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# A genetic strategy involving a glycosyltransferase promoter and a lipid translocating enzyme to eliminate cancer cells

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**Abstract** The most common therapeutic strategy for the treatment of cancer uses antimetabolites, which block uncontrolled division of cancer cells and kill them. However, such antimetabolites also kill normal cells, thus yielding detrimental side effects. This emphasizes the need for an alternative therapy, which would have little or no side effects. Our approach involves designing genetic means to alter surface lipid determinants that induce phagocytosis of cancer cells. The specific target of this strategy has been the enzyme activity termed aminophospholipid translocase (APLT) or flippase that causes translocation of phosphatidylserine (PS) from the outer to the inner leaflet of the plasma membrane in viable cells. Efforts to identify the enigmatic, plasma membrane APLT of mammalian cells have led investigators to some P-type ATPases, which have often proven to be the APLT of internal membranes rather than the plasma membrane. By measuring kinetic parameters for the plasma membrane APLT activity, we have shown that the P-type ATPase Atp8a1 is the plasma membrane APLT of the tumorigenic N18 cells, but not the non-tumorigenic HN2 (hippocampal neuron × N18) cells. Targeted knockdown of this enzyme causes PS externalization in the N18 cells, which would trigger phagocytic removal of these cells. But how would we specifically express the mutants or antisense *Atp8a1* in the cancer cells? This has brought us to a glycosyltransferase, GnT-V, which is highly expressed in the transformed cells. By using the *GnT-V* promoter to drive a luciferase reporter gene we have demonstrated a dramatic increase in luciferase expression selectively in tumor cells. The described strategy could be tested for the removal of cancer cells without the use of antimetabolites that often kill normal cells.

**Keywords** Plasma membrane flippase

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## Introduction

The plasma membrane has been referred to as the "barrier" that controls the entrance of hydrophilic solutes and transmits information through signal binding. These crucial roles of the plasma membrane depend primarily upon its lipid composition and distribution. The lipids, which include glycerophospholipids, sphingolipids and cholesterol, are asymmetrically distributed across the two leaflets. In the plasma membrane, phosphatidylserine (PS) and phos-

phatidylethanolamine (PE) are mainly located in the cytosolic leaflet, while phosphatidylcholine (PC), sphingomylein (SM) and glycosphingolipids, reside in the outer leaflet [1, 2]. The asymmetric distribution is presumably maintained by the regulation of three families of enzymes: scramblases, floppases and flippases [1]. Scramblases that are activated by $Ca^{2+}$ play an important role in platelet activation and apoptosis upon cell stimulation. Rather than assisting, scramblases disrupt membrane asymmetry by ATP-independent bidirectional transportation of phospholipids across the membrane. A second family of translocases, termed floppases, conducts an outward ATP-dependent, but head-group independent translocation of phospholipids. A third family of enzymes, flippases or aminophospholipid translocases (APLT), which have attracted much attention in the recent years, catalyzes inward translocation of aminophospholipids such as PS and PE.

It has been known for almost fifteen years that the inner-leaflet PS translocates to the outer leaflet of the plasma membrane during apoptosis [3, 4]. This PS externalization causes activation of blood clotting factors and also macrophage recognition [3, 5, 6]. Although such PS-labeling of apoptotic cells prepare them for phagocytosis by PS-receptor-bearing macrophages and microglia [7–9], quite surprisingly, why PS remains in the inner leaflet of the plasma membrane of healthy cells and how it is translocated to the outer leaflet during apoptosis have remained controversial. Attempts to understand this process have led to the identification of a P-type ATPase (Atp8a1), which is believed to be an APLT or flippase [10–13]. Further studies identified the genes for other homologous P-type ATPases, which, because of their sequence similarity to Atp8a1, were named as putative APLTs [14, 15]. Four isoforms of Atp8a1 have been purified and antibodies have been prepared against them [12, 16]. Also, Atp8a1 is homologous to a yeast ATPase, which is encoded by the DRS2 gene [11]. Even though these initial studies identified Atp8a1 as a plasma membrane APLT, more recent *in vitro* analysis of the purified protein, Atp8a1, submitted that it was similar to but not the same as the plasma membrane flippase [17]. Züllig and coworkers demonstrated that RNAi-mediated suppression of the *C. elegans* analog of Atp8a1 (Tat-1) causes stimulation (rather than inhibition!) of plasma membrane flippase activity [18]. Contradicting the previous report, Darland-Ransom and coworkers have published that suppression of Atp8a1 in the same *C. elegans* germ cells causes externalization of PS [19]. Thus, so far, there is no consensus on the identity of any protein that harbors plasma membrane flippase activity.

The current study shows that hippocampal neuron-derived cells as well as neuroblastoma cells harbor this protein, but only the neuroblastoma N18 cells use this protein as plasma membrane flippase. Additionally, by demonstrating cancer cell specificity of a glycosyltransferase (GnT-V) promoter we also submit an idea of using this promoter to drive expression of antisense or mutant Atp8a1, thus causing targeted exposure of PS in the cancer cells.

