FUN26 (Function Unknown Now 26) Protein from *Saccharomyces cerevisiae* Is a Broad Selectivity, High Affinity, Nucleoside and Nucleobase Transporter*

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**Background:** FUN26 is a nucleoside transporter expressed in yeast vacuoles.

**Results:** Proteoliposome studies of purified FUN26 reveal broad nucleoside and nucleobase uptake that is sensitive to C(2\')-ribose modifications.

**Conclusion:** FUN26 is a high affinity and broad selectivity nucleoside and nucleobase transporter.

**Significance:** FUN26 has a unique substrate transport profile relative to other ENTs and retains activity following detergent solubilization and purification.

Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins that transport nucleosides and, to a lesser extent, nucleobases across cell membranes. ENTs modulate efficacy for a range of human therapeutics and function in a diffusion-controlled bidirectional manner. A detailed understanding of ENT function at the molecular level has remained elusive. FUN26 (function unknown now 26) is a putative ENT homolog from *S. cerevisiae* that is expressed in vacuole membranes. In the present system, proteoliposome studies of purified FUN26 demonstrate robust nucleoside and nucleobase uptake into the luminal volume for a broad range of substrates. This transport activity is sensitive to nucleoside modifications in the C(2\')- and C(5\')-positions on the ribose sugar and is not stimulated by a membrane pH differential. \[^{3}\mathrm{H}]\text{Adenosine nucleobase transport efficiency is increased ~4-fold relative to nucleosides tested with no observed ~}^{3}\mathrm{H}\text{adenosine or ~}^{3}\mathrm{H}\text{UTP transport. FUN26 mutational studies identified residues that disrupt (G463A or G216A) or modulate (F249I or L390A) transporter function. These results demonstrate that FUN26 has a unique substrate transport profile relative to known ENT family members and that a purified ENT can be reconstituted in proteoliposomes for functional characterization in a defined system.}

Nucleosides and nucleobases serve essential roles in a wide array of biological processes (1), characterized initially in eukaryotic cells through studies of *Saccharomyces cerevisiae* (2). These studies demonstrated that membrane flux of nucleoside and nucleobase substrates was a mediated process involving IMP transporters (3–7). Definitive identification of specific transporters and their associated substrate specificities has proven challenging due to overlapping functional roles and hurdles associated with IMP expression and/or purification. Mammalian nucleoside transporters (NTs)\(^2\) are divided into two distinct families based upon primary sequence: concentrative nucleoside transporters and equilibrative nucleoside transporters (ENTs) (8). ENTs, only found in eukaryotic organisms, are of clinical relevance because they modulate efficacy for a wide array of human therapeutics (e.g. anticancer, antiviral, antiarrhythmia, and antihypertensive medications), and ENT expression is a predictive biomarker for drug efficacy in cancer treatment (9, 10). The molecular basis for ENT substrate transport, recognition, and inhibition is undefined.

There are three definitive human ENT isoforms (hENT1–3) with 11 predicted transmembrane domains (TMDs) and a large hydrophilic loop at either the NH\(_2\) terminus (hENT3) or in the C-region between TMD5 and TMD7 (hENT1–2) (11). ENTs are broadly expressed in human tissues with distinct cellular localization profiles; ENT1 and ENT2 are localized in plasma membranes and broadly expressed in tissues, whereas ENT3 is resident in endosomal/lysosomal membranes and broadly expressed with higher abundance in placenta (8, 12). Functional studies of mammalian ENTs in cell-based systems have demonstrated that hENT1–3 transport purine and pyrimidine nucleosides and, to a lesser extent, nucleobases (13–16). Passive transport is a hallmark of the ENT family, although active, proton-linked, equilibrative transporters have been identified in protozoa (17). Indeed, transport activity in hENT3 is stimulated

\[^{2}\]The abbreviations used are: NT, nucleoside transporter; DDM, n-dodecyl-3\(\beta\)-d-maltoside; ENT, equilibrative nucleoside transporter; hENT, human ENT; IMP, integral membrane protein; PL, proteoliposome; SEC, size exclusion chromatography; SeHAS, *S. equisimilis* hyaluronan synthase; TMD, transmembrane domain.

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at lower pH (13, 14), which suggests a proton-coupled transport mechanism or activation via side chain modifications in an extramembrane regulatory domain. A definitive transport mechanism has not been elucidated for any of the ENT proteins, yet the working hypothesis is that they function through an alternating access model via passive diffusion along a concentration gradient (8, 18).

Modern genomic sequencing methods have increased the number of putative NTs, with the vast majority lacking definitive functional characterization (8, 19). Indeed, S. cerevisiae was one of the first systems from which detailed cell-based studies provided clear evidence of a saturable, specific, and energy-independent transport process for nucleosides and nucleobases, yet definitive identification of all yeast NTs has remained elusive (3, 7). Thus far, only one putative EN1 yeast ortholog (YAL022c) has been identified, tentatively named FUN26 (function unknown now 26) (20, 21). FUN26 is 19% identical to hENT1–hENT3 with known functional roles (8, 19). Indeed, S. cerevisiae FUN26 can be purified and functionally reconstituted into PLs; 2) FUN26 transports both nucleosides and nucleobases but does not transport nucleotides or C(2')-modified nucleosides tested in this study; 3) FUN26 is a high affinity [3H]uridine, [3H]cytidine, and [3H]adenine transporter; 4) the glycine side chain of Gly-463 is an absolute requirement for FUN26 transport activity; 5) residues Leu-390 and Phe-249 are capable of modulating substrate flux; and 6) FUN26 transport activity is not influenced by a membrane proton potential (ΔµH+). Because FUN26 is localized to yeast vacuoles, the observed broad substrate selectivity and affinity profile suggests involvement in nucleoside and nucleobase salvage from vacuoles to cytoplasmic pools. Furthermore, this work demonstrates reconstitution and functional characterization of a purified ENT family member.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning and FUN26 Protein Expression—Native FUN26 open reading frame was PCR-amplified from S288C S. cerevisiae genomic DNA (Novagen, catalog no. 69240-3) using FUN26-FOR (GGTAGCGCCGAGGAGGCAGGCG-ACTAGTCCCAAGGATGAGTAGTACTAGTGCGGAC-AC) and FUN26-REV (AATGTAAGCGTGACATAACTA-ATTACATGACTGAGTCACCCCTGATTAATAGT-GATCAI) primers. The resulting PCR product was cloned into the “83x” (26, 27) plasmid by homologous recombination in yeast, resulting in an NH2-terminal 10-histidine-thrombin-GSS-FUN26 protein expression construct, and was sequence-verified. Mutations were introduced into the 83x-FUN26 template using the QuikChange site-directed mutagenesis kit (Agilent, catalog no. 200523) following the manufacturer’s protocol. All plasmid maintenance and propagation was conducted in XL2Blue Ultracompetent E. coli cells (Stratagene, catalog no. 200150). Protein expression work utilized W303-Δrep4 (leu2-3,112 trp1-1 can1-100 his3-11,15 Δrep4 MATa)
Functional Characterization of S. cerevisiae FUN26

