Npr1 Ser/Thr Protein Kinase Links Nitrogen Source Quality and Carbon Availability with the Yeast Nitrate Transporter (Ynt1) Levels*

Received for publication, May 25, 2011 Published, JBC Papers in Press, June 7, 2011, DOI 10.1074/jbc.M111.265116

Yusé Martín, Yelvis V. González, Elisa Cabrera, Celia Rodríguez, and José M. Siverio

From the Department of Biochemistry and Molecular Biology, Institute of Biomedical Technologies, Nitrogen Metabolism Group, Universidad de La Laguna, E-38206 La Laguna, Tenerife, Canarias, Spain

Ynt1, the single high affinity nitrate and nitrite transporter of the yeast Hansenula polymorpha, is regulated by the quality of nitrogen sources. Preferred nitrogen sources cause Ynt1 dephosphorylation, ubiquitinylation, endocytosis, and vacuolar degradation. In contrast, under nitrogen limitation Ynt1 is phosphorylated and sorted to the plasma membrane. We show here the involvement of the Ser/Thr kinase HpNpr1 in Ynt1 phosphorylation and regulation of Ynt1 levels in response to nitrogen source quality and the availability of carbon. In \( \Delta npr1 \), Ynt1 phosphorylation does not take place, although Ynt1 ubiquitin conjugates increase. As a result, in this strain Ynt1 is sorted to the vacuole, from both plasma membrane and the later biosynthetic pathway in nitrogen-free conditions and nitrate. In contrast, overexpression of \( NPR1 \) blocks down-regulation of Ynt1, increasing Ynt1 phosphorylation at Ser-244 and -246 and reducing ubiquitinylation. Furthermore, Npr1 is phosphorylated in response to the preferred nitrogen sources, and indeed it is dephosphorylated in nitrogen-free medium. Under conditions where Npr1 is phosphorylated, Ynt1 is not and vice versa. We show for the first time that carbon starvation leads to Npr1 phosphorylation, whereas Ynt1 is dephosphorylated and degraded in the vacuole. Rapamycin prevents this, indicating a possible role of the target of rapamycin signaling pathway in this process. We concluded that Npr1 plays a key role in adapting Ynt1 levels to the nitrogen quality and availability of a source of carbon.

Plants, filamentous fungi, and certain yeasts can use nitrate as the sole nitrogen source. In all of them, the nitrate assimilation pathway is highly conserved. Nitrate enters the cells via nitrate transporter(s), and it is then reduced to nitrite and then to ammonium by nitrate and nitrite reductase, respectively. Because reduction of nitrate to ammonium is a highly energy-demanding process and nitrite is potentially toxic to the cells, several mechanisms have been found that regulate this pathway in response to nitrogen and carbon sources (1). In most organisms, genes encoding the enzymatic machinery for nitrate assimilation require nitrate for their induction and are subject to nitrogen catabolite repression (2, 3). In fact, nitrate acts as an inducer once it enters the cells (4–6), besides being the substrate. Thus, nitrate transporter regulation plays a key role in modulating nitrate assimilation gene expression. In this context, the Arabidopsis thaliana nitrate transporter CHL1 (NRT1.1) acts as a nitrate sensor independently of its nitrate transport activity (7). CHL1 undergoes phosphorylation at Thr-101, in response to low nitrate concentration. This phosphorylation is carried out by a calcineurin B-like-interacting protein kinase (CIPK23) that is involved in the primary nitrate response (8). Ynt1, the sole high affinity nitrate transporter in the yeast Hansenula polymorpha (9, 10), is regulated according to the quality of nitrogen source. In the presence of preferred sources, such as glutamine or ammonium, Ynt1 is ubiquitinylated mainly at Lys-253 and Lys-270. This modification triggers Ynt1 endocytosis and vacuolar degradation (11). In contrast, when the cells are subjected to nitrogen deprivation, Ynt1 phosphorylation is essential for sorting it to the plasma membrane. The nonphosphorylated forms of Ynt1 are thus delivered to the vacuole from the biosynthetic pathway (12). Evidence so far obtained on Ynt1 does not allow us to conclude that it behaves as a nitrate sensor, in the same way as CHL1. However, when nitrate is scarce, its fine regulation by Npr1-mediated phosphorylation targets it to the plasma membrane, facilitating nitrate uptake and consequently nitrate assimilation gene induction (12). There is also strong evidence on the post-translational regulation of A. thaliana NRT2.1 and Aspergillus nidulans NrtA (13, 14).

Plasma membrane permeases involved in nutrient transport have been extensively studied in Saccharomyces cerevisiae. They are internalized and degraded in the vacuole or stabilized at the plasma membrane in response to the quality and quantity of nutrients available in the medium. Ubiquitinylation via E3-ubiquitin ligase Rsp5p and phosphorylation are involved in cellular trafficking of such plasma membrane permeases (15–19). The Ser/Thr protein kinase termed nitrogen permease
Npr1 Protein Kinase Regulates Ynt1 Levels

reactivator 1 (Npr1) plays an outstanding role in regulating nitrogen-related permeases such as Bap2 (19), Mep2 (20), Tat2, and Gap1. In this way, Npr1 is required for Gap1 plasma membrane sorting in the presence of nonpreferred nitrogen sources (15), while under the same conditions, Tat2 is degraded in the vacuole (21). Npr1 is also involved in the ubiquitinylation of Gap1 in an Rsp5-dependent manner (15). The knowledge of Npr1 beyond S. cerevisiae is very scarce. In addition to our work in H. polymorpha, in Candida albicans Npr1 has been reported to regulate the ammonium permease Mep2, unlike Mep1 (22).

