Yeast Npi3/Bro1 is involved in ubiquitin-dependent control of permease trafficking

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Abstract The membrane traffic and stability of the general amino acid permease Gap1 of *Saccharomyces cerevisiae* are under nitrogen control. Addition of a preferential nitrogen source such as ammonium to cells growing on a poor nitrogen source induces internalization of the permease and its subsequent degradation in the vacuole. This down-regulation requires ubiquitination of Gap1 through a process involving ubiquitin ligase Npi1/Rsp5, ubiquitin hydrolase Npi2/Doa4, and Bul1/2, two Npi1/Rsp5 interacting proteins. Here we report that yet another protein, Npi3, is involved in the regulation of Gap1 trafficking. We show that Npi3 is required for NH_4^+-induced down-regulation of Gap1, and particularly for efficient ubiquitination of the permease. Npi3 plays a pleiotropic role in permease down-regulation, since it is also involved in ubiquitination and stress-induced down-regulation of the uracil permease Fur4 and in glucose-induced degradation of hexose transporters Hxt6/7. We further provide evidence that Npi3 is required for direct vacuolar sorting of neosynthesized Gap1 permease as it occurs in *npr1* mutant cells. *NPI3* is identical to *BRO1*, a gene encoding a protein of unknown biochemical function and recently proposed to be involved in protein turnover. Npi3/Bro1 homologues include fungal proteins required for proteolytic cleavage of zinc finger proteins and the mouse Aip1 protein involved in apoptosis. We propose that proteins of the Npi3/Bro1 family, including homologues from higher species, may play a conserved role in ubiquitin-dependent control of membrane protein trafficking. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Npi3/Bro1; Ubiquitin; Permease; Traffic; Yeast

1. Introduction

Ubiquitin is a 76-amino acid polypeptide highly conserved in all eukaryotic cells. Besides its role in tagging proteins for degradation by the cytosolic 26S proteasome [1], modification by ubiquitin is involved in endocytotic internalization of plasma membrane transporters and receptors, and their subsequent vacuolar/lysosomal degradation [2]. For instance, a number of yeast cell surface proteins, including the general amino acid permease Gap1, undergo ubiquitin-dependent down-regulation [3]. We use Gap1 as a model to investigate this process because it is particularly suitable for genetic studies and responds tightly to physiological constraints.

The synthesis, intracellular trafficking, and stability of Gap1 are regulated according to the used nitrogen source. In yeast cells growing on a poor nitrogen source like proline or urea, Gap1 is abundantly synthesized and reaches the plasma membrane where it is highly active [4–6]. Under these growth conditions, a small fraction of Gap1 is mono-ubiquitinated on one or both of two acceptor lysines (K_9 and K_{16}) [7,8]. Upon addition of NH_4^+, a preferential nitrogen source, the *GAP1* gene is repressed; pre-synthesized Gap1 present at the cell surface is endocytosed and targeted to the vacuole for degradation [9,10]. Addition of NH_4^+ triggers an increase of the fraction of ubiquitin-conjugated Gap1, with concomitant formation of poly-ubiquitin chains where ubiquitin is linked to the lysine-63 residue of the previously attached ubiquitin molecule [7]. Ubiquitination of Gap1 is required for down-regulation of the permease. The essential HECT-type ubiquitin ligase Npi1/Rsp5 [9,11] is one of four proteins shown so far to be required for efficient Gap1 ubiquitination and down-regulation [10]. Npi1/Rsp5 is structured in three domains: an N-terminal C2 domain, three central WW(P) protein–protein interaction domains, and a C-terminal HECT catalytic domain [12]. As deletion of the C2 domain prevents internalization of Gap1 without affecting its ubiquitination, it has been proposed that Npi1/Rsp5 also participates in the internalization step of the permease [13]. Npi1/Rsp5 is a pleiotropic factor involved in the down-regulation of other permeases such as the uracil permease Fur4, the maltose permease Mal61, and the hexose permeases Hxt6/7 [9,14–16]. It is also involved in the down-regulation of the alpha pheromone receptor Ste2 [17]. The homologous Bul1 and Bul2 proteins, shown to interact with Npi1/Rsp5 possibly by binding with their PY motif to WW(P) domain(s) in the ligase [18,19], are also essential to NH_4^+-induced Gap1 ubiquitination [8]. The ubiquitin hydrolase Npi2/Doa4 [20] is required for efficient ubiquitination of Gap1, because of its role in maintaining the intracellular pool of ubiquitin by recycling the molecule from substrates marked for degradation [7,21].