*S. cerevisiae* cells transformed with sheared salmon sperm DNA (Invitrogen, catalog no. 15632-011). Positive yeast transformants were selected in 1× synthetic complete histidine dropout medium to select for epistol HIS3 expression. 1× synthetic complete histidine dropout medium contains 1× CSM-HIS (Sunrise Science, catalog no. 1023), 0.67% (w/v) yeast nitrogen base without amino acids (RPI, catalog no. Y20040), 1% (w/v) d-glucose (Sigma-Aldrich, catalog no. G8270), and 1% (w/v) d-raffinose (Carbosynth, catalog no. OR06197) for a final carbohydrate concentration of 2% (w/v). Each round of protein expression contained 15-liter working volumes in liter baffled shaker flasks with each containing 375 ml of 1× SC-His and 10 ml of starter culture. These flasks were grown for 24 h (30 °C and 220 rpm) until optical density of the culture reached ~18. Protein expression was induced by adding 125 ml of 4× yeast extract-peptone-galactose containing 8% (w/v) yeast extract (RPI, catalog no. Y20020), 16% (w/v) peptone (RPI, catalog no. P20250), and 8% (w/v) galactose (Sigma-Aldrich, catalog no. G0625). Following induction, cells were grown for 16 h at 30 °C, 220 rpm, and harvested by centrifuging in 1-liter volumes at 3600 × g for 30 min at 4 °C.

**FUN26 Membrane Preparation, Solubilization, and Purification**—Native and mutant FUN26 proteins were purified using the same protocol. Yeast cells were resuspended in 60 ml of 50 mM Tris–HCl, pH 7.4, 10% (v/v) glycerol, 150 mM NaCl, 10 mM EDTA, 2 mM PMSF, 1× DHALT protease inhibitor mixture, and 5 mM β-mercaptoethanol (Resuspension Buffer) per 100 g of wet cell pellet. All Tris buffer pH values listed in this study are at room temperature. Suspended cells were lysed by three passages at ~28,000 p.s.i. in an Avestin C-3 Emulsiflex and centrifuged at 7500 × g for 1 h at 4 °C. FUN26 expression was not detected in the low speed spin pellet via Western blot or SDS-PAGE. Total cell membranes were collected by centrifugation at 101,000 × g for 1 h at 4 °C. Pelleted membranes were resuspended in 50 mM Tris–HCl, pH 7.4, 400 mM NaCl, 5% (w/v) glycerol, 2 mM PMSF, 1× DHALT protease inhibitor mixture, and 5 mM β-mercaptoethanol (Solubilization Buffer) at a ratio of 16 ml of buffer/g of cell membrane. In addition, 150 mg of n-dodecyl-β-D-maltoside (DDM; RPI, catalog no. D12000) is added per 2 g of membrane pellet (~9 mM DDM). Membranes were solubilized for 1 h at 4 °C with heavy stirring and then cleared of unsolubilized material by centrifugation at 101,000 × g for 1 h. Solubilized membranes were incubated with 10 mM imidazole and 6 ml of TALON cobalt resin (Pierce, catalog no. 89965) slurry (50%, v/v) for 1 h at 4 °C. Histidine-tagged protein was then purified via sequential washes with 10 bed volumes of Solubilization Buffer containing 2 mM DDM and either 10 mM imidazole (wash 1), 20 mM imidazole (wash 2), or 200 mM (elution) imidazole. Eluted protein was immediately (FUN26 is sensitive to imidazole) exchanged into Solubilization Buffer containing 2 mM DDM, 1 mM PMSF, and 5 mM β-mercaptoethanol and concentrated in a 50,000 molecular weight cut-off centrifugal concentrator (Millipore, catalog no. UFC903096) to a final concentration of 0.7–1.0 mg/ml. Detergent-solubilized protein was further purified using size exclusion chromatography (SEC) on a Superdex 200 10/300 (GE Healthcare, catalog no. 17-5175-01) column running at 0.5 ml/min (50 mM KH2PO4 at pH 7.4, 50 mM NaCl, 1 mM MgCl2, 1 mM PMSF, 2 mM DDM, and 5 mM β-mercaptoethanol) with 750-μl injection volumes (Fig. 2). Peak fractions were collected in a Foxy R1 fraction collector, pooled, and then concentrated using a centrifugal concentrator to a final concentration of 0.9 mg/ml. Protein purity was assessed by SDS-polyacrylamide gels and Western blots using an anti-His antibody (Qiagen, catalog no. 35370). The hyaluronan synthase from *Streptococcus equisimilis* (SeHAS) was expressed, solubilized, and purified as described previously (28) and incorporated into PLs using the same FUN26 incorporation protocol and detergent developed for this study.