Nutritional starvation is the most common stress that yeast cells face in nature. They use several mechanisms to counteract nutritional starvation and to restart the cell cycle when nutrients are newly available. Plasma membrane permeases undergo internalization and vacuolar degradation in response to carbon, nitrogen, or phosphate starvation. This has not been thoroughly studied. However, it is rather well established that tryptophan permease (Tat2) and uracil permease (Fur4) are degraded in response to carbon and nitrogen starvation (23, 24). The high affinity phosphate transporter Pho84 is also degraded in external phosphate below 30–40 μM and under carbon starvation conditions (25–27). In contrast, Gap1 and HpYnt1 are remarkably stable under nitrogen starvation conditions (11, 12, 17).

Hall and co-workers (21) showed that Npr1 activity is controlled by the TOR signaling pathway in response to nitrogen source quality. The inactivation of TOR by incubation in poor nitrogen sources provided a nonphosphorylated active form of Npr1. However, in good nitrogen sources where TOR is active, Npr1 is phosphorylated and inactive. Indeed, Npr1 has been shown to have 20 rapamycin-sensitive phosphorylation sites clustered in two regions within the N-terminal serine-rich domain (28). Growing evidence suggests that TORC1 is involved in vesicle trafficking and may also sense the cellular nutrient status in endogenous membrane structures. Accordingly, several components of TORC1 have been localized to the Golgi, endosome, or other vesicle compartments by different methods. In this sense, TORC1 affects late steps of endocytosis, and Pmr1, a Golgi-localized ATPase that transports Ca^{2+} and Mn^{2+} ions from the cytoplasm into the Golgi, down-regulates TORC1 but also Npr1, which is mostly cytosolic, although a portion is localized at the Golgi. Consistent with this, an increase in Gap1 was observed in npr1 mutants at the plasma membrane (29–33). Moreover, in addition to nutrients, K^{+} has also been found to be involved in plasma membrane permease stability (34).

The post-translational regulation of nitrate transporters reveals its importance in modulating nitrate assimilation genes (8, 12). Therefore, the study of nitrate transporter modifications in response to environmental and nutritional conditions is essential to achieve further understanding of nitrate assimilation control. Indeed, in H. polymorpha, an ORF encodes for a protein with a high similarity to ScNpr1, and its deletion abolishes Ynt1 phosphorylation (12). However, the role of HpNpr1 kinase in the post-translational regulation of Ynt1 was totally unknown until now. We found a new connection between carbon sources and Npr1; indeed, Npr1 is closely involved in the cellular fate of Ynt1, according to the nitrogen quality and carbon source availability. Npr1 undergoes a basal phosphorylation under nitrogen deprivation, which increases in good nitrogen sources and carbon starvation. Rapamycin also affects the phosphorylation of Npr1 and Ynt1 under carbon starvation, suggesting that the TOR kinase pathway is involved in Npr1 regulation in response to carbon availability.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The H. polymorpha strains used in this study (supplemental Table SI) are all derivatives of NCYC495 leu2 αra3. Cells were grown in YPD media (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) or synthetic media containing 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (YNB), 2% (w/v) glucose, and the appropriate nitrogen source for each experiment. In K^{+} experiments, cells were grown in synthetic K^{+}-free medium (YNB-F) (0.17% (w/v) yeast nitrogen base without amino acids, ammonium sulfate, and potassium (ForMedium™ United Kingdom, CYN7505) supplemented with 2% glucose, 5 mM ammonium sulfate, and 20 mM potassium chloride buffered at pH 5.5 with 50 mM MES-Tris. To induce nitrate assimilation genes at restricted K^{+} conditions, cells were then incubated in a homemade yeast nitrogen base following the Difco recipe but without amino acids, ammonium sulfate, or monobasic potassium phosphate. This was brought to pH 5.5 with 50 mM MES-Tris; 2% glucose and the indicated nitrogen source were then added.

To obtain yeast cultures bearing Ynt1, cells were grown in 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (YNB), 2% (w/v) glucose, 5 mM ammonium chloride, centrifuged, and resuspended at 10 mg/ml in the same medium but without nitrogen for 1 h. After that, cells were incubated in 5 or 10 mM sodium nitrate for 1.5–2 h. In the experiments where the effect of carbon starvation on Ynt1 was studied, once the cells were incubated in nitrate they were transferred to fresh medium (YNB) without nitrate in the presence of 2% glucose (YG) or absence (Y). The presence or absence of nitrate did not affect these results. Any modification of this procedure was indicated for each experiment. Whenever necessary, media were supplemented with 30 μg/ml l-leucine, 20 μg/ml uracil, or 100 μg/ml Zeocin.

**Npr1 Tagging**—To tag Npr1 with an HA epitope, we constructed the pHANPR1LEU2 plasmid following the strategy described previously (21). First, we cloned a PCR fragment containing the NPR1 promoter and the initiation codon in pGEM-T easy (Promega, Madison, WI). Then the HA-NPR1 open reading frame without initiation codon but with the NPR1 terminator was cloned in the plasmid derived from this first step. This NPR1-tagged version was made by PCR with a primer containing the HA sequence at the 5’ end. The LEU2 gene, used as auxotrophic marker, was inserted into the last plasmid.

**Nitrate Uptake**—High affinity nitrate uptake was determined by measuring depletion of extracellular nitrate as described previously (9). To determine the nitrate concentration enzy-

---

5 The abbreviation used is: TOR, target of rapamycin.

6 Y. V. González, unpublished results.
matically, the purified *H. polymorpha* nitrate reductase enzyme was used (NECl, Lake Linden, MI).

**Yeast Cell Extracts and Immunoblotting—**Cell extracts were prepared as described previously (9). For Ynt1 and Gap1 immunoblots, plasma membrane-enriched fraction was resuspended in 70 μl of 0.25 mg/ml Triton X-100 and then mixed with 4× Sample Buffer (4×: 12% (w/v) SDS, 6% (v/v) 2-mercaptoethanol, 30% (w/v) glycerol, 0.05% (w/v) Serva Blue G, 150 mM Tris-HCl, pH 7). Samples were heated at 40 °C for 30 min and then subjected to SDS-PAGE. Ynt1 was probed with 1:1500 anti-Ynt1 antiserum and Gap1 with 1:10,000 anti-ScGap1 antiserum (gift of Dr. B. André, Brussels); Pma1, used as a loading control and plasma membrane marker, was immunodetected with rabbit anti-ScPma1 antiserum (gift of Dr. R. Serrano, Valencia, Spain).

