30 min with Msx and then harvested (lanes E and F). The samples in lanes C and D were cultured at 24 °C to mid-log phase and transferred to pre-aerated, pre-warmed (37 °C) medium for 60 min. The sample in lane C was harvested at that time, whereas the one in lane D was treated with Msx (2 mM final concentration) for an additional 30 min before harvest. Panel D, cells were cultured as in panel A and extracts prepared as described under “Materials and Methods.” Extracts were treated with calf intestinal alkaline phosphatase (CIP) with and without sodium pyrophosphate (Na4P2O7).
Gln3-Myc<sup>13</sup> Phosphorylation Increases in Response to Osmotic Stress—Next, we evaluated the effects of osmotic stress on Gln3-Myc<sup>13</sup> phosphorylation. Addition of 1 M NaCl to proline-, ammonia-, or glutamine-grown cultures strongly decreased Gln3-Myc<sup>13</sup> mobility (Fig. 5, A–C, lanes A versus B). With proline, a slower migrating Gln3-Myc<sup>13</sup> species appeared in the extract from NaCl-treated cells (Fig. 5A, lanes A and B, black dot). With ammonia and glutamine, the fastest migrating Gln3-Myc<sup>13</sup> species observed in the untreated extract disappeared altogether (Fig. 5, A and B, lane A, bottom black dot), and the relative amounts of the remaining species shifted toward the slowest migrating species (lanes A and B, top black dot). We tested whether the decreased Gln3-Myc<sup>13</sup> mobilities noted above derived from increased phosphorylation by assaying their sensitivity to alkaline phosphatase. Alkaline phosphatase sensitivity similar to that observed with increased temperature occurred here as well (Fig. 5, D and E). Therefore, osmotic stress increased Gln3-Myc<sup>13</sup> phosphorylation. Also, as reported earlier, sodium pyrophosphate addition sometimes inexplicably results in a lower protein signal (19, 32).

As occurred with increased temperature, the most highly phosphorylated Gln3-Myc<sup>13</sup> species in extracts from NaCl-treated ammonia- or glutamine-grown cells were similar to that observed with Msx-treated, ammonia-grown cells (Fig. 5, B and C, lanes B and C). With proline, the Gln3-Myc<sup>13</sup> phosphorylation level did not reach that observed with extracts of the Msx-treated cells (Fig. 5A, lanes B and C). The data in Figs. 4 and 5 also demonstrated that stress-generated Gln3-Myc<sup>13</sup> phosphorylation was independent of the nitrogen source provided, because phosphorylation occurred with proline, ammonia, or glutamine medium.

To further test the hypothesis that osmotic stress increased Gln3-Myc<sup>13</sup> phosphorylation in NaCl-treated cells, we assayed cells treated with a different osmotic agent, sorbitol. As shown in Fig. 5F, Gln3-Myc<sup>13</sup> phosphorylation increased within 5 min of sorbitol addition (lanes A and B). The effect began to moderate by 30 min and was less noticeable when extracts from ammonia-grown cells were analyzed following 45 min of treatment (Fig. 5G, lanes A and B). Gln3-Myc<sup>13</sup> phosphorylation profiles observed in glutamine-grown cells (Fig. 5H) were similar to those seen with ammonia (Fig. 5G). Irrespective of the nitrogen source, the level of Gln3-Myc<sup>13</sup> phosphorylation attained was similar, but not identical, to that observed with Msx-treated, ammonia-grown cells (Fig. 5F, lanes E and F, Fig. 5G, lanes B and C, and Fig. 5H, lanes C and D). Together, the above data demonstrate Gln3-Myc<sup>13</sup> phosphorylation increases following the onset of osmotic stress.