**Proteoliposome Preparation**—Liposomes were prepared by mixing 28.25 mg of *Escherichia coli* polar lipids (Avanti Polar Lipids, catalog no. 100600C) and 1.6 mg of chicken egg 1-α-Phosphatidylcholine (Avanti Polar Lipids, catalog no. 840051C) chloroform stocks dispensed into glass tubes, mixed, dried under a stream of liquid nitrogen, and then lyophilized under a vacuum for 2 h to ensure that any excess solvent is removed. This lipid mixture was used immediately or stored at −20 °C. Prior to use, the mixture was resuspended in 830 μl of 50 mM KH2PO4 at pH 7.4, to reach a final lipid concentration of ~30 mg/ml and then subjected to five freeze/thaw cycles in liquid nitrogen. The final thaw cycle was conducted slowly over ~15 min, followed by six complete passes of liposome extrusion through a 0.4-μm Nucleopore (Whatman, catalog no. 800282) membrane. This step was then repeated using a 0.2-μm Nucleopore membrane (Whatman, catalog no. 800281). The 0.2-μm extruded liposomes were now ready for immediate use.
or storage at 4 °C. The calculated phospholipid content per liposome was ~169,000. One ml of PL was prepared by adding 200 μl of the above liposomes (at ~30 mg/ml), 400 μg of purified FUN26 (unless otherwise noted), 39 μl of 100 mM KH2PO4 (~3.9 mM final [DDM]) and brought up to a final 1-ml volume by the addition of 50 mM KH2PO4 at pH 7.4, 100 mM NaCl, and 1 mM MgCl2 ("Transport Buffer"), unless otherwise noted. The process results in the formation of unilamellar vesicles. The pH of this final buffer addition step may change, depending on the individual assay in which the PL preparation is utilized (e.g. low pH). Empty PLs (negative control) were prepared by adding 200 μl of liposomes to 800 μl of the desired Transport Buffer. Once prepared, the mixture was then incubated with gentle agitation on a rocking platform for 1 h at 23 °C. PLs were purified from unincorporated protein and excess detergent using SEC on a Superdex 200 10/300 column flowing at 0.5 ml/min that was pre-equilibrated with the desired PL Transport Buffer with peak fractions collected, pooled, and concentrated to 1 ml (Fig. 2). PLs were stored at 4 °C until use.

Proteoliposome Functional Assays—Tritium-labeled drug (Table 1) uptake into FUN26 (both wild type and mutant)-containing PLs was determined by measuring retained radioactive drug content on filtered membranes. Transport assays were conducted with 10 μl of PL (in 50 mM KH2PO4 at pH 7.4 with 100 mM NaCl and 1 mM MgCl2) and 100 nM (2.5 μl, 500 nM) radioactive substrate unless otherwise specified. The calculated total luminal volume in one 12.5-μl final volume assay was ~8.7 × 10⁻⁴ ml at 30 mg/ml liposome and 4 μg of reconstituted FUN26. Following the substrate addition, samples were incubated for 3.5 h at 4 °C for bulk transport assays using either 100 or 200 nM substrate unless otherwise noted. Transport reactions were stopped by filtering the PLs through GF/C 25-mm diameter filters (GE Healthcare, catalog no. 1822-025) on a vacuum manifold, followed by rinsing with 750 μl of 50 mM KH2PO4 at pH 7.4 with 100 mM NaCl (Wash Buffer) under vacuum. Low pH experiments used Transport Buffer and Wash Buffer at pH 5.5. Membranes were washed a total of six times for each measurement. Following membrane washing, the filter was placed in a scintillation vial and incubated with 750 μl of 1% (v/v) scintillation grade Triton X-100 (Eastman, catalog no. 13075) at room temperature for 10 min to ensure that retained PLs are fully permeabilized in the vial prior to the addition of scintillation mixture. Each individual substrate transport assay is conducted relative to an empty liposome negative control, and all assays are performed between days 8 and 11. All time course measurements are the time between radioactive substrate addition and filtration. Specific substrate transport, in pmol of substrate/mg of FUN26, was calculated by (CPMsubstrate - CPMempty)/(specific radioactivity/mg protein), as described previously (29, 30).

**Statistical Analysis**—The significance of substrate transport was determined by comparing mean substrate uptake (pmol of substrate/mg of FUN26) relative to [³H]UTP uptake by FUN26 using one-way analysis of variance and Dunnett’s multiple-comparison tests with significance denoted as follows: ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. All data in the current study were fitted directly using nonlinear regression (e.g. Vₘₐₓ and apparent Kᵣₘ determinations). The number of trials for each observation for classification of substrate transport was generated from multiple PL preparations across multiple experiments. All statistical analysis was conducted using version 6.03 of GraphPad PRISM 6 for Windows and Microsoft Excel version 14.0.7106.

**RESULTS**

Functional Reconstitution of S. cerevisiae FUN26 into Proteoliposomes—S. cerevisiae FUN26 was expressed as an NHL₂-terminal 10-histidine fusion construct in W303 yeast using an episomal galactose-inducible system. The expressed protein was extracted from total cell membrane pellets following DDM solubilization and purified to homogeneity (Fig. 2a). The detergent-solubilized and purified protein is stable in dilute form at 4 °C and runs in the included volume during SEC in a non-overlapping elution volume relative to protein-free detergent micelles. Purified FUN26 was inserted into partially solubilized liposomes composed of 5.4% (w/v) L-α-phosphatidycholine (egg, chicken), 9.3% (w/v) cardiolipin, 21.9% (w/v) L-α-phosphatidylylglycerol, and 63.4% (w/v) L-α-phosphatidylethanolamine with an average diameter of 200 nm following sequential extrusion. Quantitative FUN26 insertion into prepared liposomes was observed at protein concentrations up to 400 μg of FUN26, corresponding to ~3% of the total lipid bilayer volume. Once incorporated, FUN26 is stable for ~15 days at 4 °C before any detectable loss in transport activity or protein precipitation is observed. We observed that some FUN26 PL preparations never show signs of transport activity. We hypothesize that this is due to conformational malleability of the transporter, as previously suggested for detergent-solubilized ENT-containing membrane PL preparations (31), resulting in a subpopulation of non-functional protein in reconstituted PL systems. Negative control liposomes (i.e. liposomes without FUN26 protein) were generated by mixing liposome vesicles with Transport Buffer and purified using the same protocol as described above. SDS-PAGE and Western blot analysis, using an anti-His primary antibody, were used to verify FUN26 liposome incorporation (Fig. 2b, inset) and the absence of protein in negative control liposomes (data not shown). Thus, FUN26 can be purified to homogeneity in a detergent-containing buffer and inserted into

