

The Deubiquitinating Enzyme Dot4p Is Involved in Regulating Nutrient Uptake

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In yeast, several membrane-bound nutrient transporters have been shown to be regulated by the covalent attachment of ubiquitin, a signal for internalization and degradation. The yeast gene *DOT4* encodes one of a family of enzymes which remove ubiquitin from proteins to which the peptide has been attached. Mutations in *DOT4* cause a growth defect that is particularly severe when combined with mutations in nutrient biosynthetic enzymes (1). These results suggest that nutrient transport or utilization may be compromised in *dot4* mutants. We now report that preventing the down-regulation by endocytosis of membrane proteins partially suppressed the *dot4Δ* growth defect. We also show that the activity of the amino acid permease Gap1p is reduced in *DOT4* mutants. This correlates with a reduction in Gap1 protein level, while *GAP1* mRNA levels remains unchanged. We conclude that Dot4p is involved in posttranscriptionally regulating Gap1p, and possibly other transporters as well. © 2001

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The covalent attachment of the 76-amino-acid peptide ubiquitin to cellular proteins is a well-studied signal for protein turnover (2). Ubiquitination marks both soluble and membrane-bound proteins for degradation, the former by the 26S proteasome, and the latter through internalization and targeting to lysosomes (3).

In yeast, several membrane-bound nutrient transporters have been shown to be regulated by ubiquitin-mediated internalization and degradation, including the general amino acid permease Gap1p, the uracil permease Fur4p, the maltose permease Mal6p, and the galactose permease Gal2p (3). Since organisms from yeast to humans share a requirement for nutrient up-

take, advances in understanding of the yeast model system lend important clues to our general understanding of the regulation of nutrient uptake.

The yeast gene *DOT4* encodes one of a family of enzymes which remove ubiquitin from proteins to which the peptide has been attached (1). Our earlier studies demonstrated that mutations in *DOT4* result in loss of transcriptional silencing at telomeres and the silent mating-type loci (1, 4). Mutants lacking functional Dot4p not only are impaired in silencing, but also show reduced cellular growth rate. The growth rate is particularly reduced by combining a mutation in *DOT4* with mutations in nutrient biosynthetic pathway enzymes, even when cells are grown on nutritionally complete medium (1). Our initial work showed that this growth defect is partially relieved by deletion of Silent Information Regulator (*SIR*) genes, indicating that the silencing complex, probably through dysregulated activity, contributes to the impaired growth of *dot4* mutants. However, additional pathways must play a role in the *dot4* growth defect since *dot4* mutants were moderately growth-retarded even in the absence of a functional silencing complex (1). These results suggested that nutrient transport or utilization may be compromised in *dot4* mutants.

We now report that preventing the down-regulation by endocytosis of membrane transporters suppressed the *dot4Δ* growth defect, suggesting that the accumulation of a membrane protein can overcome the growth defect of a *DOT4* mutant. We then use the general amino acid permease Gap1p, which is known to be regulated by the ubiquitin system, to show that Gap1p cellular activity and protein levels are reduced in *DOT4* mutants, while *GAP1* mRNA levels remain unchanged.

MATERIALS AND METHODS

Strains and growth media. All strains were grown in standard culture media and standard yeast genetic methods were used (5, 6). Yeast strains were based on the YPH (7) and BY (8) strain backgrounds, which are *per1* mutant. Strains YMW499-U and YMW81-U were the generous gift of B. Winsor (9).

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DOT4 was replaced with *KanMX* by PCR-mediated gene disruption as described (8, 10). The reaction contained pRS400 as template, and primers (dot4 RS(+): TCC AGG AAT ATC GAG TTT TTT CAT TTG GTG AAC CTG TGC GGT ATT TCA CAC CG; dot4 RS(-): TCC AGG AAT ATC GAG TTT TTT CAT TTG GTG AAC CTG TGC GGT ATT TCA CAC CG). Transformants were selected using G418 and confirmed by PCR as described (Kahana and Gottschling, 1998).