Gln3-Myc<sup>13</sup> Phosphorylation Increases in Response to Oxidative Stress—The final stress condition we evaluated was that caused by hydrogen peroxide. In a manner similar to other stress conditions, 0.3 mM hydrogen peroxide (30 min.) increased Gln3-Myc<sup>13</sup> phosphorylation in extracts from glutamine-grown cells (Fig. 6A, lanes A and B). The fastest migrating Gln3-Myc<sup>13</sup> species was absent (Fig. 6A, lanes A and B, bottom black dot), and the slower migrating species were much more prominent (top black dot). Similar results occurred in ammonia-grown cells (Fig. 6B). The extent of phosphorylation, however, did not occur to the extent observed following Msx.
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Treatment (Fig. 6, A and B, lanes B and D). It is pertinent, in this regard, to note that we used a relatively low concentration of hydrogen peroxide (0.3 mM) compared with those we had used in studies of Ure2 function (2 or 3 mM) (30, 31). This was done to avoid cell injury and possible secondary effects derived from it. The somewhat diminished phosphorylation response correlated with the fact that Gln3-Myc<sup>13</sup> relocalization to the cytoplasm following hydrogen peroxide addition appeared less strong than observed with NaCl (Fig. 3, A and B).

Effects of Caffeine Treatment on Gln3-Myc<sup>13</sup> Phosphorylation and Intracellular Localization Are Similar to Those Elicited by Rapamycin—

Kuranda et al. (39), investigating the mechanisms of caffeine toxicity, reported results of a transcriptome experiment in which treating cells with caffeine elicited increased expression of many NCR-sensitive genes. The transcription profile of caffeine-treated cells resembled that observed following rapamycin treatment (39, 40). These observations prompted us to determine whether caffeine-induced Gln3-Myc<sup>13</sup> phosphorylation and localization paralleled the profile seen in rapamycin-treated cells or, alternatively, Msx-treated cells. Both inhibitors induce increased NCR-sensitive transcription and nuclear Gln3-Myc<sup>13</sup> localization (1–4, 18, 19) but in our hands oppositely affect its phosphorylation. Rapamycin decreases Gln3-Myc<sup>13</sup> phosphorylation, whereas Msx increases it (19). As shown in Fig. 7, A and B, caffeine addition, like rapamycin treatment, elicited Gln3-Myc<sup>13</sup> relocalization from the cytoplasm to the nucleus of glutamine-grown cells. Neither inhibitor elicited nuclear Gln3-Myc<sup>13</sup> localization in a sit4Δ (Fig. 7, A and B).

The effects of caffeine addition on Gln3-Myc<sup>13</sup> localization extended to, and were more easily observed with, ammonia-grown cells. Gln3-Myc<sup>13</sup> was highly nuclear in nearly all caffeine-treated cells, compared with only about half of those treated with rapamycin (Fig. 7, C and D).
Additionally, as we observed with Msx (19), the Sit4 requirement for nuclear localization of Gln3-Myc13 in ammonia-grown cells was weaker for caffeine than rapamycin treatment (Fig. 7, C and D).

Responses to caffeine and rapamycin treatment were indistinguishable in wild type cells provided with proline as nitrogen source (Fig. 7, E and F). The two inhibitors also elicited localization profiles that were indistinguishable from one another in a proline-grown sit4Δ. However, Gln3-Myc13 was less nuclear in caffeine-treated sit4Δ cells than wild type and than observed earlier when sit4Δ cells were treated with Msx (compare Fig. 7, E and F, with Fig. 6D of Ref. 33).

The similarity of Gln3-Myc13 intracellular localization in caffeine- and rapamycin-treated cells (Fig. 8A) suggested the transcription factor might also be dephosphorylated in caffeine-treated cells. As shown in Fig. 8B, Gln3-Myc13 was dephosphorylated (higher electrophoretic mobility) within 5 min of adding caffeine to ammonia-grown cells. The phosphorylation profile was indistinguishable from that observed following rapamycin treatment (Fig. 8B, lanes D and E). Also like rapamycin treatment (19, 32), Gln3-Myc13 dephosphorylation did not reverse even after extended treatment (Fig. 8C, lanes B and E).