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**TABLE 1**

| Radiolabeled substrates | Source-catalog no. | Specific activity Ci/mmol |
|-------------------------|-------------------|--------------------------|
| [³H]Cytidine            | ARC-0146          | 20                       |
| [³H]Guanosine           | ARC-0545          | 15                       |
| [³H]Inosine             | ARC-0738          | 20                       |
| [³H](2`)-deoxyuridine   | ARC-0281          | 20                       |
| [³H]Uracil              | ARC-1782          | 40                       |
| [³H]Thymidine           | ARC-1241          | 33.9                     |
| [³H]Uridine             | ARC-0154          | 20                       |
| [³H]Uridine-C(5`)        | ARC-0241          | 55                       |
| [³H]Adenosine           | ARC-0287          | 40                       |
| [³H]Gencitabine         | ARC-1784          | 20                       |
| [³H]Adenine             | ARC-0143          | 20                       |
| [³H]Cytosine            | ARC-1720          | 19                       |
| [³H]Cytosine-β-α-arabinofuranoside | MB-631 | 26 |
| [³H]Uridine-monohydrrochloride | ARC-0266 | 20 |
Functional Characterization of S. cerevisiae FUN26

artificial lipid bilayers in a defined buffer system. We did not observe any impact on FUN26 stability in solution as a result of pH changes, between 5.5 and 8.2, or monovalent cation concentration (between 0 and 300 mM NaCl).

Nucleoside and Nucleobase Uptake by FUN26-containing Proteoliposomes—FUN26-containing PLs showed time-dependent accumulation of [3H]uridine and [3H]cytidine across 60-min incubations (Fig. 3a). Substrate uptake by PLs in these assays was observed by the presence of radioactive signal on borosilicate filters following vacuum filtration; the filters captured PLs while allowing non-luminal substrate to pass through. Substrate uptake was observed to be linear over 8 h when incubated with 100 nM [3H]uridine, which was subsequently determined to be about one-half of the observed Km.

To verify that the observed signal for FUN26 PLs was FUN26-dependent and not a result of the protein incorporation protocols, we expressed and purified SeHAS from E. coli and incorporated it into PLs using the same protocol as followed for FUN26. SeHAS is an IMP that synthesizes hyaluronic acid and functions as a negative control in this study because it does not have any nucleoside or nucleobase transport activity (32). [3H]uridine uptake by SeHAS containing PLs was 2.15 ± 0.46 pmol of [3H]uridine/mg of SeHAS (mean value of five separate trials from multiple PL preparations) versus 85.8 ± 3.1 pmol of [3H]uridine/mg of FUN26 (mean value of six separate trials from multiple PL preparations) (Fig. 3b). Thus, the observed [3H]uridine uptake by FUN26 PLs is not an artifact of the protein incorporation protocol or presence of membrane protein. Uptake was abrogated when permeabilizing concentrations of DMSO, ethanol, or PEG 400 (Fig. 3b) were incorporated into [3H]uridine uptake assays to further demonstrate that radiolabeled substrate accumulates in the PL lumen.

ENTs are known to differentiate between nucleosides and nucleotides because no ENT family member has been shown to transport substrates containing C(5)-phosphorylation on the ribose sugar (33, 34). FUN26-containing PLs do not transport [3H]-uridine-C(5)-phosphorylated uridine in the presence of 3.4% (v/v) PEG 400, 7.4% (v/v) ethanol, or 7.4% (v/v) DMSO to demonstrate that disruption of PL integrity leads to a loss of observed substrate uptake into the luminal volume. SeHAS PLs served as a negative control for protein incorporation into PLs because SeHAS does not transport nucleosides or nucleotides. [3H]Uridine uptake was not observed in SeHAS PLs, reported as pmol of substrate/mg of FUN26 (ordinate), was measured at 10, 20, 30, 40, 50, and 60 min time points (abscissa). A linear fit to each data set was obtained using a nonlinear regression model in GraphPad Prism, version 6.0, with R2 values indicated (0.0 not included in fit).

FIGURE 3. Substrate uptake in FUN26 PLs is protein-mediated and subject to competition. a, [3H]Uridine (●) and [3H]cytidine (□) time-dependent uptake into FUN26 PLs, reported as pmol of substrate/mg of FUN26 (ordinate), was measured at 10, 20, 30, 40, 50, and 60 min time points (abscissa). A linear fit to each data set was obtained using a nonlinear regression model in GraphPad Prism, version 6.0, with R2 values indicated (0.0 not included in fit). b, [3H]Uridine uptake was measured from FUN26 PLs in Transport Buffer as a positive control and FUN26 PLs incubated with [3H]uridine in the presence of 3.4% (v/v) PEG 400, 7.4% (v/v) ethanol, or 7.4% (v/v) DMSO to demonstrate that disruption of PL integrity leads to a loss of observed substrate uptake into the luminal volume. SeHAS PLs served as a negative control for protein incorporation into PLs because SeHAS does not transport nucleosides or nucleotides. [3H]Uridine uptake was not observed in SeHAS PLs. n = 2 for each experiment unless otherwise noted. c, FUN26 PL [3H]uridine uptake is subject to competition (dilution of measured signal) by the addition of increasing concentrations of non-radiolabeled uridine (●, blue) but not UTP (△, red). [3H]Uridine uptake (pmol/mg) is reported for each point determined at n = 2 and plotted against log of substrate concentration. Bars, mean substrate uptake ± S.D. (error bars); not shown where values are smaller than individual data point markers.

