The Fenpropimorph Resistance Gene FEN2 from Saccharomyces cerevisiae Encodes a Plasma Membrane H⁺-Pantothenate Symporter*

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The product of the FEN2 gene of Saccharomyces cerevisiae has previously been described as a protein conferring sensitivity to the antifungal agent fenpropimorph. Fen2p was postulated to act as a common regulator of carbon and nitrogen catabolite repression and of amino acid and ergosterol biosynthesis. In this paper, we present experimental evidence characterizing Fen2p as a plasma membrane-localized transporter for the vitamin pantothenate. The high affinity transport system (K_m = 3.5 μM) is sensitive to uncouplers, suggesting a H⁺-pantothenate cotransport. Pantothenate transport rates in yeast are modulated by extracellular pantothenate, being maximal at low pantothenate concentrations. It is demonstrated that β-alanine can suppress the growth defect of FEN2 wild-type and fen2 mutant cells on pantothenate-free medium. Evidence is presented that β-alanine is transported by the general amino acid permease Gap1p. The relation among pantothenate transport, nitrogen catabolite repression, and sensitivity to the antifungal agent fenpropimorph is discussed.

The vitamin pantothenate is essential for the synthesis of coenzyme A, a universal carrier of activated acyl groups. Many organisms, such as most bacteria and higher plants, synthesize pantothenate de novo. Mammals (including man) or the bakers’ yeast Saccharomyces cerevisiae, however, have lost this ability and depend on the supply of pantothenate with their diet or growth medium. The pantothenate dependence of bakers’ yeast was first reported in 1942 (1), when the effects of different vitamins, such as pyridoxine (vitamin B₆), thiamine (vitamin B₁), biotin (vitamin H), and pantothenate, on the growth of S. cerevisiae were analyzed. Whereas all strains analyzed grew equally well in the absence of pyridoxine or thiamine, growth was drastically retarded when biotin or pantothenate was omitted from the growth medium. This suggested that biotin and pantothenate cannot be synthesized by S. cerevisiae and that both vitamins have to be taken up from the medium by plasma membrane-localized vitamin transporters.

Only recently has the VHT1 gene encoding the H⁺-biotin symporter of the S. cerevisiae plasma membrane been identified (2). This transporter belongs to the major facilitator superfamily (3) and is part of a subfamily of eight S. cerevisiae genes encoding proteins sharing between 20 and 30% amino acid identity. This subfamily was named the allantoate transporter family (4) after the product of the DAL5 gene, which was the first member of this family that has been assigned a specific transporter function (5). Dal5p transports allantoate and ureidosuccinate across the yeast plasma membrane, two compounds that can be used as sole nitrogen sources.

The physiological functions of the remaining six putative transporters of this family are not known, although mutations in two of these open reading frames confer resistances against ethionine sulfoxide (6) and the antifungal substance fenpropimorph (7). The responsible genes were named SEO1 (for suppressor of ethionine sulfoxide resistance 1) and FEN2 (for fenpropimorph resistance 2) and correspond to the open reading frames YCR028C and YAL067C, respectively.

Fungal resistance to the fungicide fenpropimorph has been the object of intensive studies by several laboratories (e.g. Refs. 8–11), and the common result of these studies was that fenpropimorph impairs sterol biosynthesis by inhibition of sterol Δ⁵,7-isomerase and C14 sterol dehydrogenase. Treatment of S. cerevisiae wild-type cells with fenpropimorph results in a drastic reduction of the ergosterol content, the major sterol of yeast cells, and in growth inhibition. This could be overcome with bulk levels of exogenous unsaturated C₅,₆ sterols (8).

When the open reading frame YCR028C was first characterized (12), it was found that disruption of YCR028C caused sensitivity to 3-aminitriazole, an inhibitor of histidine biosynthesis. The FEN2 gene of S. cerevisiae was shown to be allelic to open reading frame YCR028C after its identification by complementation cloning in the fen2-1 mutant strain CM2 (7). The fen2-1 mutation caused a 3-fold decrease in the ergosterol content and reduced growth on complete medium (7). However, no intermediates of ergosterol biosynthesis accumulated in CM2, suggesting that none of the biosynthetic steps between squalene and ergosterol was impaired. Therefore, the specific activities of all enzymes involved in squalene biosynthesis were analyzed in vitro, but again no impaired step was identified. Surprisingly, the specific activity of acetocacetyl-CoA thiolase was increased in CM2 (7).