As down-regulation of Gap1 is induced by addition of NH_4^+ to the medium, factors responding to the nitrogen status of the cell must also be involved. A likely candidate is Npr1, a protein kinase homologue essential to the activity of Gap1 and other NH_4^+-sensitive permeases during growth on proline or urea [22,23]. Npr1 prevents cell surface Gap1 from being internalized and targeted to the vacuole for degradation [6].

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Phosphorylation of Npr1 is regulated by nitrogen through the Tor signaling pathway: nitrogen starvation or growth on proline results in Npr1 dephosphorylation, whereas growth on ammonium or on rich medium triggers Tor-dependent phosphorylation of Npr1 [24]. It is noteworthy that the tryptophan transporter Tat2 is regulated by Npr1 in the opposite way from Gap1, i.e. it is stabilized under conditions where Npr1 is phosphorylated.

The nitrogen source and Npr1 also control Gap1 trafficking late in the secretory pathway. In the presence of favored nitrogen sources or in cells lacking Npr1, newly synthesized Gap1 is sorted directly to the vacuole without ever reaching the plasma membrane [6,25]. This direct sorting of Gap1 to the vacuole requires ubiquitination of the permease on at least one of the lysine residues 9 and 16, and is also dependent on Npi1/Rsp5, Npi2/Doa4, and the Bul proteins [8]. It was further reported that mono-ubiquitination is not sufficient and that poly-ubiquitination is essential for the vacuolar sorting of Gap1 [26]. The same study reported that the specific role of the Bul proteins is to promote poly-ubiquitination of Gap1.

Here we identify a novel element involved in ubiquitin-dependent control of permease trafficking: the *NPI3* gene. We show that *NPI3* is identical to *BRO1*, a gene encoding a protein structured in distinct domains and conserved in higher eukaryotes including human. Among the homologues of Npi3/Bro1 is mouse Aip1, a protein involved in apoptosis and recently found to interact with the multifunctional adapter SETA/CIN85 [27].

2. Materials and methods

2.1. Strains and growth conditions

The *Saccharomyces cerevisiae* strains 23344c (*ura3*), 27038a (*npi1 ura3*), 25173a (*npr1 npi3 ura3*), 27086c (*npi3 ura3*), 21994b (*npr1 ura3*), 27092a (*npi3Δ ura3*), and EN031 (*yor275cΔ ura3*) used in this study are all isogenic with the wild-type Σ1278b except for the mutations mentioned [28]. *NPI3* and *YOR275c* were deleted in the 23344c background according to the PCR-based gene replacement procedure [29] using the following primers: D5NPI3 5′-CGGCTGACT-GGAGCATTACTAGGTTCATTACTCACTGGTTGCGGCCGCA-TAGGCCACTAGTGGATCTG-3′ and D3NPI3 5′-CAGAGGG-TCTATTGTAAAAGGACGTAGGATTCTCTTGCAGGCGGCCG-CCAGCTGAAGCTTCGTAGC-3′; D5YOR275c 5′-GAGAGA-GTATTATTATCTATGGAGATCCAGGCAGTGTGACGCGGCC-GCCAGCTGAAGCTTCGTAC-3′ and D3YOR275c 5′-CAAG-CTCAAAATATACTTTATAGATGAATGAGGACAGGTAGCG-GCCGCATAGGCCACTAGTGGAT-3′. Unless otherwise specified, cells were grown in minimal buffered medium (pH 6.1) with 3% glucose as the carbon source [30]. Nitrogen sources were added as indicated to the following final concentrations: $(NH_4)_2SO_4$ 10 mM, proline 0.1%, urea 0.1%, glutamine 0.1%, serine 0.1%. Fur4 was examined in cells grown in minimal medium containing $(NH_4)_2SO_4$ (10 mM) as the sole nitrogen source. Hxt6/7 was detected in cells grown as previously described [16]. The *Escherichia coli* strain used was JM109.

2.2. Cloning of the NPI3 gene

Strain 27086c was transformed with a low-copy-number library representing the whole genome of strain Σ1278b [31]. Among the Ura3+ transformants, one displayed the Npi3+ phenotype on selection media (see text). DNA was isolated from this clone and used to transform *E. coli*. The recovered plasmid (YCpJYS-13) contained a DNA fragment of about 7.5 kb. A 6-kb subclone (YCpJYS-19) constructed in the centromere-based vector pFL38 [32] restored the Npi3+ phenotype in an *npi3* mutant.