[3H]Uridine uptake rate is ~24% faster relative to [3H]cytidine. Thus, detergent-solubilized and purified FUN26 can be reconstituted in a PL system with retention of transport activity.
ENTs are classified based upon their sensitivity to transport inhibition and selectivity for substrates (8, 23). FUN26 is the only putative ENT family member in *S. cerevisiae*, so we predicted that it would transport a broad range of nucleoside substrates. To test this hypothesis, we utilized FUN26-containing PLs to determine uptake for nine pyrimidine-containing and five purine-containing substrates (Fig. 4). Several of these substrates are transported by hENTs (8). Pyrimidine-containing substrates that showed statistically significant transport relative to [³H]UTP were [³H]uridine, [³H]cytidine, [³H]uracil, [³H]cytosine, and [³H]thymidine (Figs. 4 and 5). All purine-containing substrates, except [³H]adenosine, showed statistically significant uptake and included [³H]guanosine, [³H]inosine, [³H]adenine, and [³H]hypoxanthine (Figs. 4 and 5). Five substrates showed no significant transport: [³H]UTP (negative control), [³H](C(2′)-deoxy)uridine, [³H]gemcitabine, [³H]cytarabine, and [³H]adenosine (Figs. 4 and 5). [³H]C(2′)-deoxyuridine and [³H]adenosine measurements showed increased variability between experiments, relative to other non-transporting substrates, although none were determined to be significant relative to empty liposome or [³H]UTP-negative controls and [³H]uridine- or [³H]cytidine-positive controls. [³H]C(2′)-deoxyuridine and [³H]adenosine concentrations used for these experiments ranged from 100 to 2000 nM. Based upon these data, FUN26 can be classified as a broad selectivity ENT that includes both purine and pyrimidine nucleosides and nucleobases.

**FUN26 Is Sensitive to Modifications at the C(2′)- and C(5′)-positions of the Ribose Ring**—Human ENT proteins are sensitive to molecular and conformational changes in the sugar moiety of nucleoside substrates, with hENT1–3 preferring C(2′)-endo sugar puckers and the presence of a C(3′)-oxygen atom (33, 35). FUN26 is sensitive to changes at the C(2′)-position of the ribose ring (Figs. 4 and 5). Removal of the C(2′)-OH group from uridine (i.e. C(2′)-deoxyuridine) results in loss of statistically significant transport. This contrasts with hENT1–2, which are...
relatively insensitive to removal of the C(2')-OH group and have robust C(2')-deoxyuridylate transport (33, 36, 37). Inversion at the C(2') tetrahedral stereocenter of ribose generates the epimer arabinose that is identical to ribose except for orientation of the C(2')-OH group. Replacing ribose in cytidine with arabinose produces cytosine arabinoside, or Ara-C, which is an anticancer therapeutic used in the treatment of hematologic cancers and a known hENT1–2 substrate (38, 39). Incorporation of an arabinose sugar into cytosine, instead of ribose, was a significant negative effector of FUN26 transport, as demonstrated by comparing [3H]cytosine versus [3H]cytarabine uptake (Figs. 4 and 5). These data would suggest that a C(2') hydrogen bond donor is a requirement for FUN26 nucleoside substrate recognition or transport. If hydrogen bonding between the C(2')-OH position and FUN26 amino acid side chains is a key mediator of substrate binding, then substitution of the C(2')-OH (Fig. 5) group with a more electronegative fluorne atom may facilitate binding stabilization via halogen bond formation (40, 41). Replacing the C(2')-H and C(2')-OH of the ribose ring in cytosine with fluorne resulted in no observed substrate influx into FUN26-containing PLs (Fig. 5). This C(2'),C(2')-difuoroxyctydine substrate (i.e. gemcitabine or Gemzar®) is a pyrimidine analog of deoxyctydine used widely in the treatment of human cancers and is a known substrate of hENT1–3 (8). The total absence of [3H]gemcitabine transport by FUN26 suggests key differences in the underlying mechanism of substrate transport or binding between hENTs and FUN26. We see no evidence of gemcitabine binding to FUN26, which suggests that the electronegative fluorne atoms may disrupt substrate recognition or binding versus allosteric binding to a regulatory domain. Furthermore, hENT1–2 nucleoside transport is abrogated by the addition of a bulky substituent at the C(5')-ribose position, such as -(PO₃)₂PO₄ in [3H]UTP, but unlike FUN26, hENT1–2 transport is attenuated by the removal of the C(3')-OH instead of the C(2')-OH (36). Lack of nucleotide transport activity was one of the first observations related to ENT function (34). As expected, FUN26-containing PLs showed no [3H]UTP uptake across all concentration ranges and incubation times attempted in this study, which is consistent across the ENT family. These data demonstrate that FUN26 transport activity is sensitive to modifications at the C(2')-ribose and C(5')-ribose positions and that the C(2')-hydroxyl group is probably involved in FUN26 substrate binding. Further investigation is required to determine whether FUN26 preferentially transports North (C3'-endo) or South (C2'-endo) ribose conformations. The current study demonstrates that the ribose sugar, for nucleoside substrates, is a strong mediator of FUN26 nucleoside transport activity.

**FIGURE 6.** FUN26-mediated [3H]uridine uptake is not pH-dependent. [3H]Uridine uptake is not affected by pH 5.5 on the external and luminal surface or by a pH gradient between the external and luminal volumes when compared with PLs prepared with pH 7.4 buffer in both volumes. Error bars, S.E. for n = 3 trials for each sample. No significance (ns) was determined using an unpaired t test relative to the pH 7.4 in/out sample.

**NH₂-terminal domain of FUN26 may serve a pH-dependent, regulatory role in modulating FUN26 transport activity.** No significant changes in [3H]uridine uptake was observed in the presence of a pH gradient or acidic luminal pH (Fig. 6), which suggests that FUN26 transport activity is not modulated by membrane ΔpH₁+ and thus lacks luminal specific regulatory elements that are present in hENT3 (14).