It was also observed (7) that on minimal medium, the CM2 mutant strain was phenotypically indistinguishable from the wild-type strains. Fenpropimorph resistance, reduced ergosterol content, and growth retardation of CM2 were seen only on complete medium. After further analyses of this effect on different growth media, it was finally concluded that the FEN2 gene product is involved in the coregulation of carbon catabolite repression, nitrogen catabolite repression, and the

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general control of amino acid and ergosterol biosynthesis (7). In this paper, we show that the *S. cerevisiae* FEN2 gene encodes a plasma membrane-localized, high affinity H⁺-pantothenate symporter. We also present experiments explaining the different phenotypic properties of fen2 mutant strains on minimal and complete media and discuss the potential mechanism of fen2-derived fenpropimorph resistance.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, Plasmids, and Transformation**—For characterization of the FEN2 gene, we used yeast strain 711/1a (MATα, ura3-52, trp1Δ1) and its isogenic fen2 disrupting 711/1c (MATa, ura3-52, trp1Δ1, fen2::URA3). The FEN2-overexpressing strain was derived from strain 711/1c by transformation with the plasmid pFEN2mc. This plasmid was generated by cloning the HINDIII insert of plasmid pLA514 (12) that was shown to complement the fen2 mutation (7) into the HINDIII site of the plasmid YEplac112 (13). For analysis of β-alanine transport, we used strain Δ1278b (MATa, wild type) (14), strain 2512c (MATa, GAP1-1) (15), and strain 22557d (MATa, gap1-1, put4-1, uga4-1, ura3-1) (16).

Standard minimal medium contained yeast nitrogen base without amino acids (Difco, Augsburg, Germany) and 2% d-glucose. The pantothenate concentration of this medium was 0.84 mM. For other pantothenate concentrations, the medium was made from analytical grade chemicals according to the formula of Difco for yeast nitrogen base without amino acids plus 2% d-glucose, but omitting pantothenate, dissolved at 10 times the final concentration; filter-sterilized; and stored at 4 °C. Pantothenate or β-alanine was added to this medium from filter-sterilized stocks (100 mM) to give the desired final concentrations. Yeast transformation was carried out as described (17).

**Transport Assays**—Yeast cells were grown to mid-logarithmic phase (A600 nm ~ 1.0 in minimal medium and ~ 3.0 in YPD medium (1% yeast extract, 2% bactopeptone, 2% d-glucose)), harvested by centrifugation, and washed once with ice-cold water. After washing in 50 mM sodium phosphate buffer, pH 5.5, the cells were resuspended in this buffer to give 10 A units/ml and incubated in a rotary shaker at 30 °C. Cells were energized with glucose (1% final concentration), and the assay was started by the addition of [14C]-labeled pantothenate (1.7 μM initial concentration; American Radiolabeled Chemicals, St. Louis, MO), β-[14C]alanine (2.1 μM initial concentration; Sigma, Deisenhofen, Germany), t-[14C]lysine (100 μM initial concentration; NEN Life Science Products, Köln, Germany), or [14C]biotin (10.7 μM initial concentration; Amersham Pharmacia Biotech, Braunschweig, Germany). Uptake of β-[14C]glucose (100 μM initial concentration; Amersham Pharmacia Biotech) was determined without the addition of unlabelled glucose. At time points, cells were filtered on nitrocellulose filters and washed with excess water. Incorporated radioactivity was quantified by liquid scintillation counting. Inhibitors were added 1 min before the addition of labeled substrate at the given concentrations. All inhibitors were purchased from Sigma.

**RESULTS**

**FEN2 Encodes a Pantothenate Transporter**—The facts that the yeast fen2 mutation caused a reduction in the ergosterol content (7), that FEN2 is a member of the allantoate permease family (4), and that VHT1 (another member of this gene family) encodes the H⁺-biotin symporter of the yeast plasma membrane (2) led us to the assumption that Fen2p may catalyze the uptake of a compound essential for sterol biosynthesis. As the specific activities of the enzymes involved in sterol biosynthesis were not impaired in the fen2 mutant (7), as coenzyme A is a carrier of activated C₂ units in sterol biosynthesis, and as pantothenate is a biosynthetic precursor of coenzyme A, we speculated that Fen2p might be a plasma membrane-localized pantothenate transporter.