2.3. Permease assays

Gap1 permease activity was determined by measuring the initial rate of incorporation of ^{14}C-labeled citrulline (20 μM) as described by Grenson et al. [33]. Gap1 was inactivated by adding $(NH_4)_2SO_4$ (10 mM) to the culture. Fur4 permease activity was determined by measuring the initial rate of incorporation of ^{14}C-labeled uracil (20 μM) into cells transformed with plasmid YEp352fF promoting Fur4 overexpression. The Fur4 inactivation was followed after adding cycloheximide (100 μg/ml) to the culture.

2.4. Yeast cell extracts and immunoblotting

Crude extracts were prepared as previously described [9]. Membrane-enriched preparations were obtained as described elsewhere [10]. For Western blot analysis, 10 μl of solubilized proteins was loaded onto a 10% SDS–polyacrylamide gel in a Tricine system [34]. After transfer to nitrocellulose, the proteins were probed with rabbit antisera raised against Gap1 (1:20 000) [6], Fur4 (1:30 000) [35,36], or against Hxt6/7 (1:500) [16].

3. Results and discussion

3.1. Npi3 is a novel factor involved in ubiquitination and down-regulation of the Gap1 permease

The *npi1/rsp5* and *npi2/doa4* mutations impair ubiquitination of the Gap1 permease and its sorting to the vacuole. They were initially selected for their ability to suppress the loss of Gap1 activity and the resulting growth defect that would normally be caused by an *npr1* mutation in cells growing on minimal medium supplemented with tryptophan and citrulline as sole nitrogen sources [22]. In the same genetic screen, another recessive mutation was isolated, belonging to a third distinct complementation class we named *NPI3*. Since Npi1/Rsp5 and Npi2/Doa4 are both involved in NH_4^+-induced down-regulation of Gap1 [7,9,10], we investigated whether the *npi3* mutation affects this process. The fate of the permease was monitored after addition of NH_4^+ to proline-grown wild-type and *npi3* cells. In wild-type cells, NH_4^+ addition caused a loss of Gap1 activity and a parallel decrease in the level of Gap1 protein (Fig. 1A,B), reflecting the endocytosis and progressive vacuolar degradation of the permease [10]. In *npi3* cells, in contrast, Gap1 remained active and stable, showing that Npi3 is required for NH_4^+-induced down-regulation of Gap1.

The resistance of Gap1 to down-regulation in *npi3* cells could be due to impaired ubiquitination of the permease, as in *npi1/rsp5* and *npi2/doa4* mutants, or to a defect in a step such as internalization, as in the *act1-1* mutant altered in actin [7,10]. To address this issue, we used immunoblotting of membrane-enriched fractions to compare the efficiency of Gap1 ubiquitination in wild-type and *npi3* cells, as well as in *npi1* cells used as a control (Fig. 1C). The Gap1 signal detected with proline-grown wild-type cells consists of a major band around 60 kDa plus minor bands of higher molecular weight, corresponding to mono-ubiquitinated forms of the permease [10]. In the early minutes after NH_4^+ addition, the fraction of ubiquitin-conjugated Gap1 increased markedly in wild-type cells. In proline-grown *npi3* cells, ubiquitin–Gap1 conjugates were hardly detectable (these forms could be observed only upon blot overexposure; not shown). After addition of NH_4^+, the intensity of these bands did not increase (Fig. 1C), a phenotype very similar to that of *npi1* cells. These results show that ubiquitination of Gap1 is impaired in *npi3* cells.

3.2. Cloning of the NPI3 gene

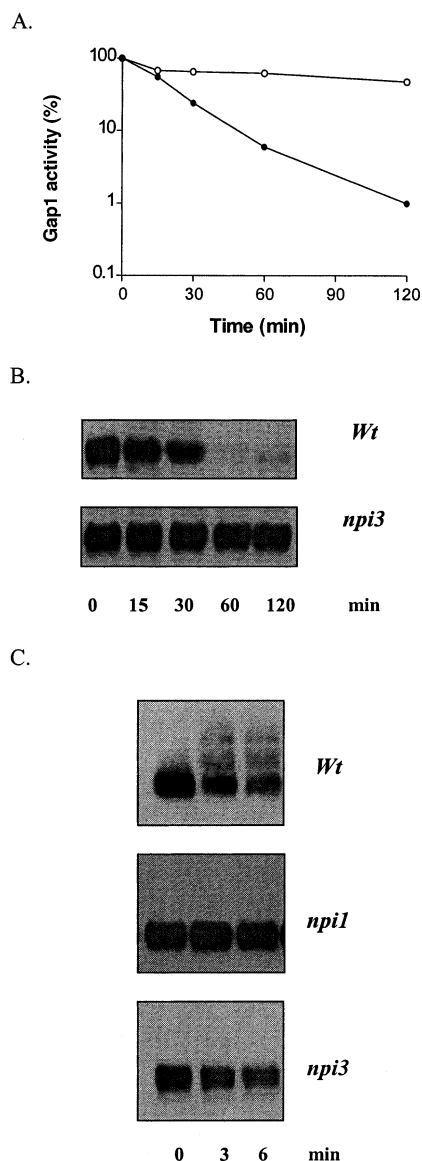The *npi3* mutant was found to grow slowly on minimal medium containing L-serine as the sole nitrogen source.

