

# Novel green fluorescent polyamines to analyze ATP13A2 and ATP13A3 activity in the mammalian polyamine transport system

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**Abstract:** Cells acquire the polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) *via* 21  
the complementary action of polyamine uptake and synthesis pathways. The endosomal P<sub>5B</sub>-type 22  
ATPases ATP13A2 and ATP13A3 emerge as major determinants of mammalian polyamine uptake. Our 23  
biochemical evidence shows that fluorescently labeled polyamines are genuine substrates of ATP13A2. 24  
They can be used to measure polyamine uptake in ATP13A2 and ATP13A3-dependent cell models 25  
resembling radiolabeled polyamine uptake. We further report that ATP13A3 enables faster and stronger 26  
cellular polyamine uptake than ATP13A2. We also compared the uptake of new green-fluorescent PUT, 27  
SPD and SPM analogs using different coupling strategies (amide, triazole or isothiocyanate) and 28  
fluorophores (symmetrical BODIPY, BODIPY-FL and FITC). ATP13A2 promotes the uptake of 29  
various SPD and SPM analogs, whereas ATP13A3 mainly stimulates the uptake of PUT and SPD 30  
conjugates. However, the polyamine linker and coupling position on the fluorophore impacts the 31  
transport capacity, whereas replacing the fluorophore affects polyamine selectivity. The highest uptake 32  
in ATP13A2 or ATP13A3 cells is observed with BODIPY-FL-amide conjugated to SPD, whereas 33  
BODIPY-PUT analogs are specifically taken up *via* ATP13A3. We found that P<sub>5B</sub>-type ATPase 34  
isoforms transport fluorescently labeled polyamine analogs with a distinct structure-activity relationship 35  
(SAR) suggesting that isoform-specific polyamine probes can be designed. 36

**Keywords:** Fluorescently labeled polyamines, mammalian polyamine transport systems, P<sub>5B</sub>-type 38  
ATPases, radiolabeled polyamines. 39

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## Introduction

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Polyamines such as putrescine (PUT), spermidine (SPD) and spermine (SPM) are ubiquitous and 43  
physiologically important organic polycations found in every living cell. Polyamines are implicated in 44  
a broad range of cellular processes, ranging from cell proliferation to signaling, but their levels decline 45  
with aging [1,2]. Conversely, polyamine supplementation increases lifespan of model organisms such 46  
as mice and fruit fly [3,4]. At the molecular level, polyamine biosynthesis and catabolism pathways are 47  
well understood, but a clear knowledge gap remains regarding the molecular characteristics of the 48  
mammalian polyamine transport systems (mPTS). Three main mechanisms have been proposed to be 49  
involved in cellular polyamine uptake [5]: (i) a direct transport at the plasma membrane, (ii) a glypican- 50  
mediated uptake *via* endocytosis and (iii) a caveolin-1-mediated endocytosis [5-10]. Recently, we 51  
identified two key players of the mPTS that belong to the P<sub>5B</sub>-type ATPases (ATP13A2 and ATP13A3) 52  
[11-13]. 53

In humans, ATP13A2 and ATP13A3 are ubiquitously expressed with highest expression in the brain 54  
and the liver, respectively ([14] and <https://www.proteinatlas.org>). At the subcellular level, both proteins 55  
are localized in the endosomal pathway with a preferred late endo-/lysosomal distribution for ATP13A2 56  
[12-14] and early/recycling endosomes for ATP13A3 [12,13,15]. We provided biochemical evidence 57  
that human ATP13A2 (hATP13A2) transports polyamines from late endo/lysosomes to the cytosol with 58  
a high affinity for SPM and SPD [13], which was confirmed at the structural level [16-20]. ATP13A2, 59  
and also the closely related ATP13A3, fulfill their polyamine transport function downstream of 60  
polyamine internalization *via* endocytosis [11,13]. Both transporters are implicated in disease, since 61  
genetic mutations in *ATP13A2* have been linked to neurodegeneration, whereas ATP13A3 is genetically 62  
implicated in pulmonary arterial hypertension and may play a role in cancer (see [14] and references 63  
therein). Studying the transporters of the mPTS in cells typically relies on the use of radiolabeled (<sup>3</sup>H 64  
and/or <sup>14</sup>C) or fluorescently labeled polyamines. We recently reported fluorescent polyamine conjugates 65  
incorporating a symmetrical BODIPY fluorophore (referred to as ‘BODIPY’ throughout the 66  
manuscript). These have proven invaluable to characterize the P<sub>5B</sub>-type ATPases [11,21]. Indeed, 67  
ATP13A2 contributes to the cellular uptake of BODIPY-SPM and BODIPY-SPD in human 68  
neuroblastoma (SH-SY5Y) cells [13] corresponding to the substrate specificity of purified ATP13A2 69

for SPM and SPD [13]. We further demonstrated that ATP13A3 is responsible for the impaired BODIPY-PUT uptake observed in the CHO-MG cell line with a deficient mPTS [11,22].