GAP1 ORF was replaced with *HIS3* using PCR-mediated transformation with pRS303 as template. Primer 1: 5'-AGT AAT ACT TCT TCG TAC GAG AAG AAT AAT CGA TTG TAC TGA GAG TGC ACC. Primer 2: 5'-ACA CCA GAA ATT CCA GAT TCT ATA CCA TCT CTG TGC GGT ATT TCA CAC CG. The PCR fragment was then used to transform UCC4788, which contains only the *HIS3* auxotrophy. Correct integration was checked by PCR, and confirmed by testing for growth on minimal media containing 0.1% citrulline as the sole nitrogen source.

Citrulline uptake assays. To measure Gap1p activity, citrulline uptake was measured as previously described (11) using the isogenic strain pair UCC4786 (*DOT4*) and UCC4794 (*dot4::KanMX*). Briefly, cells grown to mid-logarithmic phase in minimal ammonium sulfate media were harvested, washed and resuspended in citrulline uptake buffer (2% glucose, 1× nitrogen-free Difco yeast nitrogen base). Uptake rates were standardized by cell density. Radio-labeled ¹⁴C-citrulline (ARC, MO) was added to a final concentration ranging from 10 μM to 70 μM, and time-points were taken in triplicates. Maximal rates of uptake (V_{max}) were achieved at citrulline concentrations of >3 μM per million cells.

Gap1p immunoprecipitation and Westerns. Immunoprecipitation was used to concentrate Gap1p from total protein extracts as described (12). pPL257 and pPL247, a generous gift of C. Kaiser, are pRS316 derivatives carrying HA-tagged or untagged *GAP1*, respectively (13). Strains UCC4776 (*DOT4*) and UCC4799 (*dot4Δ*) were transformed with pPL257 and pPL247, and total protein extracts were made and incubated with antibody 12CA5 specific for the HA tag (BABCO, CA), followed by addition of Protein G-Sepharose (GammaBind, Pharmacia, IN) and analysis on a 10% gel. Membranes were developed with HA.11 antibodies (BABCO, CA), and HRP-coupled goat anti-mouse IgG1-specific antibodies (Boehringer-Mannheim, IN), to reduce the signal of the 12CA5 fragments used for the immunoprecipitation (IgG2_b subtype). ECL (Amersham-Life Sciences, IL) was used to detect the HRP signal.

***GAP1* Northern analysis.** Prototrophic strains UCC4786 (*DOT4*) and UCC4794 (*dot4::KanMX*) were grown in nutritionally complete media and cells collected at mid-logarithmic growth phase. RNA was extracted by a phenol-freeze protocol (14), and Northern analysis was performed as previously described (1). Membranes were developed using digoxigenin-labeled riboprobes to *GAP1* and *PDA1* (Boehringer-Mannheim, IN). Primers for a *PDA1* probe were described (1). Primers for a *GAP1* probe (5'-ATC GAT AAT ACG ACT CAC TAT AGG GAA AGG ACT CAT CAA TAA ATC TGG) and (5'-ACT TCT TCG TAC GAG AAG AAT AAT CC).

RESULTS

The Growth Defect of a dot4Δ Strain Can Be Suppressed by Inhibition of Endocytosis

Even when nutrients are provided, cells with an impaired biosynthetic pathway will nevertheless show diminished growth if uptake of the nutrient is inefficient (15–17). Amino acids enter the cell via transporters located in the plasma membrane (18). The concentration of transporters in the membrane, and consequently the rate of amino acid uptake, is determined by an equilibrium between (1) transporter syn-

