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NPR1 Kinase and RSP5-BUL1/2 Ubiquitin Ligase Control GLN3-dependent Transcription in *Saccharomyces cerevisiae**

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The GATA transcription factors GLN3 and GAT1 activate nitrogen-regulated genes in *Saccharomyces cerevisiae*. NPR1 is a protein kinase that controls post-Golgi sorting of amino acid permeases. In the presence of a good nitrogen source, TOR (target of rapamycin) maintains GLN3 and NPR1 phosphorylated and inactive by inhibiting the type 2A-related phosphatase SIT4. We identified NPR1 as a regulator of GLN3. Specifically, loss of NPR1 causes nuclear translocation and activation of GLN3, but not GAT1, in nitrogen-rich conditions. NPR1-mediated inhibition of GLN3 is independent of the phosphatase SIT4. We also demonstrate that the E3/E4 ubiquitin-protein ligase proteins RSP5 and BUL1/2 are required for GLN3 activation under poor nitrogen conditions. Thus, NPR1 and BUL1/2 antagonistically control GLN3-dependent transcription, suggesting a role for regulated ubiquitination in the control of nutrient-responsive transcription.

In yeast, the quality of the available nitrogen source controls the expression of genes encoding proteins required for the uptake and assimilation of nitrogenous compounds. This regulatory process is mediated by four GATA transcription factors, the activators GLN3 and GAT1/NIL1 and the repressors DAL80 and DEH1/NIL2/GZF3. GLN3 is the major activator of nitrogen-regulated genes (for recent reviews, see Refs. 1 and 2). URE2 is a GLN3-binding protein that retains GLN3 in the cytoplasm to prevent its nuclear translocation and activation during growth under good nitrogen conditions.

The two homologous kinases TOR1[1] and TOR2 are central controllers of cell growth, and their inactivation with rapamycin causes physiological changes characteristic of nutrient-starved cells (3, 4). A TOR signaling pathway regulates the expression of nitrogen-regulated genes by inhibiting GLN3 and GAT1 (5). In the presence of a preferred nitrogen source such as NH_4^+ or glutamine, TOR promotes complex formation between GLN3 and URE2 by maintaining GLN3 in the phosphorylated state necessary for URE2 binding (5). Upon nitrogen limitation or rapamycin treatment, the TOR-controlled phosphatase SIT4 causes GLN3 dephosphorylation, and GLN3 dissociates from URE2 and translocates into the nucleus to activate target genes (5).

NPR1 encodes a Ser/Thr kinase that controls the post-Golgi sorting and degradation of amino acid permeases. NPR1 inversely regulates GAP1, the general amino acid permease, and TAT2, a tryptophan permease (2). In cells grown in poor nitrogen sources, NPR1 is dephosphorylated by the TOR-modulated SIT4 phosphatase to become active (6). In turn, active NPR1 allows GAP1 to reach the plasma membrane and causes the rapid degradation of TAT2. In cells shifted to a good nitrogen source medium, plasma membrane GAP1 is internalized and pre-existing permease is no longer delivered to the plasma membrane, whereas TAT2 is stabilized at the plasma membrane (7–9). GAP1 down-regulation occurs via its ubiquitination by the E3 ubiquitin protein ligase RSP5 in conjunction with the RSP5-binding proteins BUL1 and BUL2 (8, 10, 11).

Here, we identify NPR1 as a negative regulator of GLN3-dependent transcription. Deletion of *NPR1* causes nuclear localization and activation of GLN3 during growth on a good nitrogen source. Activation of GLN3 in *npr1* mutant cells requires the presence of functional RSP5 and BUL1/2 proteins, and BUL1/2 proteins are necessary for the activation of GLN3 under nitrogen starvation conditions. Thus, a ubiquitin-dependent signaling pathway appears to control GLN3-dependent transcription.

MATERIALS AND METHODS

Strains, Media, and Reagents—The complete genotypes of the yeast strains used in this study are listed in Table I. Standard techniques and media were used (22). The SD medium contained NH_4^+ as nitrogen source and the amino acids required for auxotrophies. All cultures were incubated at 30 °C. Rapamycin was used at a final concentration of 200 ng/ml.

Plasmids—pJC10 (YCplac111-based), used to screen for regulators of GLN3, was obtained by PCR amplification of the *MEP2* promoter (from nucleotide −827 to −1) and the *HIS3* gene (from nucleotide 1 to 913). pJC20 expressing functional single HA-tagged NPR1 under control of own promoter was obtained by cloning a 2.2-kb fragment containing the *NPR1* promoter and a 2.6-kb fragment from pAS103 (6) containing HA-NPR1 into YCplac33. pJC21 expressing kinase-dead single HA-tagged NPR1 was constructed by replacing the 2.6-kb fragment from pJC20 by a 2.6-kb fragment from pAS104 containing HA-NPR1 with the mutation K467R. YCpMEP2-lacZ was described previously (12).