This assumption was further supported by the fact that the specific activity of acetoacetyl-CoA thiolase, the first enzyme in the squalene biosynthetic pathway, was increased in the fen2 mutant (7). An increased activity of this first enzyme in the squalene biosynthetic pathway has previously been reported for wild-type cells grown under limiting pantothenate concentrations (18).

Initial support for our hypothesis was obtained from growth analyses of a FEN2 wild-type strain (711/1a) (12) and a fen2 disrupting (711/1c) (12) on media with different pantothenate concentrations (Fig. 1). Obviously, neither the wild-type nor the mutant cells were able to grow in the absence of pantothenate, but as little as 10 mM pantothenate was sufficient to support the growth of FEN2 wild-type cells. In contrast, growth of the fen2 disruptant was detected only at pantothenate concentrations being at least 2000-fold higher, indicating a potential defect in the pantothenate transport.

In the next step, the pantothenate uptake rates of the FEN2 wild-type strain, of the fen2 disruptant, and of a strain carrying FEN2 on a multicopy plasmid (711/1c transformed with pFEN2mc) were measured. Fig. 2 shows the results of these experiments performed with [14C]-pantothenate. Uptake rates of FEN2 wild-type cells were determined both in the absence and presence of 1% d-glucose. As transport rates were reproducibly higher when d-glucose was added, [14C]-pantothenate transport by the disruptant and the overexpressing strain was also determined in the presence of d-glucose.

The [14C]-pantothenate uptake rate determined for the FEN2 wild-type strain in Fig. 2 was 75 nmol/h/g (fresh weight). The rates for the fen2 disruptant and the FEN2-overexpressing strain were 1 and 240 nmol/h/g, respectively (all rates were determined in the presence of d-glucose). This shows that a fen2 gene disruption leads to a complete loss of [14C]-pantothenate transport and that overexpression of the FEN2 gene results in a 3-fold increase in [14C]pantothenate transport, confirming our assumption that FEN2 encodes a plasma membrane pantothenate transporter.

**Fen2p Is a High Affinity, H⁺-dependent Sympporter with No Sequence Homology to Mammalian or Bacterial Na⁺-dependent Vitamin Transporters**—Initial evidence that Fen2p may represent an energy-dependent H⁺-pantothenate symporter came from the glucose stimulation of pantothenate transport shown in Fig. 2. This stimulation is due to a glucose-dependent stimulation of the plasma membrane H⁺-ATPase (19). Activation of the H⁺ pump leads to an increase both in the plasma membrane electrical potential (Δϕ) and in the transmembrane proton gradient (ΔpH). Eventually, this results in enhanced transport rates of endogenous yeast H⁺ symporters (2) and of foreign H⁺ symporters expressed in yeast (20). For further support of this assumption, we tested the effect of carboxyl cyanide m-chlorophenylhydrazone, an uncoupler of transmembrane proton gradients, on Fen2p-dependent pantothenate transport (Table I). Carboxyl cyanide m-chlorophenylhydrazone inhibited uptake of pantothenate by >70%, confirming the H⁺ dependence of pantothenate transport.

If the physiological function of Fen2p is the supply of yeast cells with pantothenate, the Km of this transporter should allow the efficient use of this vitamin at naturally occurring pantothenate concentrations ranging from 4 to 80 μM in different plant materials (21). Analyses of [14C]-pantothenate trans-
strain, a fen2 mutant strain, and a fen2-overexpressing strain of S. cerevisiae. Cells of the FEN2 wild-type strain 711/1a (● and □), of the fen2 mutant strain 711/1c (○), and of the strain 711/1c expressing FEN2 from a multicopy plasmid (■) were grown to the same density, harvested, and washed, and uptake of \( ^{14} \text{C} \) pantothenate was determined. Pantothenate uptake by strain 711/1a was measured in the absence (■) or presence (○) of 1% D-glucose. Transport of the mutant (○) and of the overexpressing strain (■) was analyzed only in the presence of D-glucose. The dashed line indicates the intracellular pantothenate concentration corresponding to the initial outside concentration of 1.7 \( \mu \) M (concentration equilibrium).