## Methods

*Aminophospholipid translocase assay* APLT activity is measured using NBD-PS (Avanti Polar Lipids, Alabaster, AL) by modifying reported procedures [20–23]. Cells ($10^6$) were washed and resuspended in 1 ml ice-cold incubation buffer (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 500 μM PMSF, 10 mM HEPES, pH 7.5). Increasing concentrations of NBD-PS in 12 μl ethanol (ice-cold) were added to the 1 ml cell suspension to obtain the following concentrations: (in μM) 0.35, 0.5, 0.75, 1, 2, 4, 10. Next, triplicate sets of samples were gently mixed and incubated on ice for 10 min. Cells in the sample were then centrifuged at 7,000 rpm (Eppendorf centrifuge 5415C) within 1 min, the supernatant removed, the pellet gently resuspended in ice-cold incubation buffer, and the suspension placed in a 28°C water bath for 10 min to allow internalization of PS. It should be noted that a time course analysis performed earlier had shown that this enzyme reaction is linear up to 15 min. After the 10-min incubation, the cell suspension was placed back on ice. To remove NBD-PS fluorescence from the outer leaflet of the membrane, cells were incubated on ice in 10 mM sodium dithionite for 30 s (10 μl of a 1 M solution of dithionite was added to the chilled cell suspension, followed by gentle mixing). The cells were then centrifuged within 1 min, the supernatant removed, and the pellet washed once by resuspending in ice-cold PBS followed by centrifugation. The pellet obtained was resuspended in 200 μl of PBS and placed in a 96-well plate for the analysis of fluorescence. Triplicate samples were analyzed using a fluorescence plate reader (FLX800) set at 485 nm (excitation) and 530 nm (emission).

To record the background fluorescence, $10^6$ cells were incubated with 12 μl of ethanol without NBD-PS and then at 28°C for 10 min, followed by the same treatment as performed with the NBD-PS containing samples. To account for the loss of cells during the entire operation, they were counted again after fluorescence measurement and then all fluorescence values were normalized to $10^5$ cells. The average of the normalized background fluorescence was subtracted from the normalized fluorescence value obtained from each NBD-PS-treated sample. Finally, the fluorescence values were converted into micromoles of NBD-PS by comparing with a standard curve constructed using fluorescence readings from NBD-PS/PC vesicles

containing increasing concentrations of NBD-PS (in μM, 0.1, 0.5, 1, 2.5, and 5). The micromole values were then converted into NBD-PS molecules translocated by using the Avogadro number. Finally, Lineweaver–Burk plots were constructed by using $V_0$ in molecules/$10^5$ cells/min and [S] as concentrations of NBD-PS in μM.

*Membrane isolation using the Mem-PER kit* About $5\times10^5$ cells in microcentrifuge tubes were pelleted down, washed once with PBS, and then supplemented with 150 μl of Reagent A (lysis buffer) from the Mem-Per kit (Pierce, Rockford, IL). The mixture was incubated at room temperature for 10 min and then 450 μl of Reagent B/C was added to the mixture, followed by incubation for 30 min on ice. The mixture was then centrifuged at 10,000×*g* for 3 min. The supernatant, containing solubilized proteins, was separated and then incubated at 37°C for 10 min to separate phases. Following this, the mixture was centrifuged at 10,000×*g* for 2 min. The top layer, containing the hydrophilic proteins, and the bottom layer, containing the hydrophobic membrane proteins, were separated. The fractions separated were assayed for protein and then appropriate aliquots containing 25–50 μg protein were removed, mixed with SDS-PAGE treatment buffer, placed in boiling water for 5 min, and then subjected to SDS-PAGE followed by Western blotting as discussed in the following section.

*SDS-PAGE and Western blotting analysis* Aliquots of tissue and cell lysates prepared in RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM $Na_3VO_4$, protease inhibitor cocktail, 0.25 mM PMSF) or in a buffer from the Mem-PER kit were used. Proteins were resolved on a 7–16% gradient SDS-PAGE gel and then transferred to nitrocellulose membrane. For Atp8a1 analysis, blots were blocked overnight with 5% nonfat dry milk in 0.05% Tween-PBS and subsequently incubated overnight with Atp8a1 antibody (antibody B) (1:10,000 dilution) [16]. Following three washes with 0.05% Tween-PBS, blots were incubated for 1 h with horseradish peroxidase-labeled goat-anti-rabbit IgG (1:20,000). Protein bands were detected using Super signal West Pico kit (Pierce).

*Preparing expression vectors for Atp8a1, Atp8a2, and GnT-V* Mouse brain RNA was isolated as described earlier [24]. Using reverse transcriptase-PCR (RT-PCR) and flanking primers, in which the downstream primer (the antisense primer) included the coding sequence but not the stop codon, the cDNA sequences for both Atp8a1 and Atp8a2 were amplified and then inserted into pcDNA 6.1/myc-His A in frame with *myc-His A* sequences to eventually achieve expression of epitope-tagged Atp8a1 and Atp8a2.

The *GnT-V* gene is reportedly driven by multiple contiguous promoters [25]. A promoter segment (named as G4), which was amplified from mouse genomic DNA by the primer sequences listed below (in bold), showed selective activity in cancer cells. This sequence was PCR amplified and then inserted into the pGL3basic construct upstream of a luciferase reporter sequence. The empty vector (pGL3basic) was used a control. In our experiments, we named this promoter as "G4".

Forward: CGG *GGT ACC* **ACA CTC TGA AGT ACA GCC TGG**

Reverse: CTA *GCT AGC* **TGT GAA GGA GGA GAA GAA TTA GCC**

The underlined restriction enzyme sequences *Kpn*I and *Nhe*I were added to the forward and reverse primers, respectively, to assist in ligation into pGL3basic. The flanking sequences not in bold and not underlined were added to facilitate restriction digestion.

*Preparing constructs expressing phosphorylation site-mutants of Atp8a1* We performed mutagenesis using the GeneTailor™ Mutagenesis System (Invitrogen, Carlsbad, CA). For each mutation, Atp8a1 cDNA, which had been cloned into pcDNA 6/myc-His A, was first methylated and then subjected to PCR amplifications using two overlapping (but opposite) primers. Primers R1 and F2 were used to obtain the D409⇨E mutant and R1 and F1 were used to obtain the D409⇨K mutant.