Screen for Regulators of GLN3—TB50 cells containing pJC10 were transformed with DNA from an mTn-3×HA/GFP/URA3 library, grown in SD medium for 2 h, and plated on SD-Ura-Leu-His medium supplemented with 5 mM 3-amino-1,2,4-triazole. Colonies that formed after 4 days incubation at 30 °C were tested for growth on plates of SD medium without His containing 10 mM 3-amino-1,2,4-triazole. The genomic site of transposon insertion was identified following the "vectorette PCR" method (23).

Microarray Analysis—Total RNA was prepared by hot phenol extraction (24) from duplicate samples grown in SD medium to mid-logarith-

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[1] The abbreviations used are: TOR, target of rapamycin; E3, ubiquitin-protein isopeptide ligase; E4, ubiquitin-chain assembly factor; HA, hemagglutinin A.

TABLE I
Strains used in this study

Strain	Genotype
JK9-3d**a**	*MAT***a** *leu2–3,112 ura3–52 trp1 his4 rme1 HML***a**
TB50**a**	*MAT***a** *leu2–3,112 ura3–52 trp1 his3 rme1 HML***a**
23344c	*MAT***a** *ura3*
27038a	*MAT***a** *ura3 npi1*
30788a	*MAT***a** *ura3 npr1::kanMX2*
30788d	*MAT***a** *ura3 npi1 npr1::kanMX2*
JC19-1a	TB50**a** *npr1::HIS3MX*
JC28-1b	TB50**a** *npr1::HIS3MX sit4::kanMX*
JC31-3b	TB50**a** *npr1::HIS3MX gln3::kanMX*
JC32-2a	TB50**a** *npr1::HIS3MX gat1::HIS3MX*
JC35-1c	TB50**a** *ure2::kanMX*
JC40-1a	JK9–3d**a** *ure2::URA3 GAT1-HA-kanMX*
JC48-1c	TB50**a** *npr1::kanMX*
JC52-2a	JK9–3d**a** *npr1::URA3 GLN3-myc13-kanMX*
JC53-1d	JK9–3d**a** *npr1::URA3 GAT1-HA-kanMX*
JC54-5a	TB50**a** *ure2::kanMX sit4::kanMX*
JC59-12a	TB50**a** *npr1::kanMX bul1::kanMX bul2::HIS3MX*
JC60-4b	TB50**a** *bul1::kanMX bul2::HIS3MX*
JC62-1a	TB50**a** *lst4::kanMX*
JC63-8b	TB50**a** *GLN3-myc13-kanMX bul1::kanMX bul2::HIS3MX*
TB102-1a	JK9–3d**a** *gat1::kanMX*
TB103-1d	TB50**a** *gln3::HIS3MX*
TB106-2a	JK9–3d**a** *GAT1-HA-kanMX*
TB123	JK9–3d**a** *GLN3-myc13-kanMX*
TB138-1a	JK9–3d**a** *ure2::URA3 GLN3-myc13-kanMX*
TS64-1a	JK9–3d**a** *sit4::kanMX*

mic phase. For microarray analysis, RNA was reverse transcribed, and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen). The cDNA was used as template for the production of biotin-labeled cRNA using a RNA transcript labeling kit (Enzo). Finally, the labeled cRNA was purified using RNeasy spin columns (Qiagen), fragmented by incubation with potassium/magnesium acetate and hybridized to Affymetrix S98 Yeast GeneChips, as recommended by the manufacturer. Raw expression signals for each transcript were computed using the algorithm implemented in MAS 5.0 (Affymetrix). The robust multichip average method, implemented as a part of the BioConductor (bioconductor.org) package affy, was employed for data normalization, background correction, and summarization (25).

Indirect Immunofluorescence—GLN3-Myc and GAT1-HA were visualized by indirect immunofluorescence on whole fixed cells as described (26). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at a concentration of 1 μg/ml. Cells were visualized with a Zeiss Axiophot microscope (×100 objective).

Western Blot—GLN3-Myc was detected by Western blot as described previously (5).

Determination of Intracellular Amino Acid Pools—The intracellular levels of amino acids were determined as described (26).

RESULTS

Identification of the NPR1 as a Negative Regulator of Nitrogen-controlled Genes—To find new regulators of GATA transcription factors, we performed a transposon insertion screen to identify mutations in genes whose products are required to repress GLN3 and/or GAT1 under rich nitrogen conditions. A strain expressing the *HIS3* gene from the GLN3-/GAT1-dependent promoter of the NH_4^+ permease *MEP2* (P_{MEP2}-*HIS3*) (12) cannot survive in NH_4^+ medium lacking histidine because GLN3 and GAT1 are inactive, whereas *e.g. his3 ure2* cells can grow under the same conditions because of the constitutive activation of at least GLN3 (Fig. 1*A*). We identified a mutation in *NPR1* that allows growth of *his3* P_{MEP2}-*HIS3* cells on NH_4^+ medium lacking histidine (Fig. 1*A*), suggesting that NPR1 is normally required to repress GLN3 and/or GAT1 during growth in a good nitrogen source.