For HA-Npr1 immunodetection, whole yeast cell extracts for SDS-PAGE and immunoblot analysis were prepared by resuspending 100 mg of cells in lysis buffer (50 mM Tris-HCl, pH 7.4, 15 mM EDTA, 15 mM EGTA, 10 mM Na3P2O7, 10 mM NaF plus Complete Mini protease inhibitor mixture (Roche Applied Science), and 2 mM PMSF) homogenized in the FastPrep homogenizer device (ThermoSavant LifeSciences, Hampshire, UK) for 20 s at 6.0 m/s and removing cell debris by centrifugation at 500 × g for 10 min at 4 °C. Samples were heated at 95 °C for 5 min. To determine Ynt1-Ub conjugates by immunoblots, cells were processed as in Ref. 11. Immunoblots were prepared from a minimum of three independent experiments.

**Gene Disruption—**NPR1, END4, and VPS27 were disrupted by replacing the chromosomal copy by one containing the partially deleted gene target sequence flanking the *H. polymorpha* auxotrophic markers URA3 or LEU2. Further information on constructing vectors and cassettes is available on request.

**λ-Protein Phosphatase Treatment—**50 μg of proteins was incubated with 150 units of λ-protein phosphatase (New England Biolabs, Beverly, MA) for 25 min at 30 °C as indicated (12). A previous characterization of NPR1 suggested that the vacuolar proteinase A (Fig. 1B) or PE4 gene that encodes the vacuolar proteinase A (Fig. 1B).

As expected, Ynt1-GFP co-localizes with the vacuole in Δnpr1 cells subjected to nitrogen deprivation, whereas in a WT strain, Ynt1 remains at the cell surface even after 80 min (Fig. 1C). These results show that under nitrogen deprivation Npr1 is involved in regulating Ynt1 levels by controlling its delivery to the vacuole for degradation.

In a Δnpr1 Mutant, Ynt1 Is Sorted to the Vacuole from Endocytic and Biosynthetic Pathways—The role of Npr1 in controlling the cellular fate of Ynt1 required elucidation, in addition to the pathways involved. For this, we disrupted genes involved in targeting proteins, such as *END4* and *VPS27*, to the vacuole. *END4* is involved in actin organization and plasma membrane endocytosis (35). In Δend4, Ynt1 endocytosis was almost abolished (Fig. 2A). VPS27 is an endosomal protein required for recycling Golgi proteins, forming luminal membranes and sorting ubiquitinated proteins destined for degradation to the vacuole. As a result, Δvps27 mutation prevents endocytosis, trans-Golgi network, and resident vacuolar proteins from being delivered to the vacuole (36). When strains combining disruptions of *NPR1* with *END4* or *VPS27* were incubated in nitrate-containing medium and then transferred to one deprived of nitrogen, we observed that Ynt1 disappearance was greater in the double mutant Δnpr1Δend4 than in Δend4 but slower than in Δnpr1. However, it remained significantly slower than in WT (Fig. 1B) or Δend4 strains (Fig. 2A). These observations were consistent with those obtained by fluorescence microscopy of Ynt1-GFP. Indeed, in Δnpr1 after 40 min of nitrogen deprivation, all the fluorescence was localized in the vacuole, whereas in the Δnpr1Δend4 strain most was vacuolar but some was still at the cell surface (Fig. 2B). These results suggest that, in Δnpr1, Ynt1 is sorted to the vacuole from both the plasma membrane and the biosynthetic pathway. To further confirm this, we tested Ynt1 degradation in a Δnpr1Δvps27 strain in response to nitrogen deprivation. In contrast with Δnpr1Δend4, Δvps27
almost suppresses NPR1 deletion, and Ynt1 levels are much more stable than in \( \Delta npr1 \Delta end4 \) (Fig. 2A). Therefore, in response to nitrogen-free conditions, Npr1 is required for Ynt1 delivery to the plasma membrane and its further stabilization there.

We then asked whether the action of Npr1 on Ynt1 is restricted to nitrogen deprivation or whether it also affects the rate of Ynt1 appearance in response to nitrate. To test this, we estimated the rate of delivery of Ynt1 to the plasma membrane upon nitrate incubation. The cells were subjected to nitrogen starvation and then incubated in 0.5 mM nitrate. Afterward, nitrate uptake rate was measured, as an index of that delivery rate, showing a rapid increase (Fig. 2C) in WT. In contrast, nitrate uptake was slower to appear in \( \Delta npr1 \) and \( \Delta npr1 \Delta end4 \) mutants, being slightly higher in the latter mutant due to the stability of the Ynt1 present at the plasma membrane, as a result of \( \Delta end4 \) mutation. The behavior of the Ynt1-GFP was consistent with that observed for nitrate uptake appearance, so after incubation in nitrate, Ynt1-GFP was at the cell surface in WT. However, in \( \Delta npr1 \) and \( \Delta npr1 \Delta end4 \) mutants, Ynt1-GFP cellular distribution was heterogeneous, with a high cytosolic accumulation (Fig. 2D). Therefore, our results indicate that in nitrate and nitrogen-free conditions, Npr1 prevents sorting of newly synthesized Ynt1 to the vacuole, facilitating its delivery to the plasma membrane.