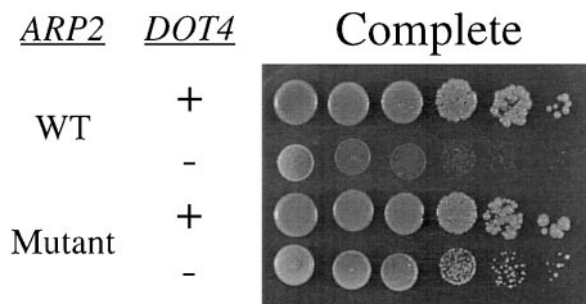


FIG. 1. The auxotrophic growth defect of *dot4Δ* strains depends on a fully functional endocytic system. In the genetic background of multiple auxotrophic mutations, deleting *DOT4* causes a severe growth defect (*ARP2*). Growth of the *DOT4/dot4Δ::KanMX* strain pair YMW499-U and UCC4806 was compared (*ura3-52 lys2-801amber ade2-101ochre trp1-Δ63 his3-Δ200 leu2-Δ1 ARP2::URA3*). The effect of combining a *DOT4* deletion with a mutation in endocytosis (*arp2-1*) was determined by comparing growth of the *DOT4/dot4Δ::KanMX* strain pair YMW81-U and UCC4807 (*ura3-52 lys2-801amber ade2-101ochre trp1-Δ63 his3-Δ200 leu2-Δ1 arp2-1::URA3*). Growth was assayed at the permissive temperature (23°C) on nutritionally complete media.

thesis and targeting to the membrane, and (2) transporter turnover by endocytosis (3). Hence, Dot4p may be required to maintain optimal transporter concentration and function. Alternatively, the utilization of imported amino acids by the cell for its metabolic needs may be compromised by a mutation in *DOT4*, resulting in a growth defect. We reasoned that disrupting endocytosis in a *DOT4* mutant background would help to distinguish a transporter defect from a utilization defect.

A point mutation in the actin-related protein *ARP2* results in a reduced rate of endocytosis at the permissive growth temperature but little effect on growth rates, leading to accumulation of transporters at the plasma membrane (9). To test whether the transporter endocytosis pathway was playing a role in the slow growth phenotype, *DOT4* was deleted in an auxotrophic strain bearing the mutant *arp2-1* mutation. The *arp2-1* mutation diminished the growth defect caused by a *dot4* deletion (Fig. 1). This result suggests that a nutrient transport defect contributes to the auxotrophy-dependent slow growth defect of *dot4* mutants, likely by decreasing the number of amino acid transporters at the plasma membrane.

Gap1p Activity Is Reduced in dot4Δ Mutants

The results above suggest a model in which Dot4p protects nutrient transporters from ubiquitin-dependent endocytosis. In the absence of Dot4p, the concentration of transporters at the cell surface would reach new steady-state levels that are insufficient to support optimal growth in cells that depend on nutrient import. This model was tested by examining the level of Gap1p activity in a *DOT4* mutant background.

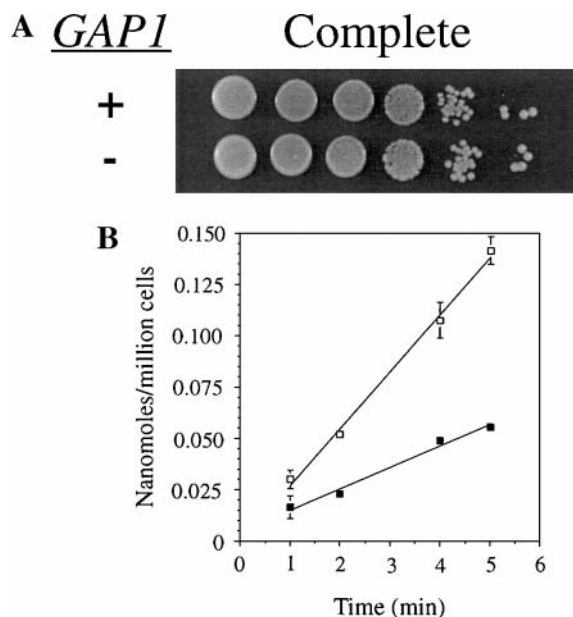


FIG. 2. Gap1p amino-acid transport activity is decreased by deleting *DOT4*. (A) Deleting *GAP1* causes no differences in growth on media containing ammonium sulfate. The congenic strain pair UCC4788 (*MAT@ his3-200*) and UCC4815 (*MAT@ gap1::HIS3*) was used. (B) Gap1p activity was measured using ¹⁴C-citrulline uptake assays. Cells were grown in ammonium sulfate and harvested at mid-logarithmic growth. The congenic strain pair UCC4786 (*DOT4*; open squares) and UCC4794 (*dot4::KanMX*; closed squares) was used.