Primers used for mutagenesis:

**R1** CTTGAACCGGTCCAATTTATGTATAAAAGA

**F1** AAATACATATTTTCT***A***A***G***AAAACTGGGACC

**F2** AAATACATATTTTCTGA***G***AAAACTGGGACC

As shown in the primers, the forward primer in each pair contained the required altered bases for a D409K mutation **(**Bold and italicized "A" and "G"**)** or the replaced base for a D409E mutation **(**italicized "G"**)** in order to produce the desired mutation within the 'DKTGTL' motif. The PCR product was transformed into mcrBC$^+$ E.coli included in the kit. In each case, the unmutated, methylated parent plasmid was digested in the mcrBC$^+$ *E. coli* and only the mutated plasmid was obtained after lysis and DNA isolation.

*Transfection of cells with plasmid DNA* Tumor cells, plated in poly-L-lysine-coated six-well plates, were allowed to grow up to 60% confluency. The non-tumor cells, which were differentiated after transfection, were allowed to grow up to 70–80% confluency. At this point, the cells were transfected as described here. Each plasmid DNA (3 μg) was diluted in 200 μl 150 mM NaCl (for all the tumor cells) and the mixture was subjected to gentle vortexing followed by brief centrifugation. Next, the ExGen transfection reagent (Fermentas, Inc., MD), at 7:1 molar equivalent

ratio for amine in polyethyleneimine (in the reagent) *vs* phosphate residues in DNA, was added to the DNA solution. This amounts to 11.52 μl of the reagent per sample per well. For HN2 cells, the DNA was dissolved 100 μl of 5% glucose and then the ExGen reagent, adjusted to 100 μl with 5% glucose, was added to the DNA solution. The mixture was vortexed for 10 s and then incubated for 20 min at room temperature. The medium in each well of cells was replaced with 1.8 ml of serum-free DMEM and then the 200 μl of DNA-ExGen complex was added to it, followed by gentle rocking to achieve even distribution of the complex. For cotransfection of pAsRed2-N1, 2.4 μg of the experimental plasmid was mixed with 0.6 μg of pAsRed2-N1 in 200 μl of 5% NaCl or 5% glucose. Typically, the cells were allowed to incubate for three hours in a 37°C $CO_2$ incubator, following which fetal bovine serum (FBS) was added to each well to a final concentration of 10%, and then the cells were allowed to incubate overnight in the incubator. The medium was then changed to DMEM containing 10% FBS and 1% Pen-Strep for tumor cells or the differentiation medium for the hybrid neuroblastoma cells. The cells were visualized using fluorescence microscopy to monitor transfection efficiency. Differentiation of the HN2 or HN2A12 cells was achieved in 1% serum-containing DMEM containing 5 μM retinoic acid for 24 h. Control cells were transfected with the same amount of empty vector, pcDNA 6.1/myc-His A.

*Monitoring PS externalization by Annexin V staining* Annexin V staining was achieved using the Vybrant apoptosis assay kit containing Biotin-X annexin V/Alexa Fluor 350 streptavidin/propidium iodide (Molecular Probes/Invitrogen detection technologies, Carlsbad, CA). Cells were grown in 6-well plates. First, cells were washed once with 1× PBS followed by a wash with 1× annexin binding buffer (component D, at pH 7.4, containing $CaCl_2$). Biotin-X annexin V (20 μl) (component A) was added to the cells in 400 μl of 1× annexin binding buffer followed by a 30-min incubation at room temperature with rocking. The solution was then removed and cells washed once with 1× annexin binding buffer. Alexa Fluor 350 streptavidin (component B, 5 μl of 1 mg/ml solution) was added to the cells in 500 μl of 1× annexin binding buffer followed by incubation at 4°C for 1 h. The solution was removed and cells were washed with 1× annexin binding buffer and then analyzed using a Nikon fluorescence microscope and imaged using a SPOT digital camera.

*Luciferase Assays* Cells grown to 30% confluence in a 12-well plate were transfected in quadruplicate wells using the following mixture: Plasmid DNA mixture in each well contained 2 μg of the pGL3-*G4-FireflyLuciferase* construct and 100 ng of the pRL-TK construct, which expresses the Renilla luciferase protein. Following transfection (as described above) and a 3-h incubation at 37°C, the medium was replaced with 1 ml of regular growth medium (DMEM plus 10% FBS, 1% PS) and the cells were allowed to grow for 48 h. Next, cells were washed with PBS and lysed in 200 μl 1× passive lysis buffer included in the DLR assay kit and 20 μl of each lysate was added to 100 μl of luciferase assay reagent II (LARII). After mixing, initial luminescence was measured within 30 s using a TD20/20 luminometer (Turner Design, Sunnyvale, CA). Subsequent to the first reading, the sample tube was removed from the luminometer and 100 μl of 'Stop & Glo' Buffer (included by the kit), was added to the mixture of LAR II and lysate. Luminescence from either firefly or Renilla luciferase (internal control) was measured using the same luminometer. Results were expressed as a ratio of Firefly to Renilla luminescence. Statistical significance of differences in transcriptional activities was analyzed using unpaired Student's *t* test.