**TABLE I**

Effect of inhibitors on the uptake of \( ^{14} \text{C} \) pantothenate by S. cerevisiae at an initial pantothenate concentration of 10 \( \mu \) M

| Inhibitor added | Conc | \( ^{14} \text{C} \) Pantothenate transport |
|-----------------|------|---------------------------------|
| None            |      | 100 ± 1.4                       |
| CCCP*           | 50   | 28.2 ± 3.2                      |
| Pantothenate     | 100  | 7.1 ± 0.9                       |
| \( \beta \)-Alanine | 100 | 89.9 ± 1.8                      |
| Biotin          | 100  | 94.6 ± 9.5                      |
| Lipoate         | 100  | 106.8 ± 8.9                     |
| Ureidopropionate| 100  | 93.9 ± 12.5                     |
| \( \gamma \)-Amino-n-butyrate | 100 | 90.8 ± 1.8                      |
| Taurine         | 100  | 91.4 ± 8.3                      |
| Gly-Asp         | 100  | 98.0 ± 9.4                      |
| Pantoylactone   | 100  | 85.2 ± 12.9                     |
| Pantoylaurine   | 1000 | 77.5 ± 16.4                     |
| Pantoylaurine   | 1000 | 68.5 ± 9.5                      |

*CCCP, carbonyl cyanide m-chlorophenyl hydrazone.

FIG. 3. Protein sequence of the Fen2p protein and prediction of putative transmembrane helices. A, the protein sequence of Fen2p is shown. Putative transmembrane helices predicted from the TMpred analysis in B are underlined and numbered. B, a TMpred hydrophilicity analysis (22) of Fen2p was performed. Shown is a plot of the Fen2p amino acid numbers (X coordinate) against the probability that a specific amino acid is part (positive values on the y coordinate) or not part (negative values on the y coordinate) of a transmembrane helix. Solid and dotted lines indicate the probability of a putative transmembrane helix to be oriented from the cytoplasm toward the outside (●→□) or in the opposite direction (□→●), respectively.

FIG. 4. Modulation of pantothenate transport activity by extracellular pantothenate. Cells of the FEN2 wild-type strain 711/1a were grown on minimal medium supplemented with 0.1 \( \mu \) M \( \square \), 1 \( \mu \) M \( \bigcirc \) pantothenate; harvested; and washed. Uptake of \( ^{14} \text{C} \) labeled pantothenate was determined.
β-Alanine Supports Growth of fen2 Mutants on Pantothenate-Free Medium—It has been suggested previously that β-alanine may substitute pantothenate in growth media of *S. cerevisiae* (23, 24). We were interested to see whether the uptake of β-alanine, which represents a significant portion of the pantothenate molecule, is also catalyzed by Fen2p and whether β-alanine allows growth of the fen2 mutant on pantothenate-free media. In Fig. 5, growth of a FEN2 wild-type strain and a fen2 mutant strain is compared at different concentrations of β-alanine. In Fig. 6, growth of a fen2 mutant strain on pantothenate-free minimal medium supplemented with the indicated concentrations of β-alanine. Cells were allowed to grow for 3 days at 29 °C.

![FEN2](image)

**Fig. 5.** β-Alanine can support Fen2p-independent growth. Cells were grown on minimal medium, harvested, washed, and resuspended in H2O. Drops containing ~2000 cells of the FEN2 wild-type strain 711/1a or of the fen2 mutant strain 711/1c were spotted on solid, pantothenate-free minimal medium supplemented with the indicated concentrations of β-alanine. Cells were allowed to grow for 3 days at 29 °C.

Fenpropimorph Causes an Unspecific Inhibition of Transport Proteins—Fenpropimorph inhibits the accumulation of the pyrimidine bases uracil and cytosine from growth media in *S. cerevisiae* (28). Uracil prototrophs of *S. cerevisiae* were more resistant to the growth inhibitory effects of fenpropimorph than were uracil auxotrophs. It was found later (7) that the product of the FEN2 gene confers sensitivity to this fungicide. The characterization of Fen2p as a pantothenate transporter suggests a more general effect of fenpropimorph on transport proteins. This is confirmed by the data presented in Table II. Fenpropimorph inhibited the transport of pantothenate by yeast cells grown on different media. Cells of the FEN2 wild-type strain 711/1a were grown on YPD complete medium (∆) or minimal medium (■), harvested, and washed, and uptake of 14C-labeled β-alanine was determined. The initial outside concentration of β-alanine was 2.1 μM.