**Environmental Stress Predominates over Nitrogen-dependent and Tor1,2 Regulation of Gln3-Myc13.—** The above results demonstrated that Gln3-Myc13 localization to be strongly responsive to environmental stresses. The fact that Gln3-Myc13 responses to stress were the opposite of those elicited by nitrogen limitation prompted us to investigate which response predominated when the two perturbations were applied together. We addressed this question by following Gln3-Myc13 localization in ammonia-grown wild type (TB123) cells initially starved for nitrogen and then subjected to osmotic stress via addition of 1 M NaCl. Gln3-Myc13 remained cytoplasmic throughout the 2-h experiment in unstarved control cells and, as expected, relocated to the nuclei of most nitrogen-starved cells within 30 min (Fig. 9, A and B). In contrast, irrespective of whether NaCl was added following 30 or 60 min of nitrogen starvation, Gln3-Myc13 became cytoplasmic (Fig. 9, A and B).

These results argued that when cells were subjected to both nitrogen starvation and osmotic stress, it was to stress that Gln3-Myc13 appeared to respond. Because the stress response (NaCl treatment) also predominated over the response to limited nitrogen (Proline, None versus NaCl in Figs. 3, A–D, wild type), we inquired whether the response to NaCl treatment predominated over that elicited by the inhibitors we had been using to alter NCR-sensitive transcription and Gln3-Myc13 localization. To this end, we treated ammonia-grown mid-log phase cells, in which Gln3-Myc13 is cytoplasmic, with rapamycin (Fig. 10, A and B), caffeine (Fig. 10, C and D), or Msx (Fig. 10, E and F). Within 7–10 min of inhibitor addition, Gln3-Myc13 became nuclear-cytoplasmic or nuclear in most cells. After being treated for 20 min with these inhibitors, NaCl (1 M) was added to each culture. Within 5 min, Gln3-Myc13 had relocalized to the cytoplasm irrespective of the initial inhibitor treatment (Fig. 10, A–F).

The uniform ability of environmental stress to predominate over the response of the cell to normal environmentally generated nutritional signals, i.e. nitrogen starvation and limitation (growth with proline as nitrogen source) or those elicited by inhibitors (rapamycin, caffeine, or Msx), argued that either (i) stress completely altered, neutralized, or overwhelmed the nutrient signals, or (ii) generated an additional signal to which Gln3-Myc13 preferentially responded. To determine whether the stress response was operating through alteration of the NCR-sensitive regulatory pathway, we eliminated the most Gln3-proximal regulatory element known to participate in nitrogen-responsive regulation, Ure2 (27). NaCl was added to a glutamine-grown ure2Δ. Gln3-Myc13 accumulated in the nuclei of most untreated ure2Δ cells (Fig. 11A and B, ure2Δ Glutamine 0 min), just as it had in the proline-grown wild type (Fig. 11, A and B, Wild Type Proline 0 min). However, within 5 min of adding NaCl to the culture, Gln3-Myc13 was cytoplasmic in both wild type and the ure2Δ (Fig. 11, A and B, +NaCl 5 min). Between 15 and 30 min post-NaCl treatment, Gln3-Myc13 was...
nuclear-cytoplasmic in a small fraction of the cells (Fig. 11, A and B, +NaCl 15 min).

**Relationship between Stress-induced Gln3-Myc<sup>13</sup> Regulation and the GATA Factor Requirement of Ena1-mediated Cation Tolerance.**—When considered together, the above data and several earlier reports describing control of ENA1 expression and cation tolerance appeared paradoxical and therefore required further investigation. There are three _S. cerevisiae_ ENA genes, ENA1, ENA2, and ENA5, whose coding sequences are nearly identical as are the upstream regions of ENA2 and ENA5. All three upstream regions contain multiple GATAA sequences, although their locations relative to the ATG are different in ENA1 than in ENA2 and ENA5. ENA1 expression is activated by Crz1, which must be dephosphorylated by calcineurin before entering the nucleus (41–44). ENA1 transcription is also repressed via Mlg1 and CRE (Skol1) sites regulated by the glucose repression and Hog1 osmotic shock signaling pathways (44–46). A third level of ENA1 regulation, which generated the paradox, is mediated by the GATA factors Gln3 and Gat1.