## Results

*Overexpression of Atp8a1 in N18 cells elicits an increase in $V_{max}$ without causing any change in $K_m$ for plasma membrane APLT* Data presented in Fig. 1 show that overexpression of Atp8a1 in the N18 neurotumor cells causes no change in the $K_m$ value of the enzyme (APLT) that catalyzes NBD-PS translocation from the outer to the inner leaflet of the plasma membrane. However, the $V_{max}$ value for this enzyme increases from $3.8\times10^7$ NBD-PS molecules translocated /min/cell to $5.8\times10^7$ NBD-PS molecules translocated/min/cell. This strongly indicates that Atp8a1 is indeed the plasma membrane APLT of the N18 cells (the same $K_m$ value) and that the process of transfection causes an overall increase in the number of APLT molecules in these cells, thereby causing an increase in the $V_{max}$ value for the enzyme. This emphasizes that Atp8a1 is identical to the plasma membrane APLT molecule of the N18 cells.

*Overexpression of Atp8a2 in N18 cells does not cause an increase in $V_{max}$ for plasma membrane APLT* Having observed that overexpression of Atp8a1 in N18 cells causes an increase in $V_{max}$ without affecting the $K_m$ for plasma membrane APLT, we had to demonstrate that this change was not an artifact that would result from expression of any P-type ATPase in N18 cells. To achieve this, we overexpressed a second class-1 P-type ATPase, Atp8a2, which is also a putative APLT. In sharp contrast to our previous observation with Atp8a1 (Fig. 1), overexpression of Atp8a2 caused a decrease in $V_{max}$ and $K_m$ for plasma membrane

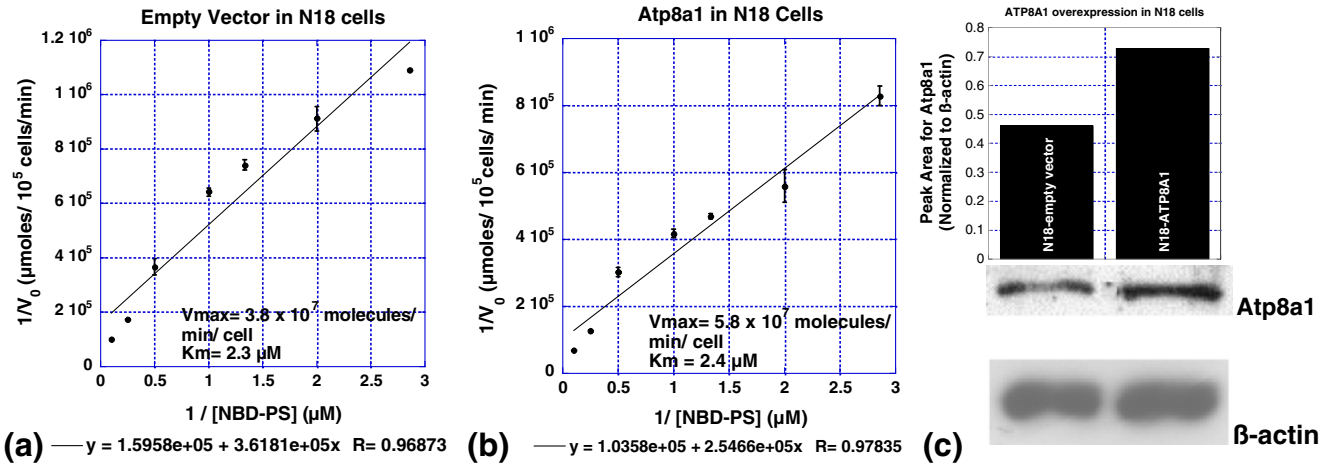


**Fig 1** Overexpression of Atp8a1 in N18 cells elicits an increase in $V_{max}$ without causing any change in $K_m$ for the plasma membrane APLT activity. To obtain PS translocase activity per $10^5$ cells from 10-min incubations, each triplicate set of samples was processed as described in the methods. **a**, **b** Lineweaver–Burk analysis showed that overexpression of Atp8a1 in N18 cells elicits an increase in $V_{max}$ without affecting the value of $K_m$. **c** Western blotting shows a 1.7-fold increase in the normalized Atp8a1 band. Kinetic data presented are representative of three experiments, each performed with triplicate samples

NBD-PS translocation in N18 cells (Fig. 2). Our results strongly indicated that overexpression of Atp8a1 in N18 cells indeed produces a specific increase in plasma membrane NBD-PS translocation in N18 cells.

*Overexpressed Atp8a1 in the non-tumorigenic hybrid neuroblastoma cells HN2 (differentiated) causes a complete change in both $K_m$ and $V_{max}$ values* As shown in Fig. 1, overexpression of Atp8a1 in the N18 neurotumor cells causes an increase in $V_{max}$ without influencing the value of $K_m$, which is expected if Atp8a1 were identical to the plasma membrane NBD-PS translocase of the N18 cells. In sharp contrast, overexpressed Atp8a1 in differentiated (non-tumorigenic) hybrid neuroblastoma HN2 cells [26] caused a complete change in both $K_m$ and $V_{max}$ values of the plasma membrane APLT activity (Fig. 3a, b). Western blot analysis of membranes isolated from these cells showed several-fold higher expression (overexpression) of Atp8a1 in the transfected clone HN1A12 (Fig. 3c). Furthermore, we do not know the identity of the plasma membrane APLT in the HN2 cells, but it could be expected that following such overexpression of Atp8a1 (Fig. 3c), the endogeneous APLT molecules will be rendered insignificant in the midst of the overwhelming number of Atp8a1 molecules, which then