A.

YPL084w gene and confirmed its allelism with *NPI3*. The *npi3Δ* and *npi3* mutants behaved similarly as regards protection against NH_4^+ down-regulation and ubiquitination of Gap1 (data not shown).

The *bro1* mutation was initially isolated as lethal when combined with *mdm1*, a mutation affecting mitochondrial and nuclear inheritance and interacting genetically with mutations affecting components of the Pkc1 mitogen-activated pro-
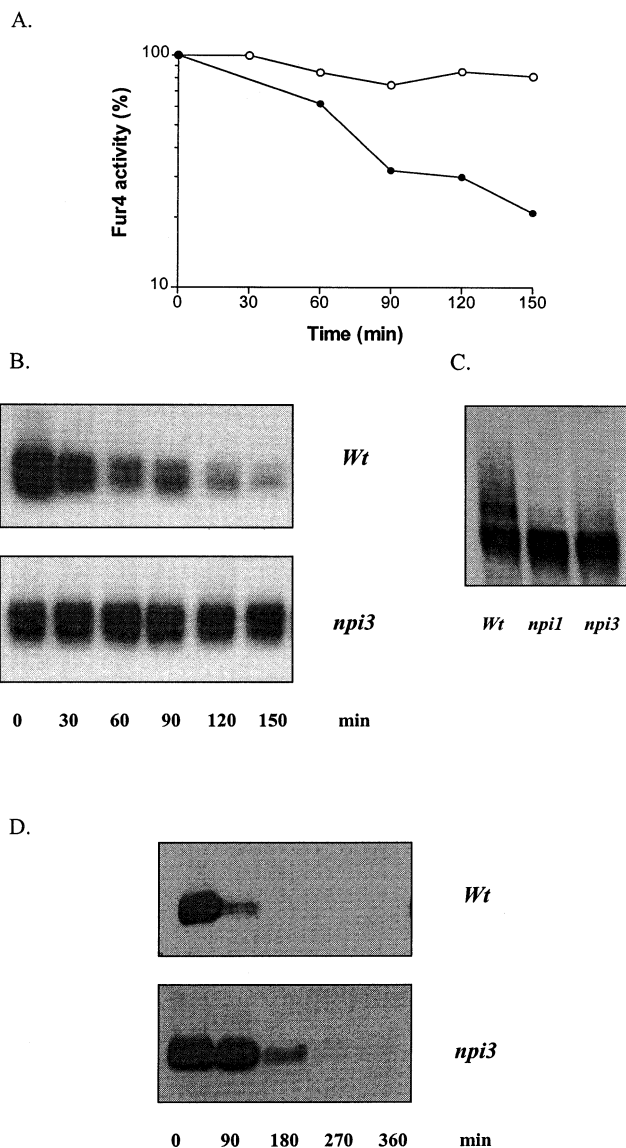
B.

A.

C.

B.   C.

Fig. 1. Ammonium-induced down-regulation of Gap1 is impaired in *npi3* cells. $(NH_4)_2SO_4$ (10 mM) was added to 23344c (Wt, black circle) and 27086c (*npi3*, empty circle) cells grown in minimal medium containing proline as sole nitrogen source. A: Gap1 activity was measured by incorporation of [^{14}C]citrulline (20 μM) before ($t = 0$) and at several times after $(NH_4)_2SO_4$ addition. Results are percentages of initial activities per ml of culture. B: Total protein extracts were prepared at the times indicated and Gap1 was detected by Western immunoblotting. C: Gap1 ubiquitination is impaired in *npi3* cells. Immunoblot of Gap1 from membrane-enriched fractions prepared from 23344c (Wt), 27038a (*npi1*) and 27086c (*npi3*) cells before ($t = 0$) and at several times after addition of $(NH_4)_2SO_4$.