Phosphorylation of Ynt1 Is Mediated by Npr1—Nitrogen limitation induced phosphorylation of Ynt1 at Ser residues 244 and 246, located in the central hydrophilic domain. Under nitrogen limitation, a nonphosphorylatable form of Ynt1 (S244A and S246A) is missorted to the vacuole (12). The similarity in behavior of the nonphosphorylatable Ynt1 with \( \Delta npr1 \) prompted us to study the relationship between Ynt1 phosphorylation and Npr1 in detail. For this, we compared the levels of Ynt1 under nitrogen deprivation in \( \Delta npr1 \) and the Ynt1\(_{2S3A}\) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown). We then studied the phosphorylation state of Ynt1 in a \( \Delta npr1 \) mutant. In both strains, Ynt1 disappearance (Fig. 3A) and the drop in nitrate uptake were very rapid. Ynt1-GFP was localized in the vacuole after a few minutes (data not shown).
Ynt1 ubiquitylation (11) were mutated to Arg (Fig. 4A). This finding suggested a close relationship between Ynt1 ubiquitylation and Npr1. To test this, the ubiquitylated forms of Ynt1 in a Δnpr1 mutant were determined by the use of Δmpr1Δynr1 bearing the Ynt1Δ232–286-Ct232–286 (Ynt1-Ct) mutation, achieved by fusing the core sequence (236–286) of the central cytosolic domain to the C terminus of Ynt1Δ232–286 (11). The mutation of the nitrate reductase gene (YNRI) ensures high levels of Ynt1, whereas Ynt1-Ct provides a robust Ynt1-ubiquitylation assay (11). When the cells were incubated in nitrate, the Δmpr1 mutant showed an increased amount of Ynt1 ubiquitin conjugates, as seen in WT cells after addition of glutamine. The nonphosphorylatable form of Ynt1 (Ynt1Δ232–286) exhibited the same ubiquitylation as the WT (Fig. 4B). These results indicate that Npr1 down-regulates the ubiquitylation of Ynt1, independently of Ynt1 phosphorylation.

We then asked whether Npr1 in multicopy (nNPR1) further stabilizes Ynt1 by lowering the amount of ubiquitin conjugates. This was assayed on Ynt1 down-regulation in response to glutamine. In WT, the amount of Ynt1 falls abruptly, but in an nNPR1 it did not (Fig. 4C). Examination of Ynt1 ubiquitylation in WT and the nNPR1 mutant revealed that the Ynt1 ubiquitin conjugates could not be detected in the nNPR1 strain, even after the addition of glutamine (Fig. 4D). These results support a close relationship between Ynt1 ubiquitylation and Npr1. However, it is worth noting that the ubiquitylation is independent of Ynt1 phosphorylation.

To gain further insights into the relationship between Npr1 and Rsp5, we constructed a loss of function rsp5-1 temperature-sensitive mutant strain (38). Most plasma membrane proteins in yeast undergo Rsp5-mediated ubiquitylation and subsequent internalization (39). However, in rsp5-1, Ynt1 down-regulation was not affected (supplemental Fig. S2), possibly because the ubiquitylation activity remaining in this temperature-sensitive mutant (rsp5-1) is enough to

FIGURE 3. Phosphorylation of Ynt1 is mediated by Npr1. A, immunoblots of Ynt1 from nitrogen-depleted cells. Previously, cells were incubated in 10 mM nitrate. B, Ynt1 mobility in SDS-PAGE of protein extracts treated (+) or nontreated (−) with γ-protein phosphatase. The indicated strains were incubated in 5 mM nitrate for 2 h. C, phosphorylation state of Ynt1 in WT, Ynt1Δ3K→R and nNPR1 strains. Protein extracts were obtained from cells incubated in 10 mM nitrate for 2 h (upper panel) or 40 min after 5 mM glutamine addition (lower panel). D, Ynt1 phosphorylation is dependent on Npr1 and takes place at Ser-244 and Ser-246. Samples were taken from cells incubated in 10 mM nitrate for 2 h and after 20 min of nitrogen deprivation.

FIGURE 4. Npr1 is involved in regulating Ynt1 ubiquitylation. A, effect of NPR1 disruption on the levels of Ynt1Δ25–286 and Ynt1Δ3K→R under nitrogen-free conditions. Cells incubated in 10 mM nitrate for 2 h were then deprived of nitrogen. B, Ynt1 ubiquitin (Ub) conjugates increase in Δnpr1. Cells were incubated in 10 mM nitrate for 2 h. C, Ynt1 levels in response to glutamine in a strain overexpressing NPR1 gene. Cells were incubated in 10 mM nitrate, and then 5 mM glutamine was added. D, Ynt1 ubiquitin conjugates in a strain overexpressing NPR1. After 2 h of incubation in 10 mM nitrate (0 min), 5 mM glutamine was added.
ubiquitinylate Ynt1 and deliver it to the vacuole for degradation. In contrast, Gap1 (supplemental Fig. S1) and Hak17 increase their stability in response to preferred nitrogen sources or K\(^+\) in \textit{rsp5-1}, confirming the phenotype of this mutant. Similarly, in the \textit{S.cerevisiae RSP5} conditional mutants \textit{rsp5-1} and \textit{spa1-1}, the high affinity iron transporter Fet1-Fet3 and Pi transporter Pho84 are unaffected in response to iron or Pi abundance, respectively (40, 41).

Ynt1 Levels Are Also Regulated by the Carbon Sources and Dependent on Npr1—It has been shown in our laboratory that, in addition to the nitrogen source, \textit{YNT1} gene expression is regulated by the carbon source. Here, we investigated whether this regulation also takes place at the post-translational level. We have found that carbon starvation also down-regulates Ynt1 (Fig. 5A), which correlates with a fast dephosphorylation of Ynt1 (Fig. 5B), as occurs with glutamine (12). The presence of different carbon sources, such as 0.5% glucose, sucrose, ethanol, and glycerol, restores the levels of Ynt1 (Fig. 5C). Therefore, we concluded that carbon starvation down-regulates Ynt1 levels. We then asked if Npr1 is also involved in regulating Ynt1 levels in a medium lacking a carbon source. To test this, we compared the levels of Ynt1 in WT and in nNPR1 strains transferred to a carbon-free medium. In the nNPR1 strain, Ynt1 was clearly resistant to degradation (Fig. 5D), indicating that Npr1 is involved in adapting Ynt1 levels to carbon source availability.