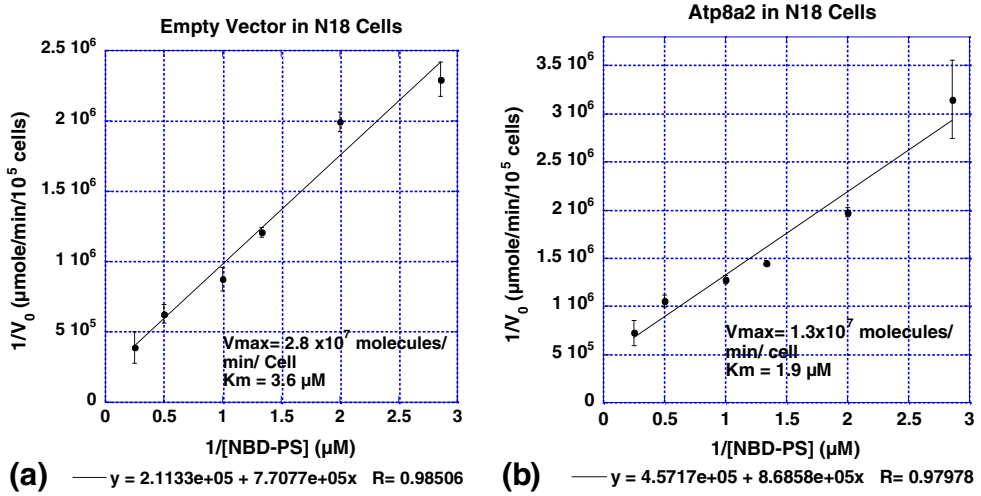


**Fig 2** Unlike Atp8a1, another putative APLT, Atp8a2, does not cause an increase in the $V_{max}$ for NBD-PS translocation in N18 plasma membranes. As described under this figure, N18 cells in six-well plates were transfected with pcDNA6.1 (empty vector) **(a)** or pcDNA6.1-Atp8a2 **(b)**. Parallel wells were transfected with pAsRed2-N1 to monitor transfection efficiency. After 48 h, the cells were dislodged by trituration and then assayed in quadruplicate samples for plasma membrane APLT as described in the "Methods". Data presented are representative of two experiments, each performed with triplicate samples

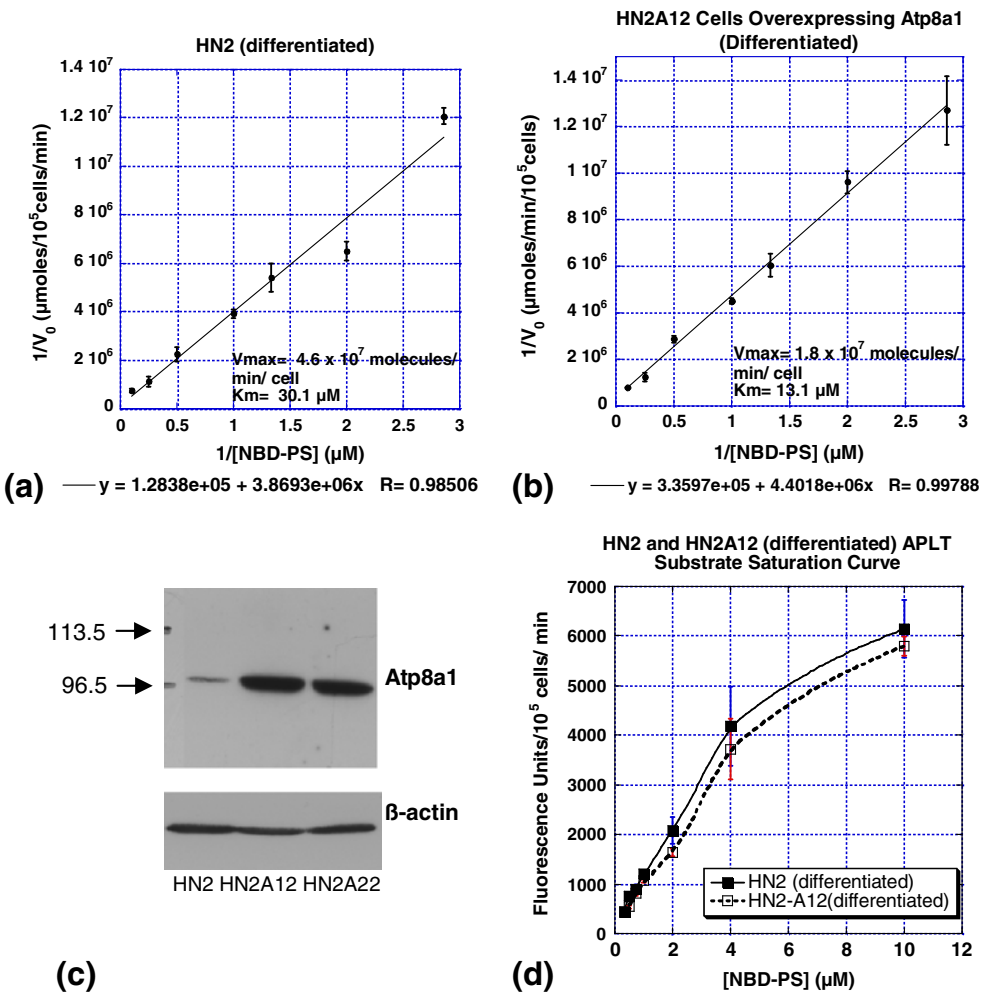


**Fig 3** Stable expression of the Atp8a1 gene in the non-tumorigenic hybrid neuroblastoma cells HN2 (differentiated) causes a complete change in both $K_m$ and $V_{max}$ values. Untransfected HN2 cells **(a)** and an Atp8a1-overexpressing clone, HN2A12 **(b)**, were differentiated by incubating in DMEM, containing 1% fetal bovine serum, 1% Pen-Strep, and 5 μM all-trans retinoic acid for 24 h. Next, the cells were harvested and subjected to APLT assays as described in the methods. **c** Western blot analysis of membrane fractions isolated using the Mem-PER kit (Pierce) shows a dramatic increase in expression of Atp8a1 in two clones (HN2A12 and HN2A22) obtained from stable expression of Atp8a1 in HN2 cells. **d** Atp8a1 overexpression did not confer a sigmoidal shape to the NBD-PS substrate saturation curve, indicating that Atpa1 overexpression does not evoke any allosteric interaction with the endogeneous plasma membrane APLT in the HN2 cells. Data presented are representative of three experiments performed with triplicate samples