Three earlier observations framed our thinking about Gln3/Gat1 participation in ENA1 regulation. (i) Withee et al. (41) reported that mutation of _URE2_ increased Na<sup>+</sup> tolerance (1.2 m) of _ena1_ena2 mutants, lacking Ca<sup>2+</sup>/calmodulin-dependent calcineurin, an outcome that required both Gln3 and cation extrusion ATPase, Ena1. _ENA1_ expression increased 20- and 10-fold (30 min after 0.8 m NaCl addition) in wild type and _ena1_ena2 strains, respectively. It did not, however, detectably increase in a _ure2_ mutant whether or not Na<sup>+</sup> was present (41).

(ii) Masuda et al. (47) reported Li<sup>+</sup>, Na<sup>+</sup>, and K<sup>+</sup> induced _SIT4_ expression 2–3-fold. However, _SIT4_ overexpression did not increase Na<sup>+</sup> or K<sup>+</sup> tolerance (only Li<sup>+</sup> tolerance increased), or ENA1 expression, nor was Na<sup>+</sup>- or K<sup>+</sup>-induced ENA1 expression diminished in a _sit4_ mutant (47). (iii) Crespo et al. (48) reported rapamycin-induced _ENA1-lacZ_ mRNA production and showed that 0.4 m NaCl induced _ENA1-lacZ_-mediated β-galactosidase production 4-fold. This induced level decreased 60–70% in a _gln3gat1_ double mutant (48). Together, these observations showed Gln3, Gat1, and Crz1 were responsible, in varying degrees, for high level ENA1 expression, but that cells could get by, albeit less well, with only one of the activation systems operating.

The above observations, and conclusions they support, raised the obvious question of how Gln3 could activate NaCl-induced ENA1 expression in a _ure2Δ_ when Gln3-Myc<sup>13</sup> exited from the nucleus when we treated wild type, and more importantly, _ure2Δ_ cells with 1 m NaCl (Figs. 3, 9, 10, and 11, A and B). Furthermore, the previous experiments cited above were performed in YPD medium where Gln3 is localized to the cytoplasm (Fig. 6B in Ref. 33 and earlier observations from multiple laboratories, e.g. see Ref. 1).

The consistent characteristics of these previous ENA1 observations are as follows: (i) the observed effects are generated by relatively low (<2-fold) to undetectable changes in NaCl-induced, Gln3-dependent levels of _ENA1_ mRNA, and (ii) the Gln3 contribution to NaCl tolerance was most convincingly observed when normal negative regulation of Gln3 was genetically eliminated by deleting _URE2_.

Of the many experiments performed throughout this work, Gln3-Myc<sup>13</sup> was cytoplasmic in nearly all NaCl-treated cells except on the following two occasions: (i) a small number of cells with nuclear-cytoplasmic Gln3-Myc<sup>13</sup> were observed 20 min after NaCl was added to wild type cells previously treated with Mxs (Fig. 10E, NaCl 20 min, and F, right histogram), and (ii) 15 or more min after NaCl was added to a glutamine-grown _ure2Δ_ (Fig. 11, A, +NaCl 15 min, and B). These data suggested that Gln3-Myc<sup>13</sup> might be re-entering the nuclei of NaCl-treated cells, which was not unreasonable given that we had seen signs, in the earlier phosphorylation experiments, that the stress response was not sustained long term (Figs. 5, F and G, and 6A). Also, cells continued to grow following addition of NaCl to the medium, albeit more slowly.