To confirm that *npr1* causes expression of *MEP2*, we assessed the expression of a second reporter, P_{MEP2}-*lacZ,* in wild type, *npr1*, and *ure2* cells. Expression from P_{MEP2}-*lacZ* was ~12 times higher in *npr1* cells and ~25 times higher in *ure2* cells compared with that from the wild-type strain (Fig. 1*B*).

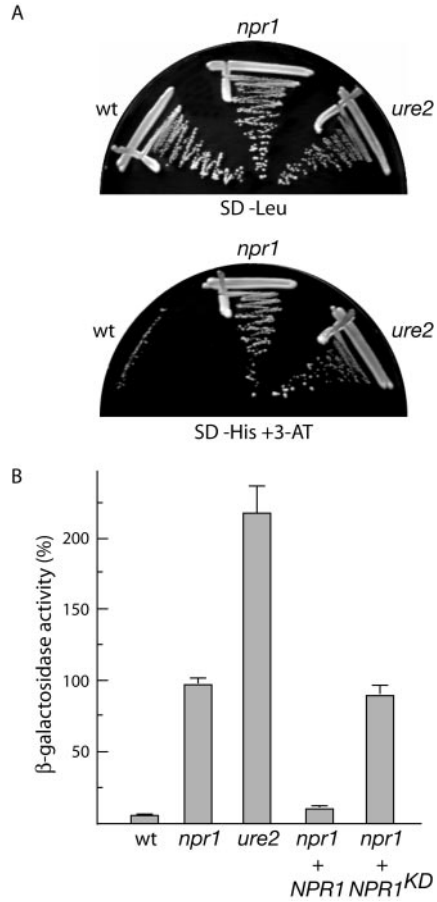

FIG. 1. **NPR1 inhibits expression of a GLN3/GAT1-regulated gene, *MEP2*.** *A*, loss of NPR1 or URE2 function allows growth on NH_4^+ medium lacking histidine in *his3*-deficient cells expressing *MEP2*-driven *HIS3*. Wild-type (*wt*) (TB50), *npr1* (JC48-1c) and *ure2* (JC35-1c) mutant cells harboring pJC10 were streaked on NH_4^+ medium lacking leucine (*SD −Leu*) or NH_4^+ medium lacking histidine (*SD −His*) supplemented with 10 mM 3-amino-1,2,4-triazole (*3-AT*). *B*, wild-type (TB50), *npr1* (JC19-1a), and *ure2* (JC35-1c) mutant cells transformed with YCpMEP2-lacZ were grown in SD medium to an A_{600} of 0.5. Cells were then collected and processed for β-galactosidase assay. *npr1* mutant cells were also transformed with pJC20 or pJC21 and processed for β-galactosidase assay. 100% of β-galactosidase activity corresponds to 1149 Miller units.

NPR1 is a protein kinase, and we transformed *npr1* cells with plasmids carrying *NPR1* (pJC20) or $npr1^{kin-}$ that encodes kinase-dead NPR1 (pJC21). Only the kinase-dead *NPR1* failed to reduce P_{MEP2}-*lacZ* expression, suggesting that the kinase activity of NPR1 is required for the repression of *MEP2* expression (Fig. 1*B*).

NPR1 Represses GLN3 but Not GAT1—To determine whether the effect of NPR1 on transcription is mediated by GLN3 and/or the GLN3-like GATA factor GAT1, we measured β-galactosidase activity in *npr1*, *npr1 gln3,* and *npr1 gat1* mutants containing P_{MEP2}-*lacZ*. Deletion of *GLN3* abolished P_{MEP2}-*lacZ* expression in an *npr1* mutant, whereas loss of *GAT1* had no significant effect (Fig. 2*A*). To extend these observations, we assessed GLN3 and GAT1 localization in wild-type and *npr1* cells. GLN3 was localized in the cytoplasm in wild-type cells but was partially nuclear in *npr1* cells (Fig. 2*B*). This nuclear accumulation of GLN3 in *npr1* cells was less pronounced than in *ure2* cells where GLN3 is strongly nuclear (Fig. 2*B*), which is consistent with the levels of P_{MEP2}-*lacZ* expression in *npr1* and *ure2* strains (Fig. 1*B*). In contrast, GAT1 was cytoplasmic in both wild-type and *npr1* cells (Fig. 2*B*). In addition, in a *ure2* strain there was only a weak accu-