make up the overall plasma membrane APLT activity. This would explain why the HN2A12 clone shows a Lineweaver–Burk plot with one slope instead of displaying two straight lines with two slopes. The substrate saturation curve was not sigmoidal in nature, thus ruling out the possibility of major allosteric interactions (Fig. 3d).

*Transient expression of Atp8a1 mutants causes externalization of PS* The ATPase activity of the P-type ATPases critically depends on the phosphorylation of the aspartic acid D409 (*i.e.* formation of relatively labile –COO–$PO_4^{2-}$ bond) at the beginning of the signature **D**KTGT[L,I,V,M][T,I,S] domain. We expected that a conservative mutation would eliminate phosphorylation of this protein and, thereby, its ATPase activity, while retaining its ATP-binding property. To this end we substituted the phosphorylation site D409 with a negatively charged amino acid, such as glutamic acid (mutant Atp8a1-D409E) (**M1**). Also, since this phosphorylation site was located in a pocket that possibly participated with the ATP-binding groove in trapping $Mg^{2+}$-ATP, a non-conservative mutation from D to a positively charged amino acid like lysine (D⇨K) (**M2**) would increase the affinity for the negatively charged ATP molecule without changing the overall conformation of the protein. Therefore, such a mutant would bind very tightly to ATP without hydrolyzing this substrate. Because the ATPase activity of the wild type P-type ATPase is required for the trans-bilayer movement of PS, such mutants could actually function as dominant negative versions of the protein by co-localizing with and sequestering ATP away from the wild type protein. To obtain the phosphorylation-site mutants, we used the *Atp8a1* constructs that had been already prepared in multiple vectors. To achieve site-directed mutagenesis of the Atp8a1 cDNA we designed two forward primers (F1 and F2) and one reverse primer (R1) (see methods). The target plasmid (*e.g. Atp8a1*-pcDNA3.1/myc-His) was methylated and amplified with primers F1 and R1 to generate mutant Atp8a1-D409K (**M2**), or with F2 and R2 to produce mutant Atp8a1-D409E (**M1**). A variant of **M2** without the myc tag, named as **M3**, was also prepared (Fig. 4a).

The proof of the principle that suppression of the activity of Atp8a1 will cause PS externalization was obtained through transient expression of the vectors for the D⇨E and D⇨K mutants in the N18 cells. To achieve effective PS

externalization in a large number of these neurotumor cells (N18), we first standardized conditions to obtain high transfection efficiency. We performed a systematic study on various cell lines using a wide range of DNA to polyethylenimine ratios in a variety of media and supplements (Sobocki and Banerjee, unpublished data) to finally arrive at conditions that allowed high transfection efficiency as judged by AsRed expression (Fig. 4) and flow cytometry. Next, we transfected the three Atp8a1 mutant vectors (M1, M2, and M3), one by one, into N18 cells and performed the standard surface-labeling assay using Alexafluor350-coupled annexin V (annexin V binds specifically to externalized PS). As shown in Fig. 4b, these experiments demonstrated widespread externalization of PS in the cells that received the mutant vectors but not in the cells that were treated with the empty vector.

*The Gn-TV protein is highly expressed in neurotumor cells* Ample literature evidence suggests that the Gn-TV protein is highly expressed only in cancer cells and suppression of this protein results in an inhibition of metastasis [27–30]. Our preliminary analysis showed that this protein is highly expressed in cancer cells, such as the metastatic human neuroblastoma (SH-SY5Y), mouse neuroblastoma (N18), human oligodendroglioma (HOG), proliferating (undifferentiated) HN2 cells, but not in normal mouse brain and human fibroblasts (Fig. 5). The Atp8a1-overexpressing and differentiated (*i.e.* non-tumorigenic) HN2A12 cells showed diminished expression of this protein.

*The Gn-TV promoter is selectively active in neurotumor cells* Having confirmed that Gn-TV was expressed highly in the neurotumor cells that were being tested in our project, we proceeded to test the efficacy of the promoter for the human *Gn-TV* gene (also known as *Mgat*) in three neurotumor cell lines, N18, HOG, and B16F10 (Mouse melanoma). As shown in Fig. 6, luciferase reporter assays showed that the presence of the *Mgat* promoter (G4) caused a dramatic increase in luciferase expression over that displayed by the promoter-less vector pGL3basic in all the cancer cell lines (Fig. 6). Quite