**Kinetics of [3H]Uridine, [3H]Cytidine, and [3H]Adenine Uptake by FUN26-containing Proteoliposomes—Substrate uptake data for FUN26 demonstrates broad selectivity (Fig. 4), whereas the initial uptake rates for [3H]uridine and [3H]cytidine suggest differences in substrate transport efficiencies (Fig. 3a). Apparent Kₘ and Vₘ₃ values were determined for [3H]uridine, [3H]cytidine, and [3H]adenine by measuring substrate influx at 10, 20, 30 ([3H]uridine only), 50, and 60 min time points using substrate concentrations ranging from 100 to 2000 nM (Fig. 7). Thir rates of initial uptake were linear for each concentration tested across 60 min (e.g. Fig. 3a) and saturable at higher substrate concentrations (Fig. 7). Assuming that FUN26 interacts with a single substrate molecule during each transport cycle, these results support analysis of the kinetic data using a simple steady-state Michaelis-Menten model (Fig. 7). FUN26 transport efficiency, as demonstrated by the ratio of Vₘ₃/Kₘ, for [3H]uridine and [3H]cytidine are similar, with [3H]uridine having a slightly lower substrate affinity (Table 2). It is interesting to note that substrate uptake results for the compounds tested demonstrate that FUN26 is both a nucleoside and nucleobase transporter (Figs. 4 and 5), with [3H]adenine showing the second highest net uptake for substrates tested in this study. FUN26 has a slightly lower affinity for [3H]adenine over nucleosides ([3H]uridine and [3H]cytidine) that is compensated for by a ~4-fold higher transport rate (Fig. 7 and Table 2). The uridine translocation rate for FUN26 PLs derived from the present study is ~80 molecules/site/s, in agreement with a previous study reporting 106 molecules/site/s for intact Ehrlich cells and 56 molecules/site/s for crude membrane preparations containing purified phospholipids (43), which demonstrates...
that these studies probably mimic FUN26 function in its natural environment.

**G463A and G216A FUN26 Mutants Result in Loss of Substrate Transport (G463A) or Protein Expression (G216A)—**

FUN26 shares a similar predicted membrane topology with hENT3, and both proteins are expressed in intracellular membranes. Non-synonymous single nucleotide polymorphisms (SNPs) in the ENT3 coding region are associated with human genetic disorders, including H and PHID syndromes (14). One of these SNPs, G427S, is located at a conserved glycine residue in hENT1–3 that abrogates hENT3 transport activity in a manner independent of cellular localization or protein abundance (14). Indeed, any minor side chain substitution, including alanine, at the Gly-427 position in hENT3 will completely disrupt substrate transport (14). The working hypothesis is that Gly-427 lines a substrate translocation pore and that minor side chain alterations in this position disrupt substrate-protein interactions. This glycine residue is also conserved in hENT1–2 as Gly-408. Sequence alignment of FUN26 with hENT3 shows that Gly-427 (or Gly-408 from hENT1–2) is conserved in FUN26 as Gly-463. To this end, a FUN26-G463A mutant was generated using site-directed mutagenesis. This mutant expressed at levels similar to native FUN26 at around 150 μg of purified protein/liter of yeast culture. The FUN26-G463A mutant was purified and inserted into PLs using the same protocols developed for native FUN26. FUN26-G463A PL incorporation was verified by anti-histidine Western blots and SDS-polyacrylamide gels (Fig. 8a, inset). No [3H]uridine transport was observed for FUN26-G463A in the current PL system (Fig. 8b). Thus, minor mutation of glycine 463 to alanine is sufficient to disrupt [3H]uridine uptake in FUN26-G463A-containing PLs. This further supports the identification of FUN26 as an ENT ortholog and suggests that Gly-463 in FUN26 may function via a conserved mechanism relative to ENT3. In addition, the absence of [3H]uridine uptake by the FUN26-G463A mutant provides further evidence that any observed substrate uptake in this PL study is a FUN26-mediated effect. The Gly-216 residue (FUN26 numbering) in FUN26 is strictly conserved in all putative ENT IMPs and is known to be essential for hENT1-mediated uridine transport (44). This residue is located in TMD5 of FUN26 and, when mutated to an alanine, results in severe attenuation of FUN26 expression (data not shown). This suggests that Gly-216 is essential for proper protein folding or membrane targeting. Indeed, Gly-184 (hENT1 numbering) is another strictly conserved glycine residue located in TMD5 that is directly involved in hENT1 targeting to the plasma membrane (44).

**Nucleoside Uptake Studies of L390A and F249I FUN26 Mutants—**

Phe-249 and Leu-390 are conserved residues in the ENT family located in TMD6 (Phe-249) and TMD8 (Leu-390). Conserved aromatic residues located near TMD ends (putative) often serve regulatory or functional roles (45). Therefore, a conservative isoleucine mutation at Phe-249 was constructed in an effort to preserve hydrophobic side chain characteristics while

![FIGURE 7. Kinetics of [3H]uridine (a), [3H]cytidine (b), and [3H]adenine uptake (c) by FUN26-containing PLs. [3H]Uridine, [3H]cytidine, and [3H]adenine uptake by FUN26 PLs were measured at 10, 20, 30 ([3H]uridine only), 50, and 60 min using the following substrate concentrations: 100, 200, 300, 500, 760, 1000, and 2000 nM.](image)

| Substrate | $K_m^{app}$ | $V_{max}^{app}$ | $V_{max}/K_m^{app}$ |
|-----------|-------------|-----------------|---------------------|
| [3H]Uridine | 0.308 pmol/mg/min | 1.92 pmol/mg/min | 6 |
| [3H]Cytidine | 0.190 pmol/mg/min | 0.947 pmol/mg/min | 5 |
| [3H]Adenine | 0.315 pmol/mg/min | 7.68 pmol/mg/min | 24 |

Substrate concentration for kinetic assays ranged from 100 to 2000 nM with uptake measured at selected time points between 0 and 60 min.
probing functional changes. The F249I mutant showed statistically significant (3–5-fold) increased transport for \[^{3}\text{H} \]uridine, \[^{3}\text{H} \]cytidine, and \[^{3}\text{H} \]hypoxanthine, but not \[^{3}\text{H} \]adenine, when compared with transport for the same substrate in native FUN26 (Fig. 9). These data indicate that Phe-249 may serve as part of a conserved hydrophobic gate because 1) Phe-249 is strictly conserved in the ENT family, 2) the F249I mutation increases the transport rate, and 3) the conserved Phe-249 residue is localized toward the distal end of a predicted transmembrane domain. Meanwhile, the Leu-390 residue is conserved in the TMD8 region of FUN26, a region previously implicated to play a functional role in substrate transport activity for the *Leishmania donovani* NT2 ENT transporter (46). Mutation of Leu-390 to alanine resulted in a statistically significant reduction in substrate transport for \[^{3}\text{H} \]cytidine and \[^{3}\text{H} \]adenine but not for \[^{3}\text{H} \]uridine or \[^{3}\text{H} \]hypoxanthine (Fig. 9). The FUN26-L390A mutant showed no signs of decreased stability or expression level relative to native FUN26. Thus, the F249I and L390A mutations had opposing effects on transport of \[^{3}\text{H} \]cytidine, a preferred FUN26 substrate.