To further analyze the role of Ynt1 ubiquitinylation in response to carbon starvation, the strain bearing Ynt1-Ct was used. Ynt1 appears clearly ubiquitinylated in response to carbon starvation (Fig. 5E); however, ubiquitinylation disappears when \textit{NPR1} is overexpressed. Altogether our results clearly indicate that Npr1 also plays a key role in adapting Ynt1 levels to carbon source availability, acting on Ynt1 via its phosphorylation and ubiquitinylation.

We also explored whether calcineurin could play some role in signaling the effect of nitrogen and carbon sources on Ynt1 post-translational regulation. To address this, the strain \textit{Δcnb1} lacking the regulatory subunit of calcineurin was used (42). In the conditions studied, the Ynt1 phosphorylation or degradation pattern shows no significant differences between \textit{Δcnb1} and WT (supplemental Fig. S3).

\textit{Npr1 Phosphorylation State Is Regulated by Nitrogen Source Quality, Carbon Source, and K\(^+\) Availability}—The effect of \textit{Npr1} on post-translational regulation of Ynt1 was clearly observed under nitrogen limitation and nitrate and carbon starvation. We also studied the Npr1 phosphorylation state to correlate it with its involvement in Ynt1 regulation, using a strain bearing a functional N-terminal HA-tagged Npr1. As shown in

---

7 Y. Martín and E. Cabrera, unpublished results.
8 M. D. Pérez and Y. V. González, unpublished results.
Fig. 6A, the SDS-PAGE mobility of Npr1 depends on the nitrogen source. Thus, Npr1 from cells incubated in glutamine presented less mobility than that from cells incubated in nitrate or nitrogen-free media. A-Protein phosphatase treatment of cell extracts confirmed the phosphorylation of Npr1 in response to glutamine. Moreover, this treatment of cell extract from nitrogen-deprived cells revealed that Npr1 migrated faster than when not treated (Fig. 6B). This suggests that Npr1 may be phosphorylated in nonpreferred nitrogen sources, and addition of glutamine causes further phosphorylation. A close correlation was observed between glutamine-dependent Npr1 phosphorylation and Ynt1 dephosphorylation, such as in a Δnpr1 mutant (Fig. 6A). Therefore, the capacity of Npr1 to directly or indirectly phosphorylate Ynt1 is regulated by the nitrogen sources, such that when Npr1 was phosphorylated no Ynt1 phosphorylation was observed.

The effect of rapamycin on the phosphorylation state of Npr1 and Ynt1 levels was tested by incubating H. polymorpha in glutamine plus 0.5–3 μM rapamycin. Npr1 mobility was not affected while Ynt1 was being degraded, unlike those cells transferred to N-free medium (Fig. 6, C and D). Rapamycin mimicked nitrogen derepression for those genes subject to nitrogen catabolite repression (6). These results contrast with those obtained for ScNpr1, where rapamycin strongly modifies the nitrogen-dependent phosphorylation pattern of Npr1 (18).

As Npr1 is modulated by phosphorylation, we studied whether Npr1 was affected by carbon starvation. In cells subject to carbon starvation, Npr1 was found to be phosphorylated (Fig. 7A), although less so than in glutamine (Fig. 6B), which strongly suggested that Npr1 is inactivated by phosphorylation. Thus, when Npr1 is phosphorylated, or disrupted, Ynt1 is not. However, Npr1 phosphorylation could also be involved in recognizing and binding its substrates. In contrast to what was observed in glutamine, 1 μM rapamycin prevents Ynt1 degradation in response to carbon starvation (Fig. 7C). Consistent with this, rapamycin also prevented phosphorylation of Npr1 in carbon starvation (Fig. 7D), as well as Ynt1 dephosphorylation (Fig. 7E). These results suggest that Npr1 responds to carbon starvation in a TOR pathway-dependent manner.

We also explored whether K⁺ levels also affect Npr1 phosphorylation and Ynt1 levels. K⁺ has been found to be involved...
in the stabilization of several permeases at the plasma membrane (34). A clear increase in the phosphorylation of Npr1 was observed in the absence of K⁺ but only in preferred nitrogen sources such as ammonium or glutamine (Fig. 8, A and B). This makes it difficult to see the effect on Ynt1, because Ynt1 is only present in cells incubated in nitrate. However, because we have consistently observed that changes in Npr1 phosphorylation affect that of Ynt1 and its levels, we studied the effect of K⁺ absence on Ynt1. No significant changes were observed in Ynt1 by Western blotting, although nitrate uptake decreased (Fig. 8C), and this alteration in uptake could be due in part to electrochemical potential change in the absence of K⁺. Indeed, addition of 20 mM KCl restored nitrate transport. We concluded that the absence of K⁺ alters the Npr1 phosphorylation state in preferred nitrogen sources; nevertheless, this condition does not seem to affect Ynt1 but does influence nitrate uptake. In Fig. 9, we summarize our current knowledge about the role of Npr1 in regulating Ynt1.

**DISCUSSION**

In this study, we uncover the role of Npr1 in regulating the Ynt1 levels in response to nitrogen source quality and carbon availability. This yeast protein kinase belongs to a subgroup involved in the regulation of several permeases (15, 17, 34, 43). *Hp*npr1 showed deficient growth in low ammonium or other nitrogen sources such as proline. However, contrary to expectations, Gap1 levels are not affected in the *npr1* strain, indicating that this protein kinase is not involved in Gap1 activation in *H. polymorpha* (supplemental Fig. S1). Nevertheless, the poor growth of *npr1* in ammonium suggests that ammonium permeases are also under control of *H. polymorpha* Npr1. The regulatory roles of HpNpr1 do not fully coincide with those observed in *S. cerevisiae*. Consistent with this, we found that...
Δnpr1 presents a high sensitivity to toxic cations such as Li⁺ or hygromycin B.⁷

We observed that Npr1 is essential to maintain Ynt1 levels in nitrate and under nitrogen deprivation. Using the Δend4 and Δvps27 mutants involved in intracellular protein traffic, we showed that in the strain lacking Npr1, Ynt1 was routed to the vacuole from the later biosynthetic pathways and the plasma membrane. Therefore, Npr1 is involved in regulating Ynt1 levels by acting on both de novo synthesized Ynt1 present in the secretion pathway and that already in the plasma membrane. Furthermore, the following two experiments shed light on the poor growth of Δnpr1 in low nitrate; (i) the amount of Ynt1 was found to be lower in Δnpr1 after incubation in nitrate for 2 h, and (ii) the kinetics of Ynt1 reaching the plasma membrane was slower in Δnpr1 than in WT (Fig. 2C).