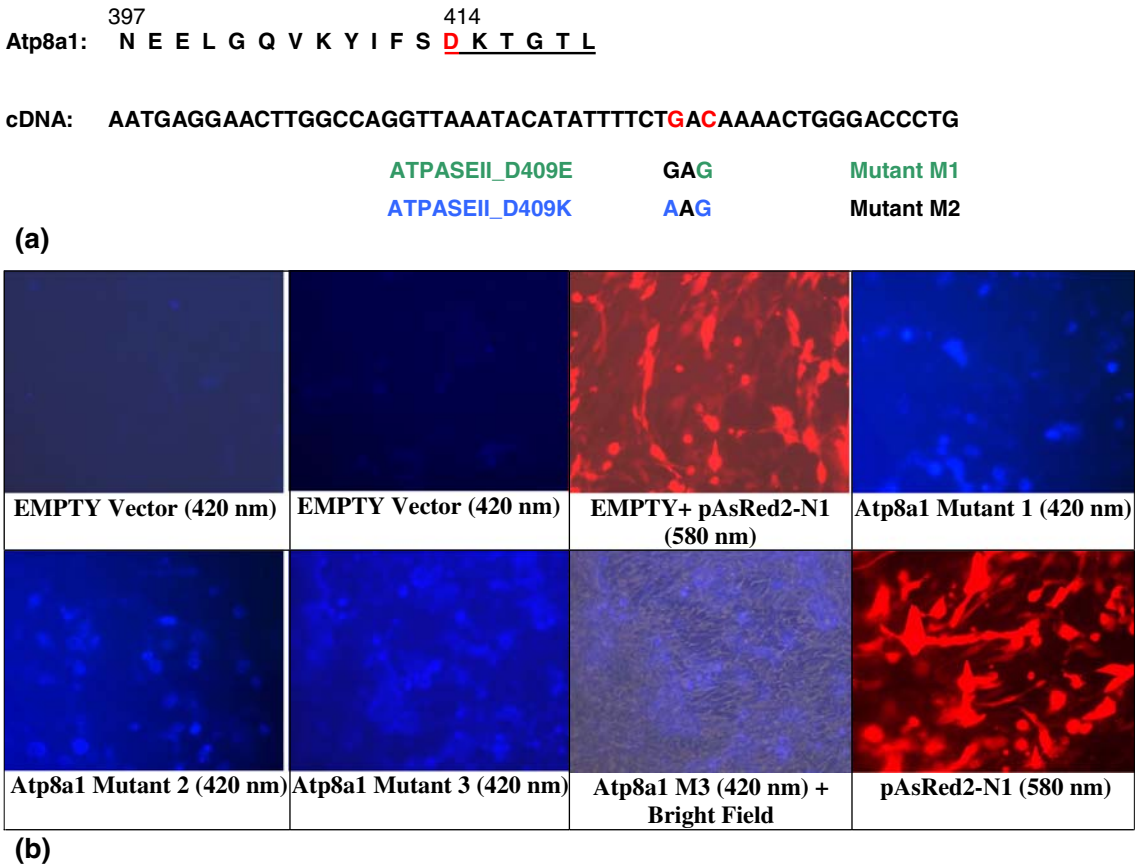


**Fig 4** Transient expression of *Atp8a1* mutants causes externalization of PS. In **a** the aspartic acid (D) that was mutated in the *DKTGTLT* sequence motif of Atp8a1 and the nucleotide bases replaced in its cDNA sequence are all shown in red. The altered bases in the conservative mutation (mutant 1) (D⇨E) are coded green and the replaced bases in the non-conservative mutation (mutant 2 and 3) (D⇨K) are shown in blue. **b** Both mutant 1 and mutant 2 were C-terminal tagged with a myc epitope. Mutant 3 was the same as mutant 2, but without the myc tag. Expression of both mutants caused externalization of PS in the N18 cells as measured by labeling with annexin V-Alexafluor 350. All the fluorescent images were captured at the sensitivity (gain) of "4"

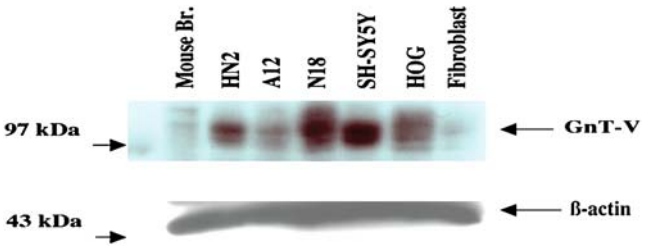


**Fig 5** Expression of GnT-V is elevated in cancer cells. About 25 μg lysate protein/ lane was resolved by SDS-PAGE, transferred to nitrocellulose and then probed with a GnT-V antibody (1:1,000) [33, 34]. Reprobing with β-actin antibody (1:1,000) (Sigma, St. Louis, MO) was performed to normalize for loading of total protein in the lanes

notably, in the non-transformed human fibroblasts, the activity of this promoter was very low (Fig. 6d), thus confirming the possibility that this *Mgat* promoter could be used for cancer cell-specific expression of genes.

## Discussion

Aminophospholipid translocase (APLT) activity of Atp8a1, its yeast homologs DRS2, DNF1, and DNF2, and its *C. elegans* orthologs have been studied extensively [10, 13, 18, 19, 31]. Some of the studies have gone to the extent of isolating the protein (*e.g.* Atp8a1), reconstituting it into lipid vesicles, and measuring its PS translocating activity. However, much controversy still surrounds the identity of the plasma membrane PS-translocase activity and some of the previous studies have presented convincing data showing that Atp8a1 is similar but not identical to the plasma membrane APLT [17]. We strongly feel that the central reason for this confusion is cell type-specific use of P-type ATPases as plasma membrane APLTs. Our studies involving transient expression of the mouse *Atp8a1* gene in the mouse neuroblastoma N18 cells demonstrate that the Atp8a1 protein is the plasma membrane APLT of these cells. Furthermore, expression of phosphorylation site mutants of Atp8a1 in N18 cells caused externalization of PS, which was monitored using fluorescence-labeled annexin V (a PS-binding protein). However, similar studies also showed that in the non-tumorigenic hippocampal neuron-derived HN2 cells (differentiated), Atp8a1 was not identical to the plasma membrane APLT. Thus, Atp8a1 may constitute the plasma membrane APLT activity only in neurotumor cells.