Although this phenotype is still unexplained, it was used to clone the *NPI3* gene by functional complementation. Strain 27086c (*ura3 npi3*) was transformed with a low-copy-number plasmid library representing the genome of strain Σ1278b, and transformants were screened for growth on serine medium. A single plasmid carrying a 7.5-kb insert complementing the *npi3* mutation was recovered. Subcloning experiments and phenotypic analysis revealed that the complementing gene corresponds with the *YPL084w/BRO1* open reading frame (ORF) [37]. We constructed a strain (27092a) lacking the entire

D.

Fig. 2. Down-regulation of Fur4 and Hxt6/7 is impaired in *npi3* cells. A: Cycloheximide (100 μg/ml) was added to 23344c (Wt, black circle) and 27086c (*npi3*, empty circle) cells transformed with plasmid YEp352fF and grown in minimal medium containing ammonium as sole nitrogen source. Fur4 activity was measured by incorporation of [^{14}C]uracil (20 μM) before ($t = 0$) and at several times after cycloheximide addition. Results are percentages of initial activities per ml of culture. B: Total protein extracts were prepared at the times indicated and Fur4 was detected by Western immunoblotting. C: Fur4 ubiquitination is impaired in *npi3* cells. Immunoblot of Fur4 from membrane-enriched fractions from 23344c (Wt), 27038a (*npi1*) and 27086c (*npi3*) cells. D: 23344c (Wt) and 27086c (*npi3*) cells grown on YEPD plus raffinose were transferred to a nitrogen-free minimal medium supplemented with glucose [16]. Membrane-enriched cell extracts were prepared at the times indicated and Hxt6/7 were detected by Western immunoblotting.

tein kinase signaling pathway such as Bck1 [37]. We deleted *BCK1* in the Σ1278b background to test whether this protein plays a role in Gap1 down-regulation. In contrast to the situation encountered in the AH216 background [37], the *bro1Δ* and *bck1Δ* mutants were not thermosensitive in the Σ1278b background, nor could we find any particular phenotype linked to concomitant mutation of *BRO1* and *BCK1* (data not shown). Furthermore, *bck1Δ* cells were not impaired in NH_4^+-induced inactivation of Gap1 (data not shown), indicating that Bck1 and the Pkc1 signaling pathway are most probably not involved in NH_4^+-induced down-regulation of Gap1.

Recently, mutations in the *BRO1* gene were also isolated as suppressors of mutations (*ssy1* and *ptr3*) decreasing the capacity of cells to take up amino acids [38]. The *npi1/rsp5*, *npi2/ doa4*, *uba1*, *bul1*, *vps20*, and *vps36* mutations were isolated in the same genetic screen, leading to the hypothesis that Npi3/ Bro1 is involved in protein turnover [38]. These mutations likely stabilize amino acid permeases at the cell surface, thereby compensating for the effect of the *ssy1* and *ptr3* mutations.

3.3. Npi3 is also involved in down-regulating the Fur4 and Hxt6/7 permeases

Since down-regulation of Gap1 is specifically induced upon NH_4^+ addition, Npi3 might be involved in the response of cells to NH_4^+. Alternatively, Npi3 could be, like the Npi1/Rsp5 HECT-type ubiquitin ligase, a component of the basic down-regulation machinery. To test these hypotheses, we investigated whether Npi3 is required for down-regulation of transporters responding to stimuli other than NH_4^+. We first tested stress-induced down-regulation of the uracil permease Fur4, a process shown to require Npi1/Rsp5-dependent ubiquitination of Fur4 [9,14]. Addition of cycloheximide to wild-type cells overexpressing Fur4 led to a loss of uracil uptake activity accompanied by degradation of the protein (Fig. 2A,B). In *npi3* cells, Fur4 remained active and stable in the presence of cycloheximide. We used immunoblotting of membrane-enriched fractions to compare the efficiency of Fur4 ubiquitination in wild-type, *npi1* and *npi3* cells (Fig. 2C). The Fur4 signal detected with wild-type cells consists of a major band plus minor bands of higher molecular weight corresponding to ubiquitinated forms of the permease. As previously shown in *npi1/rsp5* cells [14], these forms were barely detectable in *npi3* cells indicating that Fur4 ubiquitination is impaired. We next examined glucose-induced degradation of the Hxt6/7 hexose transporters (Fig. 2D). It was shown in a previous study that Hxt6/7 is degraded when raffinose-grown cells are shifted to nitrogen-free glucose medium, and that this process requires Npi1/Rsp5 [16]. The results presented in Fig. 2D show that degradation of Hxt6/7 is slowed down in the *npi3* strain. Finally, we found that Npi1/Rsp5-dependent inactivation of the proline permease (Put4) in response to NH_4^+ [4] is also strongly impaired in the *npi3* strain (not shown). Taken together, these results show that the *NPI3* gene is required for normal down-regulation of several permeases in response to diverse stimuli. Furthermore, we show that Npi3 is involved in the ubiquitination of at least two of these permeases, namely Gap1 and Fur4.