We investigated whether Gln3-Myc<sup>13</sup> could re-enter the nucleus of NaCl-treated _ure2Δ_ cells by extending the time of sampling to 6 h. As shown in Fig. 11, C and D, adding NaCl to the _ure2Δ_ resulted in Gln3-Myc<sup>13</sup> quickly (5–10 min) relocating from the nucleus to the cytoplasm. With increasing time, however, Gln3-Myc<sup>13</sup> began to reappear in the nuclei of NaCl-treated cells. Between 20 and 60 min, Gln3-Myc<sup>13</sup> was cytoplas-
mic in about half the cells and nuclear-cytoplasmic or nuclear in the other half. Thereafter, Gln3-Myc$_{13}$ was again predominantly cytoplasmic (Fig. 11C, 10, 45, and 120 min, and 11D). We anecdotally noted, both here and in other experiments, that the area of DAPI-positive material appeared to shrink transiently in cells treated with NaCl compared with those that were untreated or in those after extended treatment (Fig. 11A, +NaCl 5 min, and C, 10 min).

Finally, we investigated whether treating wild type cells with NaCl elicited detectable changes in NCR-sensitive gene expression. Wild type TB123 was grown in YNB/proline medium and treated with NaCl. Northern blot analyses were unable to detect changes in DAL5 mRNA levels following NaCl treatment (data not shown). This was not a wholly unexpected result because of the following. (i) Whithee et al. (41) did not observe detectable differences in ENA1 expression between wild type and ure2 mutant strains either in the presence or absence of NaCl under conditions that required Gln3 for NaCl tolerance. (ii) We had already observed instances in which the levels of NCR-sensitive gene expression did not closely correlate with intracellular Gln3-Myc$_{13}$ localization, i.e. the level of a gene's expression derives from the combined actions of transcription factors bound to all of the functional cis-acting regulatory elements in its promoter (35). (iii) The effects of NaCl treatment on Gln3-Myc$_{13}$ localization were transient (Figs. 5 and 11).

**DISCUSSION**

**Gln3-Myc$_{13}$ Phosphorylation and Localization Respond in Parallel to Environmental Stress**—Data presented above demonstrate that multiple environmental stresses (temperature, osmotic, and oxidative) increase Gln3-Myc$_{13}$ phosphorylation. In parallel, Gln3-Myc$_{13}$ rapidly relocalizes from the nucleus to the cytoplasm of environmentally stressed cells under conditions that would normally be highly nuclear, i.e. proline-grown, nitrogen-starved, Msx-, caffeine-, and rapamycin-treated wild type cells. The stress responses reported here join our earlier observation that centrifugation, a potentially stressful condition that increases Snf1 phosphorylation (49), also increases the Gln3-Myc$_{13}$ phosphorylation levels (19). This represents a major new type of regulation to which Gln3 is subjected and opens new insight into the regulation of intracellular Gln3 localization. Ever since the discovery of Gln3 and its requirement for NCR-sensitive gene expression, investigations have focused on elucidating how the nitrogen supply of a cell regulates Gln3 function and thereby the wide array of genes whose expression depends upon it. As important as that is, we would be unable to adequately understand Gln3 regulation without recognizing that other environmental inputs can at times predominate over nitrogen supply, if only transiently.