The negative effect observed in Δnpr1 on the levels of Ynt1 raises the question of the molecular mechanisms involved. The fact that Ynt1 was rapidly degraded in Δnpr1 at the same rate as the unphosphorylatable Ynt1²⁵⁻²⁸⁻ is highly significant. This suggests that Npr1 is involved in phosphorylation of Ynt1. The use of strains overexpressing or lacking Npr1 clearly reveals that Npr1 is involved in the phosphorylation of Ynt1, which in turn takes place at Ser-244 and Ser-246. However, unlike CHL1 (7, 8, 44), Ynt1 phosphorylation does not seem to be involved in nitrate affinity changes or nitrate signaling. Overexpression of Npr1, by increasing the NPR1 copy number, causes a typical phosphorylation of Ynt1, which takes place even in good nitrogen sources, unlike WT (Fig. 3C). This phosphorylation is not due to greater Ynt1 stability, because a highly stable nonubiquitinatable form of Ynt1, namely Ynt1²⁵⁻²⁸⁻, (11), was not phosphorylated in good nitrogen sources, unlike nNPR1 strain. Our data contrast with those observed in S. cerevisiae Gap1, which is phosphorylated and unstable in Δnpr1; however, the increased stability of Gap1 because of Rsp5 deletion causes the reappearance of Gap1 phosphorylation (15). This indicates that Npr1 phosphorylation is indirect. Using synthetic peptides, a (K/R)XXS(K/R) consensus sequence for the Npr1 phosphorylation site was found (45). This site is present in Ynt1 (Ser-244 and Ser-246). However, in S. cerevisiae, Bul proteins, components of the Rsp5 ubiquitin ligase complex, contain several perfect Npr1 consensus phosphorylation motifs (Bul1, KGWSSR55, KVKSK323, and KQHSK853, and Bul2, KRTSK450) (45). Moreover, in vitro and in vivo findings strongly suggest that the phosphorylation state and activity of the α-arrestin Aly2, involved in the intracellular traffic of Gap1, are directly regulated by Npr1 (46).

Therefore, despite the presence of a putative Npr1 phosphorylation site in Ynt1, it seems that in general Npr1 does not phosphorylate the target permease it regulates.

Concerning the relationship between Npr1 and Ynt1 ubiquitinylation, the disruption effect of Npr1 was not reversed by Ynt1²⁵⁻²⁸⁻⁻ (Fig. 4A), in which phosphorylation is functionally and constitutively mimicked by mutating Ser residues to Asp (12). This suggests that Npr1 acts beyond Ynt1 phosphorylation and that Npr1 is probably not directly involved in Ynt1 phosphorylation. In turn, the nonubiquitinatable Ynt1²⁵⁻²⁸⁻⁻ overcomes the effect of Npr1 deletion on Ynt1 degradation, pointing to a link between Npr1 and Ynt1 ubiquitinylation (Fig. 4A). Consistent with this, deletion of NPR1 causes an increment in the Ynt1 ubiquitin conjugates, even in nitrogen starvation where Ynt1 is highly stable (Fig. 4B). Furthermore, overexpression of NPR1 protects Ynt1 from ubiquitinylation, even in good nitrogen sources. As our results show that the increase in Npr1 led to high Ynt1 phosphorylation and low ubiquitinylation, and completely the opposite when NPR1 was disrupted, at first glance it could be thought that phosphorylation blocks ubiquitinylation of Ynt1. However, we have not found any evidence for this. In fact, the opposite seems to be the case, because Ynt1²⁵⁻²⁸⁻⁻ was degraded in a Δnpr1 mutant (Fig. 4A), unlike the WT strain, and the nonubiquitinatable form of Ynt1²⁵⁻²⁸⁻⁻ does not increase ubiquitinylation (Fig. 4B). Therefore, it can be concluded that the phosphorylation state of Ynt1 itself is not involved in regulating its ubiquitinylation, suggesting that Npr1 could be phosphorylating some auxiliary protein such as arrestin-related or ubiquitin-ligase adaptors or Bul1 or Bul2 (45–47), which could be blocking Ynt1 ubiquitinylation. The involvement of Npr1 in Ynt1 ubiquitinylation raises the question of the role of the ubiquitin ligase Rsp5 in this process. However, a thermo-sensitive rsp5-1 mutant in H. polymorpha affected Gap1 (supplemental Fig. S2) and the high affinity K⁺ transporter Hak1 degradation but not Ynt1. To rule out the role of Rsp5 in Ynt1 ubiquitinylation, further experiments should be undertaken. DOA4 encodes for a deubiquitinylating enzyme required for ubiquitin homeostasis in yeast (48, 49); its disruption does not affect Ynt1 down-regulation, unlike Gap1 (supplemental Fig. S2). However, as above, we cannot rule out the possibility of Ynt1 undergoing ubiquitinylation in Δdoa4.

Carbon starvation also leads to a rapid decrease of Ynt1 levels. Most likely this obeys a general program to adapt cell enzymatic machinery to the adverse nutritional state. In S. cerevisiae, carbon or nitrogen starvation induces degradation of the tryptophan permease Tat2 and uracil permease Fur4 (23, 24). Rapamycin also produces Tat2 degradation (23). We should highlight that although the precise reason why Ynt1 is degraded in response to carbon starvation is unclear, apparently the mechanism involved is the same as when cells are transferred from nitrate to glutamine (11).