Kinetic analysis is one of the rigorous biochemical methods of studying enzymes. Despite so many studies, so far there is only one report of kinetic parameters for

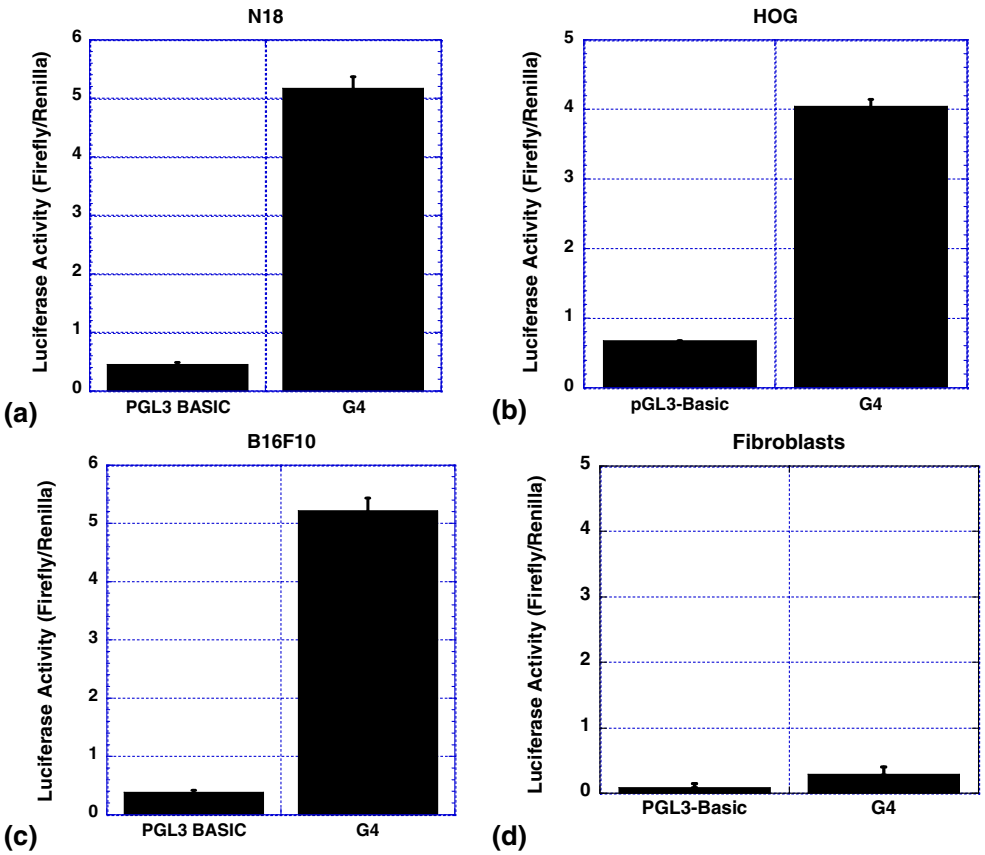


**Fig 6** The *Gn-TV* promoter is highly active in cancer cells. The *Mgat* (*GnT-V*) promoter is denoted as G4. The G4-driven increase in firefly luciferase activity was highly significant in all three cancer cells tested here ($P << 0.001$)

transmembrane movement of PS [32]. This study monitored translocation of spin-labeled PS across the plasma membrane of erythrocyte ghosts and reported a $K_m$ value of 225 μM. Our analysis in intact cells revealed values of $K_m$ that were in the range of 2–30 μM, thus showing that the plasma membrane APLTs of neural cells displayed greater affinity for PS than the plasma membrane APLTs of the erythrocyte ghosts. Thus, this is the first rigorous determination of the kinetic parameters $K_m$ and $V_{max}$ for plasma membrane APLTs in intact cells. By using these properties to compare and contrast the contribution of candidate molecules in PS translocation across the plasma membrane we infer that Atp8a1 is a plasma membrane flippase in mouse neurotumor cells. ATp8a1 is not the plasma membrane APLT of the non-tumorigenic (differentiated) HN2 cells, which may use one or more different paralog/s of Atp8a1 as plasma membrane APLT.

The final confirmation of the claim that Atp8a1 functions as plasma membrane APLT in the N18 cells came from the expression of dominant negative mutants of Atp8a1. Especially, the non-conservative mutant (D⇨K) caused pronounced exposure of PS in transfected N18 cells (Fig. 4). Such targeted externalization of PS in cancer cells could be developed into a strategy for triggering phagocytosis of cancer cells. But how could the mutants be expressed only in the cancer cells? To address this question, we have shown that a protein GnT-V, which transfers a *N*-acetylglucosamine residue to the C6 position of a branch mannose residue within the N-glycan segment of a glycoprotein, is highly expressed in a variety of cancer cells, but not in normal mouse brain or human fibroblasts (Fig. 5). If this was due to transcriptional regulation, then the promoter for the *GnT-V* gene (named *Mgat*) could be used to drive the expression of such dominant negative mutants or antisense DNA in the cancer cells. As expected, the *Mgat* promoter (G4) is highly active in a variety of cancer cells, but not in the fibroblasts (Fig. 6). Future studies will combine the two tools (1) the antisense or dominant negative Atp8a1 gene, and the (2) *Mgat* promoter to achieve targeted exposure of PS in cancer cells. In addition, it will be imperative for us to also determine the identity of the molecules (P-type ATPases) that act as plasma membrane APLT in other cell types. Collectively, this report outlines a novel strategy of targeting cancer cells for their elimination not by apoptosis, but by phagocytic uptake by the scavenger cells.

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