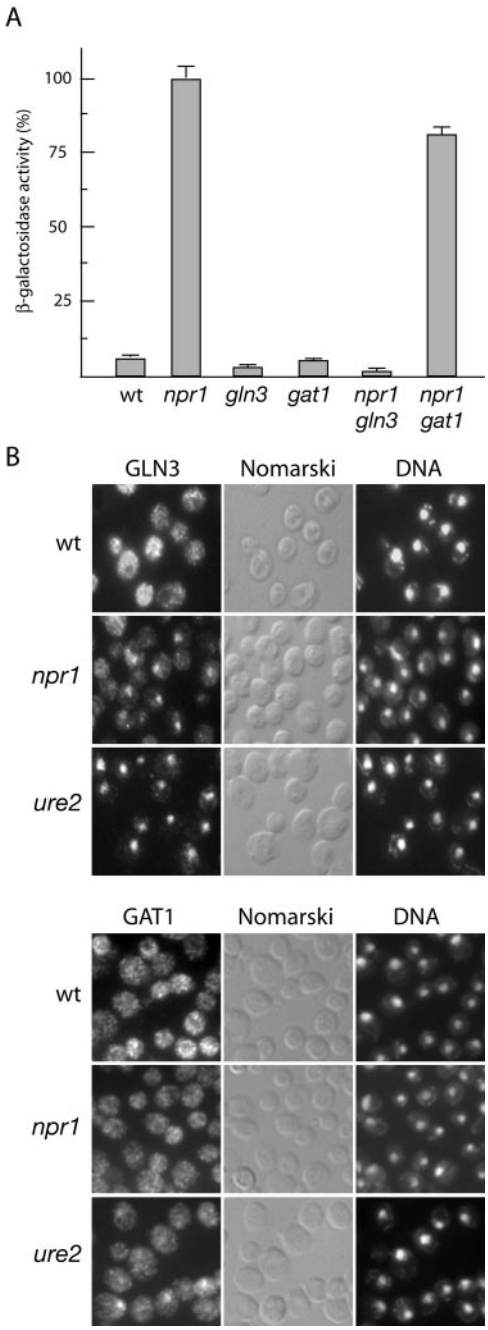

FIG. 2. **NPR1 inhibits GLN3 but not GAT1.** *A*, β-galactosidase activity in wild-type (*wt*) (TB50), *npr1* (JC19-1a), *gln3* (TB103-1d), *gat1* (TB102-1a), *npr1 gln3* (JC31-3b), and *npr1 gat1* (JC32-2a) mutant cells transformed with YCpMEP2-lacZ. All strains have similar growth rates under the test conditions. 100% of β-galactosidase activity corresponds to 1087 Miller units. *B*, localization of GLN3-Myc (*GLN3*) and GAT1-HA (*GAT1*) in wild-type (TB123 for GLN3 and TB106-2a for GAT1), *npr1* (JC52-2a for GLN3 and JC53-1d for GAT1), and *ure2* (TB138-1a for GLN3 and JC40-1a for GAT1) mutant cells. All strains were grown in SD medium at 30 °C to an A_{600} of 0.5. Cells and DNA were visualized by Nomarski optics and 4′,6-diamidino-2-phenylindole.

mulation of GAT1 in the nucleus (Fig. 2*B*), indicating that URE2 regulates the cellular localization of GLN3 but has little effect on GAT1. The above results indicate that NPR1 inhibits GLN3 but not GAT1.

NPR1-mediated Inhibition of GLN3 Is Independent of LST4, LST8, and the Intracellular Level of Amino Acids—NPR1 is a protein kinase required for post-Golgi sorting of the general amino acid permease GAP1 and the three known ammonia permeases, MEP1/2/3, to the plasma membrane. It is therefore possible that the loss of NPR1 may indirectly activate GLN3 by altering amino acid and/or ammonia metabolism. However, under the conditions in which we measured GLN3 activity, GAP1 activity is extremely low regardless of expression levels (13), and even if GAP1 is expressed significantly higher in *npr1* cells as compared with wild type, GAP1 would still not be expected to reach the plasma membrane (14).

To confirm this hypothesis, we considered other mutations known to affect Gap1p sorting to the plasma membrane. *lst4* and *lst8* mutations, like *npr1*, prevent GAP1 arrival at the plasma membrane under poor nitrogen conditions (15). We asked whether an *lst4* mutation, like an *npr1* mutation, affects GLN3 activity. Loss of LST4 failed to induce P_{MEP2}-*lacZ* expression (Fig. 3*A*). We demonstrated previously that cells depleted of LST8 express GLN3-regulated genes at levels similar to those of wild-type cells (16). Thus, although loss of NPR1, LST4, or LST8 can affect GAP1 sorting, loss of only NPR1 results in the activation of GLN3 under our experimental conditions.

Compared with wild type, cells lacking all three ammonia permeases exhibit a severe growth defect in media containing 1 mM NH_4^+ but grow normally on media containing 40 mM or more NH_4^+. Thus, at 76 mM NH_4^+, the conditions our cells experience, there are additional mechanisms to take up NH_4^+ ions, so loss of MEP1/2/3 activity caused by the loss of NPR1 does not appear to affect the availability of ammonia for intracellular metabolism. Furthermore, the *npr1* strain exhibited the same very mild slow growth defect at 1 mM as at 40 mM NH_4^+ as assessed by colony size following serial dilution spotting onto solid minimal media (data not shown). Thus, it is unlikely that GLN3 activation is occurring primarily because of reduced intracellular levels of NH_4^+.