We also studied Npr1 phosphorylation in response to nitrogen sources and carbon and K⁺ starvation. In glutamine or other preferred nitrogen sources and in carbon starvation, Npr1 is highly phosphorylated, although in nitrogen limitation conditions or in nonpreferred nitrogen sources this phosphorylation almost disappears. In contrast to Npr1, Ynt1 was phosphorylated in nitrogen-free medium and nonphosphorylated in good nitrogen sources and carbon starvation (Figs. 6 and 7). In S. cerevisiae, Npr1 phosphorylation is dependent on the TOR signaling pathway; thus, rapamycin blocks this phosphorylation in good nitrogen sources (21, 28). However, H₃Npr1 phosphorylation in good nitrogen sources is not affected by rapamycin, despite it causing de-repression of the genes subjected to nitrogen catabolite repression (6). It also does not block Ynt1 degradation in response to glutamine (Fig. 6C). However, the effect of rapamycin on plasma membrane permeases is controversial. It has been shown that Gap1 is stable in rapamycin (23). However, when cells are grown in a sublethal concentration of rapamycin, Gap1 levels were greatly reduced, and this is explained by the increase in the internal concentration of...
amino acids (50). A striking difference in the response of Npr1/ Ynt1 to carbon starvation, unlike glutamine addition, is that rapamycin prevents Npr1 dephosphorylation, leading to a lower Ynt1 degradation rate. Therefore, it seems that in *H. polymorpha* the intracellular traffic of Ynt1 elicited by carbon starvation is regulated by the rapamycin-sensitive TOR complex. Nevertheless, it cannot be ruled out that an insensitive TOR complex could also be involved in the case of glutamine. Alternatively, under nutrient stress, like that produced by carbon starvation, rapamycin-sensitive TOR complex could be more essential for endocytosis and protein trafficking, thus affecting Ynt1 degradation (29). It is noteworthy that under these conditions, HpNpr1 phosphorylation is also impeded by rapamycin (Fig. 7D). In preferred nitrogen sources, Npr1 responded to K⁺ starvation with a higher degree of phosphorylation (Fig. 8), although no changes were observed in nonpreferred nitrogen sources. However, we observed no significant changes either in phosphorylation or stability of Ynt1. However, in K⁺ starvation, nitrate uptake was significantly reduced (Fig. 8). This could be due in part to the plasma electrochemical potential alteration, although a sound conclusion on the effect of K⁺ starvation on Ynt1 would need further research.

Another unexpected result was that during nitrogen deprivation conditions, Npr1 shows a basal phosphorylation that only disappears when protein extracts are treated with phosphatase (Fig. 6B). This could indicate that some of this Npr1 phosphorylation is nitrogen-source-independent, which could explain some phenotypes of the Δnpr1 mutant not being related to the nitrogen sources, such as Li⁺ and hygromycin sensitivity. Npr1 phosphorylation events, up to 22 in *S. cerevisiae* (28), could be involved in regulating Npr1 activity and also in the recognition, binding, and phosphorylation of a broad set of substrates. Consistent with this, in the Saccharomyces Genome Data base, up to 67 records appear so far for *NPR1* genetic or physical interactions.

The current knowledge about the regulation of the yeast high affinity nitrate/nitrite transporter Ynt1 is shown in Fig. 9. In summary, Npr1 plays a pivotal role in regulating Ynt1 levels according to nitrogen and carbon availability.

**Acknowledgments—** F. Machín (Hospital Universitario Nuestra Señora de Candelaria, Tenerife) obtained the preliminary evidence on the effect of carbon starvation on Ynt1 while a member of our group. R. González (this laboratory) performed the study on the effect of rapamycin concentration and preincubation time on Ynt1 levels and phosphorylation. We thank Rhein Biotech (Germany) for providing NPR1, END4, PEP12, and VPS27 DNA sequences. We are grateful to R. Serrano (Valencia, Spain) for Pma1 antiserum and to Bruno André (Brussels, Belgium) for Gap1. We also thank Guido Jones for proofreading the manuscript and Francisco J. Navarro (Rockefeller University) for critical reading and valuable suggestions.