**DISCUSSION**

Detailed functional analysis of ENTs has been hampered by the inability to overexpress and purify full-length transporters, which are known to rapidly lose functional activity upon membrane extraction with detergents (8, 31, 47). To date, there are no published studies describing the functional analysis of a purified ENT family member, a key step for defining molecular function and transport properties and for guiding future structure determination efforts. The need for a well defined assay is supported by the observation that nucleoside transport is associated with multiple protein families (e.g. multidrug resistance transporters or concentrative nucleoside transporters) with multiple protein isoforms in each transporter family (e.g. ENT1–3). In this study, functional reconstitution of detergent-solubilized and purified to homogeneity yeast FUN26 demonstrates that ENT proteins can be detergent-extracted from membranes with retention of activity. Furthermore, functional characterization of FUN26 demonstrates novel and unique transport properties relative to known ENT proteins, including the following: 1) FUN26 transport activity is not dependent upon other protein partners; 2) FUN26 transports a broad range of purine- and pyrimidine-based nucleoside and nucleobase substrates; 3) substrate transport is sensitive to nucleoside C(2')-ribose and C(5')-ribose modifications; 4) FUN26 is a high affinity nucleoside and nucleobase transporter; 5) FUN26 transport activity is independent of a membrane proton gradient, suggesting a different regulatory mechanism relative to hENT3; and 6) mutational studies of conserved amino acid residues provide insight into FUN26 transporter function. Furthermore, this study provides definitive assignment of FUN26 as an ENT and further defines the role that FUN26 plays in *S. cerevisiae* biology and vacuole function.

FUN26 has a unique substrate transport profile relative to what is currently known about other ENT family members. hENT1–3 IMPs transport unmodified physiologic nucleosides (i.e. uridine, cytidine, thymidine, adenosine, inosine, and guanosine) and a broad range of modified nucleosides (e.g. anti-
Functional Characterization of S. cerevisiae FUN26

The mechanism by which these substrates are transported through ENTs, including FUN26, has not been experimentally defined, although an “alternating access” (or “rocking-switch”) mechanism is the prevailing hypothesis for the overall family (8, 50). This model involves substrate binding to a central cavity followed by conformational switching to release bound substrate on the opposite side of the membrane bilayer from the initial binding events (51). FUN26 mutational studies demonstrate that TMD6 and TMD8 may functionally contribute to substrate translocation because mutations at Phe-249 in TMD6 and Leu-390 in TMD8 alter bulk substrate transport for [3H]uridine, [3H]cytidine, [3H]adenine, or [3H]hypoxanthine. G463A and L390A mutations either totally disrupt (G463A; Fig. 8) or significantly attenuate (L390A; Fig. 9) FUN26 transport, which suggests that these residues are directly involved in substrate binding or conformational stability. Comparison of the spatial arrangement of Gly-216, Leu-390, and Gly-463 with corresponding residues in a published model of the LdENT1.1 ENT transporter (50) places all three of these residues in a central substrate binding cavity. This is in agreement with modeling studies of hENT3 that places G427A (Gly-463 in FUN26) in a putative central binding cavity as well (14). Localization of Phe-249 to the distal end of a putative TMD (TMD6), when coupled with its conservation and impact on substrate transport, indicates that this residue may be involved in forming a cytoplasmic gate. We suspect that the altered substrate profiles of Leu-390 and Phe-249 may be the results of changes to $V_{\text{max}}$ (F249I), $K_m$ (L390A), or potentially both if they have the proposed roles as an cytoplasmic gate (Phe-249) or are involved in directly binding substrate (Leu-390). However, further experiments are needed to determine this, and interpretation of mutagenesis data in the absence of a determined structure is difficult because mutations may simply disrupt protein secondary or tertiary structural elements and shift the protein toward a modified functional state independent of position relative to substrate transport or binding sites. Detailed structural and functional studies are needed, yet the current FUN26 mutagenesis data are not inconsistent with an alternating access model of protein function. Additionally, transport activity of purified FUN26 is not pH- (Fig. 6) or Na$^+$-dependent (23) (data not shown), which suggests a uniport mechanism.

cancer or antiviral therapeutics). Thus, hENTs are promiscuous in permeant selection within the nucleoside substrate family. Nucleobase transport in hENTs occurs with lower overall substrate affinity (measured $K_m^{\text{app}}$ values in the micromolar to millimolar range when determined) (16, 48). Indeed, of the six endogenous nucleobases, only one is transported by all three hENTs (adenine), with cytosine transport only observed in hENT2 (8). This stands in stark contrast to FUN26, which is an efficient nucleobase transporter, as demonstrated by the 4- to 5-fold increase in transport efficiency for $[^{3}\text{H}]$adenine relative to either $[^{3}\text{H}]$uridine or $[^{3}\text{H}]$cytidine (Table 2) and by all four nucleobases tested in this study showing significant substrate uptake in FUN26 PLs (Figs. 4 and 5). This increase in efficiency can be attributed to a significant increase in transport velocity ($V_{\text{max}}$) even in the presence of only a slight increase in $K_m$ (Table 2). This suggests that nucleobase substrates form fewer stabilizing interactions with FUN26 amino acid side chains during vectorial transport and, by extension, differentially impact conformational gating.

This is supported by the observation that FUN26, unlike hENTs, shows strong selectivity for nucleoside and nucleobase substrates, with known hENT substrates, such as gemcitabine, adenosine, and cytarabine, showing no uptake into FUN26 PLs (Figs. 4 and 5). The absence of statistically significant $[^{3}\text{H}]$adenosine uptake in the current system is notable because hENT1–3 transports adenosine (8), and a previous yeast transporter study observed $[^{3}\text{H}]$adenosine uptake in FUN26 expressing oocytes relative to water-injected negative controls (23) using a 10-fold higher $[^{3}\text{H}]$adenosine concentration. Discrepancies between NTs expressed in oocytes relative to their native cell environment have been observed previously (49), although the current differences may be related to variability in cell surface expression in the previous oocyte studies. In addition, expression in oocytes may result in differential carbohydrate processing, yielding $N$-glycosylation sites containing complex glycan modifications, which would not normally be found on the biological form of FUN26. These modified sugar moieties could alter FUN26 structure and function. The possibility remains that the studies mentioned above may demonstrate an altered substrate profile when compared with FUN26 in its natural biological context. Additional studies will be required to analyze FUN26 function using isolated vacuoles or organellar membranes.