**Stress and Nutrient-induced Gln3-Myc$_{13}$ Regulation Are Separable with the Stress-induced Control Being Dominant**—The insight gained from the experiments in this work concerns the overall mechanisms through which intracellular Gln3 localization is regulated. Heretofore, regulated Gln3 localization was thought to depend upon Ure2. Two alternative models suggested nuclear exclusion of Gln3 was because of phosphorylation-promoted complex formation with Ure2 or Ure2-depend-
ent stabilization of phosphorylated Gln3. However, our experiments clearly demonstrate stress-induced, Ure2-independent nuclear Gln3 exclusion. Therefore, the stress-induced Gln3-Myc\textsuperscript{13} exit from the nucleus derives from a regulatory pathway that operates in parallel with Ure2-dependent regulation, but impinges on Gln3 localization downstream of Ure2, or is capable of overwhelming responses elicited by limiting nutrients and inhibition of Tor1,2 and/or other inhibitor-sensitive regulatory molecules. These possibilities are supported by the following observations: (i) nuclear Gln3-Myc\textsuperscript{13} exit occurred when environmental stress was imposed after the onset of all five conditions previously reported to result in nuclear Gln3-Myc\textsuperscript{13} localization, i.e. nitrogen-starvation or limitation, or disruption of Tor1,2 signal transduction pathway activity, i.e. Msx, rapamycin, or caffeine treatment. (ii) Stress-induced Gln3-Myc\textsuperscript{13} phosphorylation occurred irrespective of the nitrogen source provided, i.e. similarly in proline-, ammonia-, and glutamine-grown cells. (iii) Stress-induced re-localization of Gln3-Myc\textsuperscript{13} occurred even when the most downstream regulatory event known to affect Gln3 localization, and suggested to be regulated by Tor1,2, was eliminated, i.e. loss of Ure2 in a ure2\Delta.

Although we are in the initial stages of investigating the response of Gln3-Myc\textsuperscript{13} phosphorylation and localization to multiple environmental insults, it is most straightforwardly characteristic of increased protein kinase activity. The absence of effect when HOGL\textsuperscript{1} was deleted suggests that Gln3-Myc\textsuperscript{13} phosphorylation is not occurring through that pathway and increases the likelihood of it occurring via a generalized stress-response pathway. Furthermore, the failure of osmotic stress-induced Gln3 regulation to be affected in ure2\Delta and sit4\Delta strains is consistent with the possibility of the pathway being mechanistically distinct from Tor1,2-influenced regulation. In agreement with this contention, SIT4 overexpression does not increase Na\textsuperscript{+} or K\textsuperscript{+} tolerance (only Li\textsuperscript{+} tolerance increased) or ENA1 expression, nor is Na\textsuperscript{+}- or K\textsuperscript{+}-induced ENA1 expression diminished in a sit4 mutant (47). Alternatively, the results we observed would be those expected if Ure2 functioned to stabilize the phosphorylated form of Gln3, and it was this form that was excluded from the nucleus. On the other hand, it must be remembered that Msx treatment increased gross Gln3-Myc\textsuperscript{13} phosphorylation as well as nuclear localization.

Caffeine-induced Gln3-Myc\textsuperscript{13} Dephosphorylation and Nuclear Localization—Data from experiments involving caffeine complement previously reported genome-wide transcription analyses that showed caffeine treatment increases expression of many NCR-sensitive genes (39). Those data led to the conclusion that the effects of caffeine treatment were intimately related to Tor1,2 regulation because caffeine and rapamycin treatments generated such similar outcomes. We extended those transcriptional correlations here by demonstrating that caffeine treatment induces Gln3-Myc\textsuperscript{13} dephosphorylation and nuclear localization. They do not, however, permit us to conclude that rapamycin and caffeine operate by the same bio-

![Figure 11. Panels A and B, NaCl reverses the effects of a ure2\Delta on Gln3-Myc\textsuperscript{13} localization. Wild type and ure2\Δ cultures were grown in YNB-proline and -glutamine media, respectively. NaCl (1 M) was added immediately after the zero time point sample was taken. Cultures were then sampled every 5 or 10 min thereafter and assayed as described in Fig. 2. A similar experiment was performed for panels C and D except that its duration which was much longer.](image-url)
chemical mechanisms, only that their outcomes correlate with one another. They also do not exclude the possibility that caffeine possesses more than one site of action. Multiple sites of caffeine action have been reported, the most recent of which is at TORC1 (40).