**REFERENCES**

1. Siverio, J. M. (2002) *FEMS Microbiol. Rev.* 26, 277–284
2. Crawford, N. M., and Arst, H. N., Jr. (1993) *Annu. Rev. Genet.* 27, 115–146
3. Cooper, T. G. (2002) *FEMS Microbiol. Rev.* 26, 223–238
4. Llamas, A., Igeño, M. I., Galván, A., and Fernández, E. (2002) *Plant J.* 30, 261–271
5. Narendja, F., Goller, S. P., Wolschek, M., and Strauss, J. (2002) *Mol. Microbiol.* 44, 573–583
6. Navarro, F. J., Perdomo, G., Tejera, P., Medina, B., Machín, F., Guillén, R. M., Lancha, A., and Siverio, J. M. (2003) *FEMS Yeast Res.* 4, 149–155
7. Ho, C. H., Lin, S. H., Hu, H. C., and Tsay, Y. F. (2009) *Cell* 138, 1184–1194
8. Hu, H. C., Wang, Y. Y., and Tsay, Y. F. (2009) *Plant J.* 57, 264–278
9. Machín, F., Medina, B., Navarro, F. J., Pérez, M. D., Veenhuis, M., Tejera, P., Lorenzo, H., Lancha, A., and Siverio, J. M. (2004) *Yeast* 21, 265–276
10. Pérez, M. D., González, C., Avila, J., Brito, N., and Siverio, J. M. (1997) *Biochem.* 321, 397–403
11. Navarro, F. J., Machín, F., Martín, Y., and Siverio, J. M. (2006) *J. Biol. Chem.* 281, 13268–13274
12. Navarro, F. J., Martín, Y., and Siverio, J. M. (2008) *J. Biol. Chem.* 283, 31208–31217
13. Wang, Y., Li, W., Siddiqi, Y., Kinghorn, J. R., Unkles, S. E., and Glass, A. D. (2007) *New Phytol.* 175, 699–706
14. Wirth, J., Chopin, F., Santoni, V., Viennois, G., Tillard, P., Krapp, A., Lejay, L., Daniel-Vedele, F., and Gojon, A. (2007) *J. Biol. Chem.* 282, 23541–23552
15. De Craene, J. O., Soetens, O., and Andre, B. (2001) *J. Biol. Chem.* 276, 43939–43948
16. Grenson, M., and Acheroy, B. (1982) *Mol. Gen. Genet.* 188, 261–265
17. Grenson, M. (1983) *Eur. J. Biochem.* 133, 141–144
18. Kaouss, M., Gamache, I., Ramotar, D., Audette, M., and Poulin, R. (1998) *J. Biol. Chem.* 273, 2109–2117
19. Omura, F., and Kodama, Y. (2004) *FEMS Microbiol. Lett.* 230, 227–234
20. Boeckstaens, M., André, B., and Marini, A. M. (2007) *Mol. Microbiol.* 64, 534–546
21. Schmidt, A., Beck, T., Koller, A., Kunz, I., and Hall, M. N. (1998) *EMBO J.* 17, 6924–6931
22. Neuhäuser, B., Dunkel, N., Satheesh, S. V., and Morschhäuser, J. (2011) *Eukaryot. Cell* 10, 332–342
23. Beck, T., Schmidt, A., and Hall, M. N. (1999) *J. Cell Biol.* 146, 1227–1238
24. Volland, C., Urban-Grimal, D., Géraud, G., and Huguenauer-Tsapis, R. (1994) *J. Biol. Chem.* 269, 9833–9841
25. Persson, B. L., Lagerstedt, J. O., Pratt, J. R., Pattison-Granberg, J., Lundh, K., Shokrollahzadeh, S., and Lundh, F. (2003) *Curr. Genet.* 43, 225–244
26. Lagerstedt, J. O., Zvyagilskaya, R., Pratt, J. R., Pattison-Granberg, J., Kruckeberg, A. L., Berden, J. A., and Persson, B. L. (2002) *FEBS Lett.* 526, 31–37
27. Martínez, P., Zvyagilskaya, R., Allard, P., and Persson, B. L. (1998) *J. Bacteriol.* 180, 2253–2256
28. Gander, S., Bonenhant, D., Altermatt, P., Martin, D. E., Hauir, S., Moes, S., Hall, M. N., and Jenoe, P. (2008) *Rapid Commun. Mass Spectrom.* 22, 3743–3753
29. Aronova, S., Wedaman, K., Anderson, S., Yates, J. 3rd, and Powers, T. (2007) *Mol. Biol. Cell* 18, 2779–2794
30. Devashayam, G., Ritz, D., Hellwiel, S. B., Burke, D. J., and Sturgill, T. W. (2006) *Proc. Natl. Acad. Sci. U.S.A.* 103, 17840–17845
31. Neufeld, T. P. (2007) *Cell Metab.* 5, 3–5
32. Wedaman, K. P., Reine, A. D., Anderson, S., Yates, J. 3rd, McCaffrey, J. M., and Powers, T. (2003) *Mol. Biol. Cell* 14, 1204–1220
33. Zurita-Martinez, S. A., Puria, R., Pan, X., Boeke, J. D., and Cardenas, M. E. (2007) *Genetics* 176, 2139–2150
34. Pérez-Valle, J., Jenkins, H., Merchán, S., Montiel, V., Ramos, J., Sharma, S., Serrano, R., and Yenush, L. (2007) *Mol. Cell. Biol.* 27, 5725–5736
35. Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993) *J. Cell Biol.* 120, 55–65
36. Piper, R. C., Cooper, A. A., Yang, H., and Stevens, T. H. (1995) *J. Cell Biol.* 131, 603–617
37. Avila, J., Pérez, M. D., Brito, N., González, C., and Siverio, J. M. (1995) *FEBS Lett.* 366, 137–142
38. Wang, G., Yang, J., and Huijbregts, E. J. (1999) *Mol. Cell. Biol.* 19, 342–352
39. Belgareh-Touzé, N., Léon, S., Erpapazoglou, Z., Stawiecka-Mirota, M.,
Urban-Grimal, D., and Haguenauer-Tsapis, R. (2008) Biochem. Soc. Trans. 36, 791–796
40. Estrella, L. A., Krishnamurthy, S., Timme, C. R., and Hampsey, M. (2008) J. Biol. Chem. 283, 5327–5334
41. Felice, M. R., De Domenico, I., Li, L., Ward, D. M., Bartok, B., Musci, G., and Kaplan, J. (2005) J. Biol. Chem. 280, 22181–22190
42. Rodriguez, C., Tejera, P., Medina, B., Guillén, R., Domínguez, A., Ramos, J., and Siverio, J. M. (2010) J. Biol. Chem. 285, 37551–37560
43. Goossens, A., de La Fuente, N., Forment, J., Serrano, R., and Portillo, F. (2000) Mol. Cell. Biol. 20, 7654–7661
44. Liu, K. H., and Tsay, Y. F. (2003) EMBO J. 22, 1005–1013
45. Gander, S., Martin, D., Hauri, S., Moes, S., Poletto, G., Pagano, M. A., Marin, O., Meggio, F., and Jenoe, P. (2009) J. Proteome Res. 8, 5305–5316
46. O’Donnell, A. F., Apffel, A., Gardner, R. G., and Cyert, M. S. (2010) Mol. Biol. Cell 21, 3552–3566
47. Lin, C. H., MacGurn, J. A., Chu, T., Stefan, C. I., and Emr, S. D. (2008) Cell 135, 714–725
48. Swaminathan, S., Amerik, A. Y., and Hochstrasser, M. (1999) Mol. Biol. Cell 10, 2583–2594
49. Papa, F. R., and Hochstrasser, M. (1993) Nature 366, 313–319
50. Chen, E. J., and Kaiser, C. A. (2003) J. Cell Biol. 161, 333–347