We screened up to 2000 nm $[^{3}\text{H}]$adenosine without any statistically significant uptake, which is ~8-fold higher than the observed apparent $K_m$ for $[^{3}\text{H}]$uridine. Thus, the nucleoside-specific sensitivity observed in this study for FUN26-dependent uptake is, in part, related to base substituents ($R_4, R_5$, and $R_6$ positions of purine bases), considering that $[^{3}\text{H}]$adenine has the second highest level of total uptake into FUN26 PLs (Figs. 4 and 5). One explanation is the formation of stronger binding interactions between the ribose sugar moiety and protein side chains, thereby forcing base substituents into a more defined subset of potential binding interactions and conformations. This dependence on ribose binding interactions, in particular the C(2')- and C(5')-positions, is demonstrated by comparison of $[^{3}\text{H}]$cytidine, $[^{3}\text{H}]$cytosine, $[^{3}\text{H}]$cytarabine, $[^{3}\text{H}]$thymidine, $[^{3}\text{H}]$gemcitabine, and 2-[3H]deoxyuridine. Specifically, the loss of the C(2')-OH (i.e. C(2')-deoxyuridine, thymidine) attenuates substrate transport, whereas the addition of more electro-negative atoms (gemcitabine) or inversion of the 2(R) stereo configuration (cytidine) at C(2')-OH to the 2(S) analog (i.e. cytarabine) abrogates transport (Fig. 5). These data demonstrate a novel substrate selectivity profile for FUN26 relative to hENTs with increased selectivity for the range of nucleoside substrates that may serve as FUN26 permeants. They further support the existence of ENT isomir-specific chemical compositions, or pharmacophore rule sets, that govern permeant selection and transport efficiency for ENT IMPs (as of yet undefined). A PL-based assay system is beneficial in this instance because it allows one to determine consistent basis sets of substrate transport relative to chemical composition to guide downstream ligand-based computational modeling (e.g. 3D-quantitative structure activity relationships or pharmacophore modeling).

The mechanism by which these substrates are transported through ENTs, including FUN26, has not been experimentally defined, although an “alternating access” (or “rocking-switch”) mechanism is the prevailing hypothesis for the overall family (8, 50). This model involves substrate binding to a central cavity followed by conformational switching to release bound substrate on the opposite side of the membrane bilayer from the initial binding events (51). FUN26 mutational studies demonstrate that TMD6 and TMD8 may functionally contribute to substrate translocation because mutations at Phe-249 in TMD6 and Leu-390 in TMD8 alter bulk substrate transport for $[^{3}\text{H}]$uridine, $[^{3}\text{H}]$cytidine, $[^{3}\text{H}]$adenine, or $[^{3}\text{H}]$hypoxanthine. G463A and L390A mutations either totally disrupt (G463A; Fig. 8) or significantly attenuate (L390A; Fig. 9) FUN26 transport, which suggests that these residues are directly involved in substrate binding or conformational stability. Comparison of the spatial arrangement of Gly-216, Leu-390, and Gly-463 with corresponding residues in a published model of the LdENT1.1 ENT transporter (50) places all three of these residues in a central substrate binding cavity. This is in agreement with modeling studies of hENT3 that places G427A (Gly-463 in FUN26) in a putative central binding cavity as well (14). Localization of Phe-249 to the distal end of a putative TMD (TMD6), when coupled with its conservation and impact on substrate transport, indicates that this residue may be involved in forming a cytoplasmic gate. We suspect that the altered substrate profiles of Leu-390 and Phe-249 may be the results of changes to $V_{\text{max}}$ (F249I), $K_m$ (L390A), or potentially both if they have the proposed roles as an cytoplasmic gate (Phe-249) or are involved in directly binding substrate (Leu-390). However, further experiments are needed to determine this, and interpretation of mutagenesis data in the absence of a determined structure is difficult because mutations may simply disrupt protein secondary or tertiary structural elements and shift the protein toward a modified functional state independent of position relative to substrate transport or binding sites. Detailed structural and functional studies are needed, yet the current FUN26 mutagenesis data are not inconsistent with an alternating access model of protein function. Additionally, transport activity of purified FUN26 is not pH- (Fig. 6) or Na$^+$-dependent (23) (data not shown), which suggests a uniport mechanism.
Functional Characterization of S. cerevisiae FUN26

Yeast vacuoles are functionally equivalent to mammalian lysosomes and serve as a site for catabolism of macromolecules, such as proteins and nucleic acids (52). The broad selectivity and high affinity observed for FUN26 in the current study suggest that FUN26 plays a role in recycling nucleoside or nucleobase substrates back to cytoplasmic pools. FUN26 is not stimulated by acidic pH like hENT3, yet it has a high affinity for multiple substrates and would thus be responsive to increased permeant concentrations in the vacuole lumen (even small increases, considering the high apparent affinity for some substrates). S. cerevisiae maintains viability following FUN26 knockout, suggesting overlapping substrate profiles with other vacuole transporters. This is not too surprising, considering the functional overlap in active and passive vacuole transporters that have been identified to date (53). FUN26 was also recently demonstrated to transport nicotinamide riboside, a precursor that have been identified to date (53). FUN26 was also recently demonstrated to transport nicotinamide riboside, a precursor for endogenous NAD⁺ biosynthesis, via yeast functional assays and genetic knockouts (24). Endogenous nicotinamide riboside salvage pathways are essential for maintaining NAD⁺ homeostasis and life span in calorie-restricted S. cerevisiae (54, 55).

Taken together, FUN26 is functionally distinct from previously characterized ENT IMPs. There are currently no molecular structures determined for the ENT family, and how these transporters function at the molecular level is not understood. Functional assessment of a detergent-solubilized and purified ENT family member is a significant step in facilitating structure determination efforts.

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