It is possible that GLN3 is activated in *npr1* cells because of reduced intracellular amino acid concentrations resulting from altered trafficking of permeases other than GAP1 or MEP1/2/3. To rule out this possibility, we measured the concentration of amino acids in wild type and *npr1* strains grown in minimal media (SD) containing only those amino acids required to correct for auxotrophies (His, Leu, and Trp). In the *npr1* strain the total amount of all measured amino acids remained similar (Fig. 3*B*). Importantly, glutamine and glutamate levels were only slightly altered in the *npr1* strain as compared with wild type, with glutamine levels slightly increasing and glutamate levels slightly decreasing. We demonstrated previously that GLN3 is activated by a decrease in glutamine (26). Thus, these minor alterations in amino acid concentrations in an *npr1* strain do not correlate with the greatly increased activity of GLN3 in the same strain. Thus, the activation of GLN3 in *npr1* cells does not appear to be due to an indirect effect of altered amino acid concentrations that could occur by inappropriate permease sorting.

NPR1-mediated Inhibition of GLN3 Is Independent of SIT4—NPR1 is regulated in part by SIT4 phosphatase, which, in turn, is regulated by TOR (17). The SIT4 protein phosphatase is also required for GLN3 activation following rapamycin treatment, so we asked whether GLN3 activation in *npr1* cells also requires SIT4. The expression of P_{MEP2}-*lacZ* in wild type and *sit4* strains was similarly low, and in *npr1* and *npr1 sit4* strains it was similarly high (Fig. 4*A*). This epistasis of *npr1* to *sit4* suggests that NPR1 acts downstream of SIT4 in GLN3 repression. We also examined the phosphorylation state of GLN3 in *npr1* mutant cells. In *npr1* cells GLN3 appears to be phosphorylated as in wild-type cells, because rapamycin treatment increases the mobility of GLN3 (Fig. 4*B*). The simplest model compatible with these observation is that SIT4 regulates GLN3 via NPR1, but NPR1 does not regulate GLN3 directly.

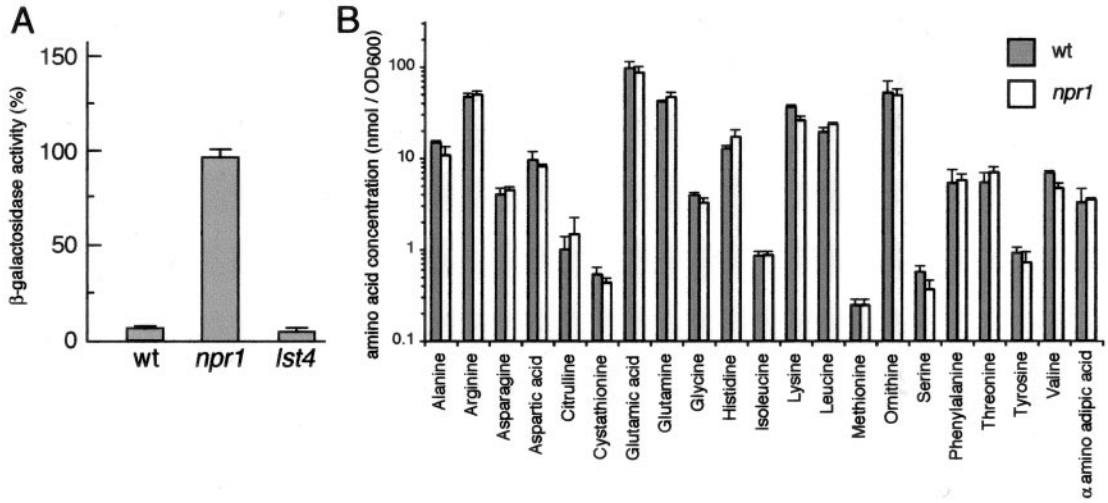

FIG. 3. **Derepression of GLN3 is not due to altered amino acid pools caused by permease sorting defects.** *A*, wild-type (*wt*) (TB50), *npr1* (JC19-1a), and *lst4* (JC62-1a) strains in the JK9 genetic background were transformed with the YCpMEP2-lacZ, grown in SD medium with His, Leu, Trp, and Ura to an A_{600} of 0.5, and processed for β-galactosidase assay. 100% of β-galactosidase activity corresponds to 1319 Miller units. *B*, intracellular pools of amino acids in wild-type (TB50) and *npr1* (JC19-1a) mutant cells grown in SD medium with His, Leu, Trp, and Ura to an A_{600} of 0.5. Units are nanomoles of amino acids per A_{600} of yeast cells; values represent mean ± S.D. of three determinations.