![FEN2](image)

**Fig. 6.** Uptake of β-alanine by yeast strains defective in different amino acid transporter genes. Cells of strain 2512c (gap1-1, PUT4, UGA4) (○), of strain 22574d (gap1-1, put4-1, uga4-1) (□), or of wild-type strain 21278b (GAP1, PUT4, UGA4) (■) were grown in minimal medium to the same density, harvested, and washed, and uptake of 14C-labeled β-alanine was determined. The initial outside concentration of β-alanine was 2.1 μM.

**DISCUSSION**

FEN2 Encodes the Plasma Membrane Pantothenate Transporter of *S. cerevisiae*—This paper describes the functional characterization of the FEN2 gene product as a H+-pantothenate symporter of the *S. cerevisiae* plasma membrane. FEN2 represents the first characterized gene encoding a H+-pantothenate symporter. Uptake of pantothenate is stimulated by D-glucose (Fig. 2), an activator of the plasma membrane H+-ATPase (19), and is inhibited by carbonyl cyanide m-chlorophenylhydrazone, an uncoupler of transmembrane proton gradi-
Table II

| Substrate       | Inhibition % |
|-----------------|-------------|
| Pantothenate    | 76.1        |
| d-Glucose       | 76.8        |
| l-Lysine        | 85.5        |
| Biotin          | 92.9        |

FIG. 8. Structural similarity of the substrates of Dal5p (allantoate and ureidosuccinate), Vht1p (biotin), and Fen2p (pantothenate).

Pantothenate Transport in Saccharomyces cerevisiae

- Fen2p is a high affinity transport protein with a $K_m$ for pantothenate of 3.5 $\mu$m, allowing optimal uptake of pantothenate at physiological concentrations (21). Fen2p is essential for growth of *S. cerevisiae* on media with low pantothenate concentrations (Fig. 1). Uptake of pantothenate is modulated by extracellular pantothenate concentrations (Fig. 4), being maximal when the concentration of the vitamin is low.

**FEN2 Belongs to a Gene Family Encoding Eight Putative Transporters**—Sequence analyses of Fen2p (Fig. 3) predicted 12 putative transmembrane helices and revealed significant sequence homology to Dal5p (5) and Vht1p (2). Fen2p shares no sequence homology with the pantothenate transporter from *Escherichia coli* (PanF) (29) or the SMVT multivitamin transporter from rat, which accepts pantothenate, biotin, and lipoate (30). These transporters are Na⁺-dependent and share homology with the Na⁺-dependent proline transporter of *E. coli* (PutP) (31) and the Na⁺-dependent mammalian glucose transporter (SGLT) (32), which are not part of the major facilitator superfamily (29).

**FEN2, DAL5, and VHT1** are likely to have evolved from a common ancestral transporter gene. Identification of common structural motifs in their substrates (Fig. 8) may reveal information on the substrates of the five additional, so far uncharacterized transporters of this family (4). Obviously, all of the substrates presented in Fig. 8 are carboxylates, and the presence of this group seems to be essential. Allantoin, which differs from allantoate (the substrate of Dal5p) by only the absence of this carboxylic group, is taken up by the product of the *S. cerevisiae DAL4* gene, which is not a member of the allantoate transporter family (33), but is closely related to *FUR4*, the gene of the *S. cerevisiae* uracil permease (34). Similarly, pantoyltaurine, the sulfonic acid analog of pantothenate, is a poor competitor for pantothenate (Table 1).

The substrates of Dal5p and Vht1p possess a ureido group, which is absent from pantothenate. However, the amide bond formed by the pantothenate synthetase between the amino group of $\beta$-alanine and the carboxyl group of pantoate forms a similar structure, which may represent the second common recognition domain within this group of transporters. This suggests that members of the allantoate transporter family transport carboxylates containing a ureido group or an amide bond. Dipeptides fulfilling these criteria, such as Gly-Asp, do not compete with pantothenate (Table 1).