3.4. Direct sorting to the vacuole of neosynthesized Gap1 involves Npi3

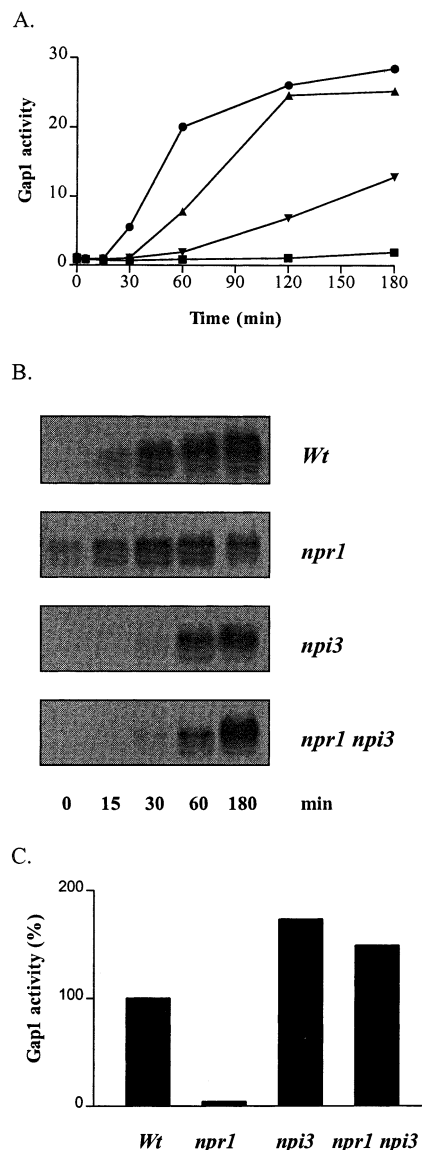On poor nitrogen sources like proline or urea, neosynthe-

Fig. 3. Sorting to the vacuole of neosynthesized Gap1 in an *npr1* mutant involves Npi3. 23344c (*Wt*, circle), 21994b (*npr1*, square), 25173a (*npr1 npi3*, inverted triangle), and 27086c (*npi3*, triangle) cells were grown on glutamine medium and transferred to urea medium at time 0. A: Gap1 activity was measured by incorporation of [14C]citrulline (20 μM) at several times after transfer. Results are expressed in nmol of incorporated citrulline per mg of protein. B: Total protein extracts were prepared at the times indicated and Gap1 was detected by Western immunoblotting. C: Gap1 activities measured in steady-state growing cells cultured on urea medium. Results are expressed as percentages of the initial activity per mg of protein.

sized Gap1 is targeted to the plasma membrane. In a mutant lacking a functional Npr1 kinase, it is directly sorted to the vacuole without ever reaching the plasma membrane [6]. Direct sorting of neosynthesized Gap1 to the vacuole also occurs in cells growing on a favored nitrogen source like glutamate [25] or NH_4^+ [6]. Ubiquitination of Gap1 is essential to sorting of the permease to the vacuole: neosynthesized Gap1 is targeted to and accumulates in the plasma membrane in *npr1* cells which additionally lack functional Npi1/Rsp5, Npi2/ Doa4 or Bul1/2 proteins, and also in *npr1* cells expressing a ubiquitination-resistant Gap1 form (Gap1^{K9K16}) [8]. In anoth-
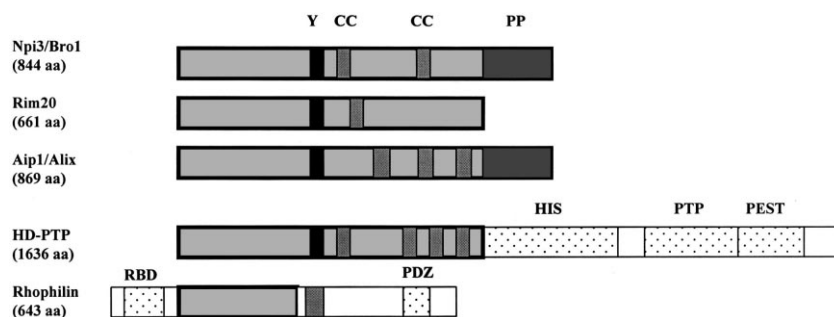
Fig. 4. Schematic representation of domains conserved in proteins of the Npi3/Bro1 family. The regions sharing homology with Npi3/Bro1 are heavily boxed. Y, Src tyrosine kinase phosphorylation site; CC, coiled coil domains; PP, proline-rich regions; HIS, his domain [52]; PTP, tyrosine phosphatase domain; PEST, PEST motif; RBD, Rho-binding domain; PDZ, PDZ domain. The proteins compared are the following: yeast Npi3/Bro1 (P48582), yeast Yor275c/Rim20 (S67177), mouse Aip1/Alix (NP_035182), human HD-PTP (BAB19280), mouse rhophilin (Q61085).