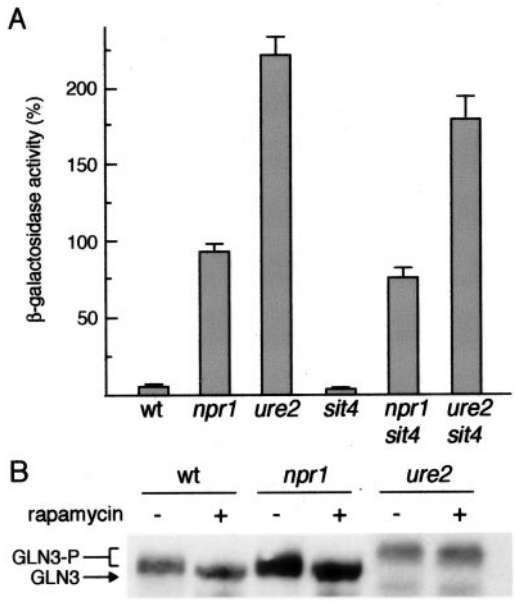

FIG. 4. **NPR1-mediated inhibition of GLN3 is independent of SIT4.** *A*, β-galactosidase activity in wild-type (*wt*) (TB50), *npr1* (JC19-1a), *ure2* (JC35-1c), *sit4* (TS64-1a), *npr1 sit4* (JC28-1b), and *ure2 sit4* (JC54-5a) mutant cells transformed with YCpMEP2-lacZ. Cells were grown and processed as described in the Fig. 1*B* legend. 100% of β-galactosidase activity corresponds to 1179 Miller units. *B*, GLN3 is phosphorylated in an *npr1* mutant and hyperphosphorylated in a *ure2* mutant. Wild-type (TB123), *npr1* (JC52-2a), and *ure2* (TB138-1a) mutant cells were grown in SD medium to an A_{600} of 0.5 and treated with either rapamycin or a drug vehicle for 30 min. GLN3 was detected by immunoblotting.

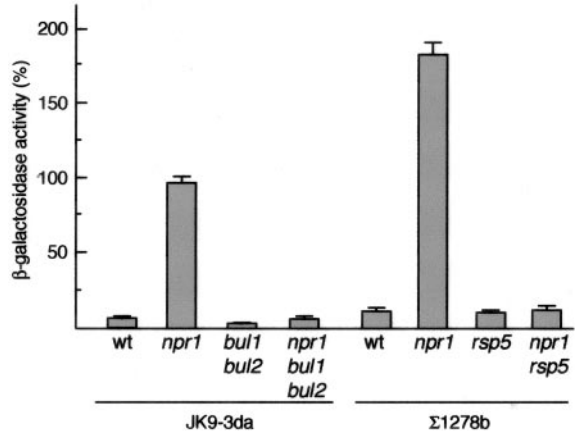

FIG. 5. **Derepression of *MEP2* in an *npr1* mutant requires RSP5, BUL1, and BUL2.** Wild-type (*wt*) (TB50), *npr1* (JC19-1a), *bul1 bul2* (JC60-4b), and *npr1 bul1 bul2* (JC59-12a) and wild-type (23344c), *npr1* (30788a), *rsp5* (27038a), and *npr1 rsp5* (30788d) strains in the Σ1278b genetic background were transformed with the YCpMEP2-lacZ. All strains were grown in SD medium to an A_{600} of 0.5 and processed for β-galactosidase assay. 100% of β-galactosidase activity corresponds to 1319 Miller units.

It has been shown that the GLN3 nuclear importin SRP1 is capable of binding only non-phosphorylated GLN3 *in vitro,* and it has thus been proposed that dephosphorylation of GLN3 is necessary for its nuclear translocation (18). However, in *npr1* and *ure2* cells GLN3 is phosphorylated and predominantly nuclear, suggesting that the phosphorylation state of GLN3 does not directly regulate its translocation. An alternative model consistent with these data is that GLN3 phosphorylation controls its binding to URE2 (5).

Activation of GLN3 in npr1 Mutants Requires RSP5, BUL1, and BUL2—NPR1 and the E3/E4 ubiquitin ligase complex composed of RSP5, BUL1, and BUL2 control nitrogen-regulated trafficking of GAP1 antagonistically (2). To investigate whether RSP5 and BUL1/2 are required for GLN3 activation in *npr1* cells, we analyzed the expression of P_{MEP2}-*lacZ* in wild type or *npr1* cells lacking *RSP5* or *BUL1*/2. The absence of RSP5 or BUL1/2 alone had no effect on *lacZ* activity. However, the mutation of either *RSP5* or *BUL1/2* in *npr1* mutant cells completely suppressed *MEP2* expression (Fig. 5*A*), indicating that RSP5 and BUL1/2 are necessary for GLN3 activation in *npr1* cells. The finding that *rsp5* and *bul1/2* mutations are epistatic to *npr1* suggests that the ubiquitin ligase is downstream of the NPR1 kinase.

BUL1/2 Proteins Are Required for Activation of GLN3 upon a Shift to Proline but Not upon Rapamycin Treatment—To better define the antagonistic role of NPR1 and BUL1/2 in the control of nitrogen-regulated genes, we assessed the relative changes in mRNA levels of a subset of genes known to be regulated by GLN3 in wild type, *npr1*, and *bul1 bul2* cells grown under three different nitrogen related conditions (see "Materials and

TABLE II

Fold-induction of known nitrogen source-regulated genes in the strains and conditions noted

Values given are relative to expression levels in wild type (WT) cells grown in ammonium media and represent the mean of duplicate microarray experiments. References (Refs.) for genes known to be a nitrogen source or GLN3-regulated (Nit./GLN3) are indicated.