**The Biosynthetic Precursor $\beta$-Alanine Can Substitute Pantothenate and Is Imported by Gap1p—Gap1p (35) catalyzes the uptake of $\beta$-alanine, and a mutation in the *GAP1* gene causes a drastic reduction in the transport rates for $\beta$-alanine (Fig. 6). In agreement with earlier reports describing the regulation of *GAP1* expression by NH4⁺ ions (26) and increased *GAP1* expression on minimal medium (27), the transport of $\beta$-alanine is repressed under optimal nitrogen supply (Fig. 7). This indicates that *S. cerevisiae* is conditionally pantothenate-auxotrophic, requiring alternatively pantothenate or $\beta$-alanine for growth.

These findings explain the previously described phenotype of the fen2-1 mutant strain CM2 (7). This strain exhibited reduced ergosterol and fatty acid biosynthesis only when grown on complete medium, but not on minimal medium. Both media contain sufficient amounts of the vitamin pantothenate. However, varying amounts of the heat-labile vitamin are degraded when the medium is autoclaved, and one product of this thermal degradation is $\beta$-alanine (36). Thus, *FEN2* wild-type cells can utilize degraded pantothenate via Fen2p (on minimal and complete media) and its degradation product ($\beta$-alanine) via Gap1p (on minimal medium only). In contrast, fen2 mutants are unable to import pantothenate and grow only on minimal medium due to Gap1p-dependent import of $\beta$-alanine. Therefore, fen2 mutants will not display the mutant phenotype on minimal medium, but will grow with low rates and reduced ergosterol content on rich medium. As a consequence of these complex interconnections, it was suggested that *FEN2* encodes a protein responsible for the coregulation of carbon catabolite repression, nitrogen catabolite repression, and the general control of amino acid and ergosterol biosynthesis (7).

**Why Does a fen2 Mutation Confer Resistance against Fenpropimorph?**—Consistent with the central position of coenzyme A as a carrier of acyl groups in metabolism, fen2 mutations leading to a reduced availability of pantothenate for coenzyme A biosynthesis have pleiotropic phenotypic effects. Mutations in *FEN2* result in a reduced biosynthesis of ergosterol and fatty acids and in retarded growth under conditions of nitrogen catabolite repression. Likewise, resistance to fenpropimorph, a further consequence of fen2 mutations, is observed only under these conditions (7).

The sensitivity of *FEN2* wild-type cells to fenpropimorph cannot be explained by a specific interaction of the fungicide with Fen2p (i) because transport of different substrates is inhibited by fenpropimorph to a similar extent (Table II) and (ii) because sensitivity to fenpropimorph is also seen in fen2 mutants grown on minimal medium when *GAP1* is expressed (7). This rather suggests that the fungicidal effect of fenpropimorph depends on the availability of intracellular pantothenate and thus of acetyl-CoA, the primary substrate of ergosterol biosynthesis, and malonyl-CoA, the substrate for fatty acid biosynthesis and elongation.

One could speculate that the inhibition of sterol $\Delta^5,7$-isomerase and C14 sterol reductase, the primary target proteins of fenpropimorph (8–11), depends on a high substrate input into this pathway. Alternatively, the sensitivity to fenpropimorph may be modulated by the membrane composition, *i.e.*, by the relative amounts of the products of acetyl-CoA-dependent biosynthetic pathways. This model is supported by the finding that *FEN1*, another yeast gene conferring fenpropimorph resistance (37, 38), is allelic to *ELO2*, which encodes a protein involved in fatty acid elongation and sphingolipid formation.

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1. J. Stolz, unpublished data.
Both the production of very-long-chain fatty acids and sphingolipid formation depend on malonyl-CoA and may therefore be reduced in fen2 mutants when pantothenate levels are low. Mutants carrying a fen1 mutation exhibit slightly increased (rather than decreased) ergosterol levels (40). We therefore speculate that the antifungal activity of fenpropimorph depends on the membrane composition, specifically on the content of very-long-chain fatty acids and/or sphingolipids, and that fenpropimorph interacts directly with these membrane components. The inhibition of fungal growth by the structurally different antifungal polyene antibiotics, such as amphotericin B and nystatin, is also due to an interaction with specific membrane components (41, 42).

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