er study, it was reported that mono-ubiquitination of Gap1 is not sufficient for sorting the permease to the vacuole. Rather, poly-ubiquitination of Gap1 would be essential and dependent on the Bul1 and Bul2 proteins [26].

To test whether Npi3 is also involved in sorting of Gap1 to the vacuole, we monitored the fate of the permease synthesized in an *npr1 npi3* double mutant. We used the wild-type and single mutants as controls (Fig. 3). The cells were first grown on a glutamine-containing medium to repress *GAP1* transcription, and then transferred to urea medium on which *GAP1* is derepressed. In both wild-type and *npi3* cells, the shift to urea led to progressive immunodetection of a high-intensity Gap1 signal with concomitant development of high Gap1 activity (Fig. 3A,B). In the *npr1* strain, in keeping with the previous finding that Gap1 is directly sorted to the vacuole in this mutant [6], Gap1 remained inactive even 3 h after the shift to urea (Fig. 3A). A low-intensity Gap1 signal was detected in glutamine-grown cells, an effect likely due to reduced glutamine uptake in this mutant leading to partial release from *GAP1* gene repression. After the shift to urea, the signal increased, but remained much weaker than that of the wild-type, showing that Gap1 does not accumulate to high levels in Npr1-lacking cells [6]. The *npr1 npi3* double mutant displayed a phenotype more similar to that of *npi3* cells: after the shift to urea, Gap1 accumulated and gradually displayed high activity in the plasma membrane, indicating that at least part of the newly synthesized Gap1 was targeted to the cell surface rather than to the vacuole. In steady-state urea-grown *npr1 npi3* cells, likewise, the *npi3* mutation completely suppressed the loss of Gap1 activity normally observed in *npr1* cells (Fig. 3C).

These data show that Npi3, like Npi1/Rsp5, Npi2/Doa4, and Bul1/Bul2, is involved both in down-regulating cell surface Gap1 and in direct sorting of the permease from the late secretory pathway to the vacuole.

3.5. Npi3/Bro1 comprises distinct domains and is homologous to fungal Rim20 and PalA proteins

Npi3/Bro1 is an 844-residue protein (97.3 kDa) containing at least three particular motifs (Fig. 4). First, it has a sequence [R,K]-X(2,3)-[D,E]-X(2,3)Y matching the consensus phosphorylation sequence for the Src family of tyrosine kinases [39]. Second, putative coiled coil domains are found in the middle of the protein. Third, Npi3/Bro1 possesses a proline-rich C-terminus containing potential SH3 binding sites and a

PPLP sequence, one of the motifs binding to WW(P) domains [40].

Analysis of sequence databases revealed that Npi3/Bro1 shares sequence homology with the product of *YOR275c/RIM20*. Yet the proline-rich region present in the C-terminus of Npi3/Bro1 is not conserved in Rim20 (Fig. 4). We isolated a *rim20Δ* mutant (see Section 2) and found Gap1 to be inactivated and degraded in this strain in the same manner as in the wild-type (not shown) indicating that Rim20 is not required for down-regulation of Gap1. Npi3/Bro1 and Rim20 are homologous to the *Aspergillus nidulans* PalA protein reported to be involved in pH-dependent gene expression [41]. Most of the other identified components of this pH-response pathway (PalB, PalH, PalI, PalF, PacC) are conserved in *S. cerevisiae* (respectively Rim13/Cpl1, Pal2, Rim9, Pal3, Rim101) and in other fungi [41–46]. However, deletion of the corresponding ORFs in yeast does not influence NH_4^+-induced down-regulation of Gap1 (our unpublished results). More recent studies showed that *A. nidulans* PalA and yeast Rim20 are required for activation of the PacC-Rim101 transcription factor by proteolytic processing of the protein's C-terminus [47,48].