Gene	Ammonium			Proline (30 min)			Rapamycin (30 min)			Nit./GLN3 (Refs.)
	WT	*npr1*	*bul1 bul2*	WT	*npr1*	*bul1 bul2*	WT	*npr1*	*bul1 bul2*	
MEP2	1	4.6	0.4	5	4	0.5	6.3	4.3	5.6	12
DUR3	1	2.7	0.2	3.5	2.9	0.2	4.6	2.2	4.2	27
DAL80	1	10.1	1.2	6.1	26.1	1.1	40.6	32.9	37.9	28
DAL5	1	4.9	0.6	8.7	13.9	0.8	21.2	16.4	18	29
DAL7	1	1.8	0.3	3.2	1.8	0.4	2.8	1.2	2.4	29
GAP1	1	2.1	0.3	2.2	2.5	0.8	2.5	2.2	2.4	30
CAN1	1	1.7	0.7	2	2	0.8	1.8	1.7	2.6	30
DAL1	1	2.8	0.7	10.4	3.5	0.9	6.1	2.2	5.2	30
PUT4	1	1.3	0.3	1.6	2.1	0.5	2.2	2.3	1.6	30
DAL3	1	4.6	0.5	10.1	3.4	1.1	8	3.1	10.1	28
OPT2	1	3.4	0.7	5.6	8.4	1	9.5	10.5	8.1	31
CPS1	1	2	0.7	2.5	3.6	0.9	6.6	4.9	3.4	32

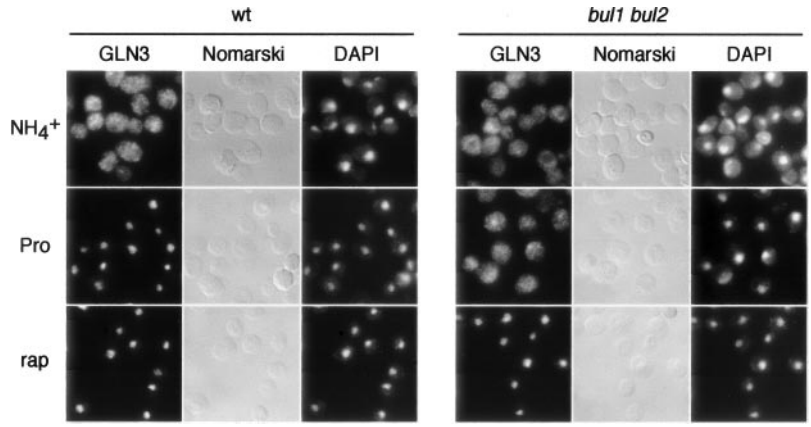

FIG. 6. **BUL1/2 proteins are required for nuclear localization of GLN3 upon nitrogen starvation.** Localization of GLN3-Myc in wild-type (*wt*) (TB123) and *bul1 bul2* (JC63-8b) mutant cells grown in SD medium (NH_4^+) to an A_{600} of 0.5 and shifted from these conditions to a proline-containing medium (Pro) for 30 min or treated with rapamycin (*rap*) for 30 min. Cells were processed as described in the Fig. 2A legend. *DAPI*, 4′,6-diamidino-2-phenylindole.

Methods"). Table II shows the relative changes of a subset of nitrogen source-regulated genes in the three strains mentioned above grown under the following conditions: (i) grown in a good nitrogen source (NH_4^+); (ii) shifted from a good to a poor nitrogen source for 30 min (NH_4^+ to proline); and (iii) grown in a good nitrogen source (NH_4^+) and treated with rapamycin for 30 min. As expected, the known GLN3-regulated genes in Table II were derepressed in *npr1* cells and unaltered or down-regulated in *bul1 bul2* as compared with wild-type, confirming the antagonistic effect of *npr1* and *bul1 bul2* mutations. These genes are also all derepressed upon shifting a wild type strain to either proline or treating with rapamycin, events known to increase GLN3-dependent transcription. Although we are preparing a comprehensive genome-wide study of NPR1/BUL-1/2-dependent gene expression,[2] the subset of known GLN3 regulated genes that we have selected here clearly demonstrates that NPR1 and BUL1/2 are antagonistically involved in the control of GLN3 activity.

Proline failed to induce a significant transcriptional response in *bul1 bul2* cells (Table II), indicating that these ubiquitination factors are an integral part of the proline-sensing transcription pathway. Curiously, rapamycin-induced transcription of the genes in Table II in *bul1 bul2* cells was similar to that of wild type cells. To further investigate the differential effect of proline and rapamycin, we examined the nuclear translocation of GLN3 in wild-type and *bul1 bul2* cells following a shift to proline or rapamycin treatment. Consistent with the above findings, both conditions induced nuclear localization of GLN3 in wild type cells, but only rapamycin induced nuclear localization of GLN3 in a *bul1 bul2* mutant (Fig. 6). Thus, the BUL1/2 proteins are required for nuclear localization of GLN3 upon a shift to proline but not upon rapamycin treatment, supporting previous suggestions that proline is sensed differently and suggesting that the RSP5/BUL1/2 complex is specifically involved in GLN3 regulation. Indeed, growth in proline specifically activates the rapamycin-sensitive proline utilization pathway, including the transcription factor PUT3 (19).