Relationship between Stress-induced Gln3-Myc\textsuperscript{13} Regulation and the GATA Factor Requirement of Ena1-mediated Ion Tolerance—Initially, past experiments describing Gln3 participation in ENA1 expression (41, 48) appeared inconsistent with the present results. We argued above that relatively low levels of nuclear Gln3 were sufficient to support the requirements of ENA1 expression required for NaCl tolerance in wild type cells and that increased tolerance occurred when Gln3 regulation was abrogated. Several observations justify this contention. (i) Pertinent earlier experiments were performed in YPD medium where Gln3 is cytoplasmic in wild type cells and hence little would be available in the nucleus to support ENA1 transcription (41, 48). This is probably why Gln3-mediated suppression of the cna1cna2 growth phenotype occurred only in a ure2 mutant where NCR-sensitive Gln3 regulation and effects of growth with a rich nitrogen source, e.g. YPD, were abrogated (41). In other words, elimination of Ure2 was only required when the ENA1 transcriptional activator, Crz1, was unable to function because it could not be dephosphorylated in the cna1cna2 mutant. (ii) NaCl tolerance was similar in YPD-grown wild type, gln3gat1, or gln3 cells and was only a little greater in a gat1 or ure2 mutant (Fig. 3A, right panel in Ref. 48). Much more important, significantly greater NaCl tolerance was seen in either ure2gln3 or ure2gat1 double mutants than any of those just mentioned (Fig. 3A, right panel in Ref. 48). In our view, the additional necessity of inactivating GLN3 or GAT1 in a ure2 mutant suggests salt tolerance observed by Crespo et al. (48) required not only increased nuclear access of Gln3 or Gat1 but also elimination of another form of negative regulation deriving from gene expression or some other process that depends upon both Gln3 and Gat1. (iii) The 30-min NaCl induction experiment of Crespo et al. (48) was performed with lower NaCl (0.4 M) than used in the growth experiments. This condition would generate less osmotic stress and quicker recovery. Reasoning that Gln3 normally plays a limited role in ENA1 expression, and the immediate response to NaCl-generated osmotic stress, coupled with the observation that NaCl-induced relocation of Gln3-Myc\textsuperscript{13} from the nucleus to the cytoplasm is transient may rectify past and present observations.

The relationship of Gln3 to LiCl tolerance possesses, with one important exception, many of the same characteristics as that of for NaCl tolerance (48). In one report, sit4 single and gln3gat1 double mutants decreased NaCl-induced ENA1-lacZ-mediated β-galactosidase to the same degree (48). We did not pursue rectifying this observation with those of Masuda et al. (47) that Li\textsuperscript{+}, Na\textsuperscript{+}, and K\textsuperscript{+} induce Sit4 expression 2–3-fold. However, Sit4 overexpression does not increase Na\textsuperscript{+} or K\textsuperscript{+} tolerance (only Li\textsuperscript{+} tolerance increased), or ENA1 expression, nor is Na\textsuperscript{+}- or K\textsuperscript{+}-induced ENA1 expression diminished in a sit4 mutant (47).

Together, these past and present data have highlighted the complex relationship that exists between NaCl tolerance and NCR-regulated Gln3. They also point to an important but unanswered question: why do we and others fail to observe steady state mRNA levels and intracellular Gln3–Myc\textsuperscript{13} localization fluctuating in parallel under conditions where Gln3 can be convincingly shown to participate in stress-induced processes? Clearly, much more remains to be learned before sufficient information exists to extend current mechanistic models of these regulatory pathways. However, knowing that stress-responsive Gln3 regulation exists is the important and successful first step required to achieve that goal.

Influence of Stress-induced Gln3-Myc\textsuperscript{13} Regulation on Experiments Employing Temperature-sensitive Mutations—Finally, our experiments were initiated to investigate a temperature-sensitive mutant. However, their outcome argues that measuring the effects of temperature-sensitive mutant phenotypes on Gln3 regulation cannot be straightforwardly interpreted, at least over short time frames, because the experiments contain two variables, i.e. possible effects of the mutation itself and those generated by the increased temperature required to alter the mutant protein.

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