3.6. Npi3/Bro1 might be involved in ubiquitin-dependent control of permease trafficking at different sorting compartments

The role of palA-Rim20 in processing of transcription factors suggests that the Npi3/Bro1 and palA-Rim20 proteins perform distinct functions. However, although palA and Rim20 lack the C-terminal proline-rich region present in Npi3/Bro1, the high degree of conservation between all three proteins is more in favor of a common biochemical function. Perhaps processing of the PacC-Rim101 transcription factor involves ubiquitin and proteins of the palA-Rim20-Npi3/Bro1 family somehow assist the process of ubiquitin fixation in various cellular mechanisms. Alternatively, processing of the PacC-Rim101 transcription factors could somehow rely on mechanisms of membrane protein trafficking. The latter view is supported by recent data obtained in large-scale analysis of protein complexes. First, a systematic two-hybrid analysis unraveled an interaction between Rim20 and Snf7/Vps32 [49], one member of the class E *VPS* family required for sorting of membrane proteins in vesicles invaginating in the lumen of the late endosome (multivesicular body pathway – MVB) [50]. Second, systematic analysis of protein complexes by mass spectrometry suggests that Npi3/Bro1, Snf7/Vps32

and Vps4 (yet another class E *VPS* protein) might be associated into a complex [51]. Third, Npi3/Bro1 is identical to Vps31 (Odorizzi and Emr, personal communication), yet another member of the class E *VPS* family involved in MVB sorting [50]. In a *vps31* mutant, the biosynthetic carboxypeptidase S as well as the endocytotic Ste2 receptor are mainly retained in the prevacuolar endosome (E compartment) instead of reaching the vacuolar lumen [50]. After NH_4^+ addition, Gap1 is in contrast stabilized and fully active at the plasma membrane in the *npi3* mutant, raising the interesting possibility that a deficiency in Npi3/Bro1/Vps31 leads to recycling of the internalized permease back to the plasma membrane.

Recently, genetic interaction between *npi1/rsp5* and *npi3/bro1* mutations has been reported, indicating a physical interaction between these proteins [38]. Furthermore, Npi1/Rsp5 can self-associate [17] and can also bind to the Bul1 and Bul2 proteins, probably via their PPXY motifs [18,19]. Together with our conclusion that the *npi3/bro1* mutant displays a phenotype similar to that of *npi1/rsp5* and *bul1 bul2* strains [8,10], these observations suggest that the Npi1/Rsp5, Bul, and Npi3/Bro1 proteins might form a multi-subunit complex involved in ubiquitination of permeases at distinct sorting compartments.

3.7. Npi3/Bro1 is conserved in higher eukaryotes

Npi3/Bro1 homologues are also found in higher eukaryotes, including mammals (Fig. 4). Some members of the Npi3/Bro1 family share high sequence similarity over their entire lengths, including the three motifs mentioned above (Fig. 4). An example of an Npi3/Bro1 homologue in a higher eukaryote is *Xenopus* Xp95 [39], a protein of unknown function. A synthetic peptide containing the putative Src kinase tyrosine phosphorylation site of this protein undergoes phosphorylation by Src kinase. Furthermore, the abundance of tyrosine-phosphorylated Xp95 increases during oocyte maturation, suggesting that Xp95 might be a component of a tyrosine signaling pathway [39]. Our current experiments aim at testing the function of the conserved tyrosine phosphorylation site of Npi3/Bro1. Another Npi3/Bro1 homologue is the human protein tyrosine phosphatase HD-PTP [52]: the N-terminal half of this protein is homologous to Npi3/Bro1, whilst its C-terminus contains a proline-rich HIS domain, the tyrosine phosphatase domain, and a PEST motif. The role of this protein remains unknown. Mouse rhophilin, a protein known to bind to the small signaling G-protein Rho [53], also contains a region highly similar to the N-terminal part of Npi3/Bro1.

Finally, mouse Aip1 is similar to Npi3/Bro1 over its entire length (Fig. 4). It is associated with both the cytoplasm and cell membranes and interacts physically with Alg2, a calcium-binding protein involved in cell apoptosis [54,55]. Interestingly, Aip1 also interacts with SETA/CIN85, a new SH3-containing adapter shown to bind to c-Cbl, a ubiquitin ligase of the RING finger family involved in ubiquitination and intracellular trafficking of receptor tyrosine kinase [27,56–59]. Aip1 might thus be part of a protein complex involved in receptor ubiquitination and/or trafficking. This raises the possibility that Npi3/Bro1 family proteins might play a conserved role in mechanisms governing the intracellular traffic of membrane proteins. Our current experiments aim at further investigating the exact role of Npi3/Bro1 in Gap1 ubiquitination and trafficking.

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