Gap1p is a low-affinity, high-capacity transporter of amino acids which are utilized as a nitrogen source (18). Recent data shows that ubiquitination plays a crucial role in the down-regulation of Gap1p by endocytosis (19, 20). By itself, a deletion of *GAP1* has no effect on growth in the presence of a rich nitrogen source such as ammonium sulfate (Fig. 2A). Therefore, Gap1p transporter activity can be measured and directly compared in strains bearing wild-type and mutant *DOT4* alleles.

Citrulline is an amino acid that is not used for protein synthesis, and its active transport requires Gap1p (11, 21). By measuring uptake of radio-labeled citrulline, the relative activity of Gap1p can be assessed. As Fig. 2B indicates, Gap1p activity, as measured by citrulline uptake, is dramatically reduced in the absence of Dot4p.

Gap1p Levels Are Reduced in *dot4Δ* Mutants

The observed reduction in Gap1p activity may be the result of either a decrease in the amount of Gap1p, or a change in Gap1p activity kinetics. The levels of Gap1p were directly assessed by western-blot hybridization. Cellular Gap1p concentrations were too low for total cellular protein detection, so immunoprecipitation was employed. In wild-type *DOT4* strains, Gap1p

was easily visualized by immunoblot detection techniques (Fig. 3, Gap1p-HA). However, in a *dot4Δ* strain, little to no Gap1p was detected (Fig. 3; asterisk marks a loading control), indicating that the lack of Dot4p resulted in a dramatic decrease in Gap1p protein levels, consistent with the observed decrease in Gap1p activity.

The Effect of a *DOT4* Mutation on Gap1p Is Posttranscriptional

The role of the ubiquitin system in protein turnover suggested that the observed decrease in Gap1p levels in a *dot4Δ* mutant strain may be posttranscriptional. However, the involvement of Dot4p in regulating transcriptional silencing raises the possibility that Gap1p activity is reduced through transcriptional changes. To distinguish between these possibilities, we used Northern blot techniques to assess steady-state *GAP1* transcript levels. While Gap1 protein activity and levels were reduced (Figs. 2B and 3), steady-state *GAP1* RNA levels were unchanged by the deletion of *DOT4*, supporting a posttranscriptional mechanism for the observed changes (Fig. 4).

DISCUSSION

The reduced growth rate observed in *dot4* mutant strains is greatly exacerbated when the mutants are dependent on an extracellular source of amino acids (1). This phenomenon occurs even when auxotrophic *dot4Δ* mutants are grown on nutritionally complete media. Although the growth defect can be partly explained by changes in the *SIR*-dependent transcrip-

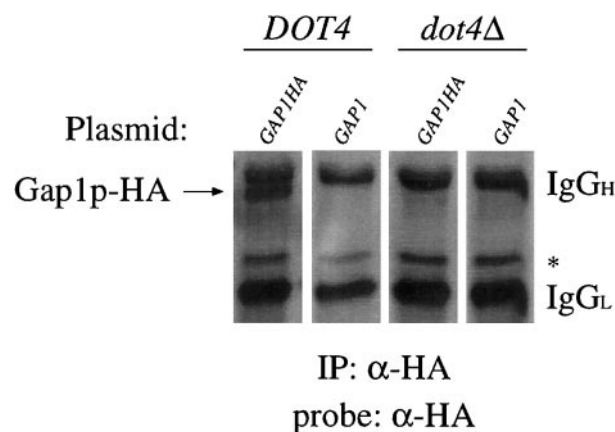


FIG. 3. In the absence of Dot4p, Gap1p is down-regulated posttranscriptionally. Western blot analysis was conducted on Gap1p immunoprecipitates. HA-tagged Gap1p or untagged controls were expressed from a plasmid, and equal numbers of cells were harvested at mid-logarithmic growth. Gap1p-HA and the Ig chains are marked as such, and the asterisk marks a cross-reacting band that serves as a loading control.