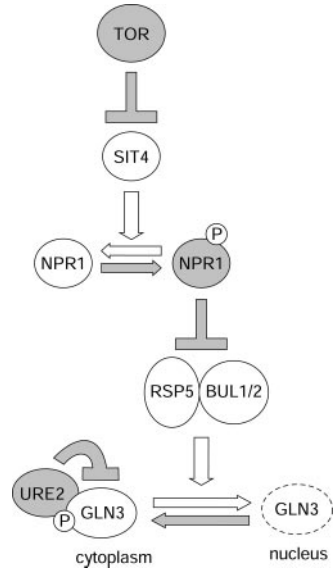

FIG. 7. **A model for the control of GLN3 activation.** *Solid gray* indicates those parts of the pathway that are active in a good nitrogen source. See "Discussion" for more details.

[2] S. Helliwell, C. Wiederkehr, P. Demougin, M. Primig, and M. Hall, manuscript in preparation.

DISCUSSION

We have shown previously that NPR1 is controlled by TOR and the phosphatase SIT4 (17). Here, we show that the kinase NPR1 and the E3/E4 ubiquitin ligase complex composed of RSP5 and BUL1/2 have antagonistic roles in the nuclear import and activation of the transcription factor GLN3. NPR1 inhibits and the ubiquitin ligase promotes GLN3 activity. Furthermore, our finding that *rsp5* (*npi1*) and *bul1 bul2* mutations are epistatic to an *npr1* mutation suggests that the ubiquitin ligase acts downstream of NPR1 in activating GLN3 (Fig. 7). Thus, this may constitute a novel ubiquitin-dependent signaling pathway controlling transcription. The membrane-associated transcription factors SPT23 and MGA1 are ubiquitinated and activated in an RSP5-dependent manner (20). However, we have been unable to detect GLN3 ubiquitination. Alternative possibilities are that the GLN3 inhibitor URE2 or the GLN3 nuclear import machinery is regulated by ubiquitination.

In yeast cells grown in a good nitrogen source, GLN3 is repressed by the nitrogen sensing TOR-SIT4 pathway (5). As shown in Fig. 7, we suggest that NPR1 acts downstream of SIT4 to repress GLN3 directly or indirectly. It has been proposed that, under nitrogen rich conditions, NPR1 is phosphorylated and thus inactive toward amino acid permeases (6, 14). The results presented here suggest that phosphorylated NPR1 is active with regard to GLN3 repression. Thus phosphorylation of NPR1 may regulate substrate specificity rather than activity *per se*.

Several lines of evidence argue against the idea that *npr1* mutation affects GLN3 activity solely indirectly, via altered nitrogen source uptake, due to incorrect nitrogen source permease sorting (21). First, a reduction in the cytoplasmic nitrogen source would be expected to activate both GLN3 and GAT1, and the *npr1* mutation causes constitutive nuclear localization and activation of only GLN3 and not GAT1 (Fig. 2). Second, under the conditions we are using for these experiments (76 mM NH_4^+), even a strain lacking all three known ammonia permeases (*mep1/2/3*) can grow perfectly well (12), and our *npr1* strain forms colonies of similar size on solid media containing concentrations of NH_4^+ ranging from 1 to 80 mM, suggesting that the availability of intracellular ammonia is not significantly compromised. Third, the other permeases that NPR1 is known to positively regulate are GAP1 and PUT4, and neither of these permeases are expected to be active under our experimental conditions regardless of their transcription; also, mutations in genes that also regulate GAP1, *LST4* and *LST8* do not cause GLN3 activation. Fourth, total and specific intracellular amino acid concentrations do not alter significantly in a *npr1* strain as compared with the wild type (Fig. 3*B*). Thus, although we cannot absolutely rule out that there is some effect due to altered permease sorting, it seems likely that a more direct pathway is involved.

Importantly rapamycin but not proline induces GLN3 activation in a *bul1 bul2* mutant. Thus, rapamycin-mediated inhibition of the TOR pathway can bypass the requirement for RSP5/BUL1/2 in GLN3 activation. Loss of the GLN3 cytoplasmic binding protein URE2 also circumvents the requirement for the ubiquitination machinery in GLN3 activation (data not shown). Thus, there appears to be two TOR-dependent inputs into GLN3 activation, depending on the nature of the signal. The first would be an RSP5- and BUL1/2-dependent input in response to proline. The second would be an RSP5- and BUL1/2-independent input in response to some other nitrogen source. The qualitative and/or quantitative differences between the signals transduced by these two inputs remains to be elucidated, but either can be sufficient for GLN3 activation. Because a *ure2* mutation has a similar effect in a *bul1 bul2* mutant as rapamycin treatment, the RSP5-BUL1/2-independent input may be the one controlling the binding of GLN3 to URE2.

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NPR1 Kinase and RSP5-BUL1/2 Ubiquitin Ligase Control GLN3-dependent Transcription in *Saccharomyces cerevisiae*

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