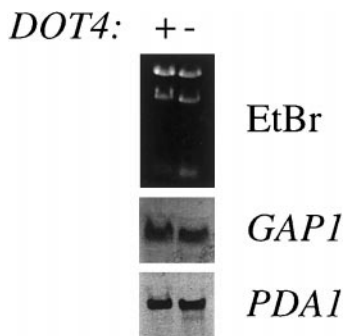


FIG. 4. Northern blot analysis of steady-state GAP1 transcript levels in *DOT4* and *dot4Δ* cells. *PDA1* was probed in a parallel loading experiment as a loading control.

tional silencing process (1), a *SIR*-independent component clearly also contributes.

Our study supports a model by which the auxotrophic *dot4Δ* mutants are nutritionally compromised because of a reduction in nutrient transport. We used Gap1p as a test case because the absence of that transporter alone does not result in a growth defect if a rich nitrogen source (e.g., ammonium sulfate) is provided, allowing for a direct comparison between wild-type and mutant *DOT4* strains. Gap1p is also a well-characterized transporter known to be regulated by ubiquitin-mediated endocytosis (19, 20). Finally, changes in Gap1p activity would be consistent with the nutritional deprivation of several different amino acids in *dot4Δ* strains. Our results showed that while *GAP1* RNA levels remained unchanged, Gap1p transport activity and protein levels were reduced in *dot4Δ* cells. These results support a posttranscriptional role for the ubiquitin-hydrolase Dot4p in regulating nutrient transporter activity.

Since ubiquitination serves both as an internalization signal for many membrane-bound transporters, as well as a signal for degradation of cytoplasmic and nuclear proteins, the mechanism for regulation by Dot4p may be direct or indirect. Dot4p may act directly on transporters as a "proof-reading" enzyme, removing covalently attached ubiquitin moieties and thereby preventing transporter internalization. In the absence of Dot4p, an increased number of transporters would be internalized and directed for degradation by the lysosome-like vacuole, resulting in decreased nutrient uptake and phenotypic starvation.

Alternatively, Dot4p may act on positive posttranscriptional regulators of transporter targeting and function, such as proteins involved in the targeting of transporters to the plasma membrane. The absence of Dot4p would result in greater degradation of such regulators, causing inappropriate targeting and subsequent degradation of nutrient transporters (11). However, the finding that a mutation in endocytosis diminished the growth defect of *dot4* mutants argues

that nutrient transporters are properly targeted to the plasma membrane.

Recently, Gap1p ubiquitination and down-regulation by endocytosis were shown to depend on Doa4p (20), a well-characterized deubiquitinating enzyme that recycles ubiquitin (2, 22). Indeed, Doa4p was shown to be required for down-regulation by endocytosis of other membrane transporters as well (23, 24). However, other deubiquitinating enzymes may inhibit transporter endocytosis by deubiquitinating specific substrates. Here we find that deleting *DOT4* led to the down-regulation of Gap1p, which is opposite from the published effect of mutating *DOA4* (20), lending further support for a more substrate-specific antidegradation activity of Dot4p.

Nutrient uptake is essential for any cell that cannot synthesize all its nutrients. Defects in transport of multiple nutrients and consequent nutritional deprivation is not limited to yeast. Such defects are proposed to form the basis of several rare human disorders, such as Hartnup's disease and Fanconi's syndrome (25–29). On the other hand, up-regulated activity of multiple transporters has been suggested as a hallmark of tumor biology (30). In both cases, multiple genetically-unrelated transporters are up- or down-regulated. Defects in transcriptional activators, as well as in ATP generation and transporter coupling, have been proposed and studied (26, 28). Impaired regulation of transporter turnover by proteins such as Dot4p and Doa4p could also contribute to transporter dysfunction.

In our earlier study, we found that Dot4p was preferentially localized to the nucleus (1). We cannot as yet determine whether Dot4p acts on nuclear proteins that influence posttranscriptional regulation of nutrient transporters, or whether a small amount of Dot4p is also present in the cytoplasm and/or at the plasma membrane and was not detectable by microscopy. Identification of those nutrient transporters responsible for the observed starvation and growth defect is the next step to understanding the mechanism by which Dot4p functions in their regulation.

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