Despite their proven value, it remains unclear how the transport of these polyamine conjugates compares with unlabeled polyamines and whether the fluorophore or its linkage to the polyamine may influence the recognition and transport by the P<sub>5B</sub>-type ATPases. Here, we report our recent work aimed at addressing these questions. Specifically, we compared uptake of BODIPY- and <sup>14</sup>C-labeled polyamines in two cell models relying either on ATP13A2 or ATP13A3 activity. Additionally, with a series of new green fluorescent analogs of PUT, SPD and SPM we tested the impact of different coupling strategies and fluorescent headgroups on transport capacities. Interestingly, we found that a broad range of fluorescent polyamine probes are taken up in cells *via* the catalytic activity of ATP13A2 and/or ATP13A3, although probe- and isoform-specific effects are observed. Overall, this work provides novel fluorescent polyamine conjugates with improved properties for the study of P<sub>5B</sub>-type ATPases in disease models, and opens the way for the design of P<sub>5B</sub> isoform-specific polyamine probes.

## Materials and Methods

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### Preparation of compounds

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All fluorescent polyamine probes were prepared in a final stock concentration of 5 mM in 0.1 M  $\gamma$ -(N-Morpholino) propanesulfonic acid (MOPS; PanReac AppliChem, A1076,1000), brought to pH 7.0 with KOH (Honeywell, 319376 Fluka) and stored at -20°C. Unclicked SPM-N<sub>3</sub>-4·HCl (azide SPM) was dissolved in MOPS-KOH to reach 20 mM, unclicked BODIPY-alkyne was dissolved in dimethyl sulfoxide (DMSO; Sigma, D5879) to a final concentration of 5 mM and they were both stored at -20°C. Unlabeled polyamines, putrescine dihydrochloride (PUT; Sigma: P7505), spermidine (SPD; Sigma: S2626) and spermine (SPM; Sigma: 85590) were dissolved in MOPS-KOH (pH 7.0) at a final concentration of 500 mM (putrescine and spermidine) or 200 mM (spermine) and stored at -80 °C.

For the biochemical ATP/NADH-enzyme coupled ATPase assay, the following stock solutions were prepared: 2.5 M potassium chloride (KCl; Sigma: P5405), 1 M magnesium chloride solution (MgCl<sub>2</sub>; Gibco: M1028), 15.625 U/ $\mu$ L lactate dehydrogenase (Sigma: L2500) and 4 U/ $\mu$ L pyruvate kinase (Sigma: P1506), and were stored at 4 °C. Additionally, 100 mM  $\beta$ -nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH; Sigma: 43420) in MOPS-KOH (pH 7.0), 50 mM phospho(enol)pyruvic acid tri(cyclohexylammonium) salt (PEP; Sigma: P7252), 500 mM dithiothreitol (DTT; PanReac AppliChem: A2948.0025), and 63 mM adenosine 5'-triphosphate disodium salt trihydrate brought to pH 7.0 (Roche: ATPD-RO), were stored at -20°C.

For the preparation of HMEC-1 cell culture medium, a 100  $\mu$ g/mL stock of human epidermal growth factor (hEGF; Sigma: E9644) was prepared in 0.22  $\mu$ m filter-sterilized 10 mM acetic acid (Chem Lab: CL00.0119.1000), supplemented with 1% albumin fraction V (Carl Roth: 8076.4), in Milli-Q, and was stored at -80°C in aliquots. Additionally, hydrocortisone (Sigma: H0888) was dissolved in absolute ethanol (VWR Chemicals: 20821.296) and diluted 1:25 in MCDB131 medium to a final concentration of 100  $\mu$ g/mL, and stored in aliquots at -20°C.

The endocytosis inhibitors Dynasore (Sigma: D7693), Genistein (Abcam: ab120112), and Pitstop-2 (Sigma: SML1169) were dissolved in DMSO to a final stock concentration of 50 mM, 25 mM and 25 mM, respectively, and stored at -20°C.

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### Lentiviral transduction and cell culture

SH-SY5Y human immortalized neuroblastoma cells (ATCC: CRL-2266<sup>TM</sup>, Lot Number 62431864) were transduced with lentiviral vectors to obtain stable overexpression of human ATP13A2 (isoform 2, wild-type (ID: NP\_001135445) or catalytically dead mutant, D508N) [13]. Lentiviral vectors were produced by the Leuven Viral Vector Core using pLenti HsATP13A2 WT (Addgene plasmid #171485; <http://n2t.net/addgene:171485>; RRID: Addgene\_171485) as described in [dx.doi.org/10.17504/protocols.io.bw57pg9n](https://doi.org/10.17504/protocols.io.bw57pg9n). SH-SY5Y cells were cultured in DMEM high glucose culture medium (Gibco: 41965), supplemented with 1% MEM Non-Essential Amino Acid Solution (Merck: M7145), 1 mM sodium pyruvate (Gibco: 11360070), 1% Penicillin-Streptomycin (Sigma: P4458), and 15% heat-inactivated Fetal Bovine Serum Standard, South America origin (FBS; PAN BioTech: P30-3306) at 37 °C with 5% CO<sub>2</sub>. After lentiviral transduction, cells were selected with 2 µg/mL puromycin (Invivogen: ant-pr-1).

HMEC-1 human immortalized microvascular endothelial cells (ATCC: CRL-3243<sup>TM</sup>, Lot Number: 70022309) were transduced with lentiviral vectors to obtain stable overexpression of human ATP13A3 (wild type or catalytically dead mutant, D498N). HMEC-1 cells were cultured in 0.2% gelatin (Sigma, G1393) coated 75 cm<sup>2</sup> flasks and in MCDB131 culture medium without L-glutamine (Gibco: 10372-019), supplemented with 10 ng mL<sup>-1</sup> hEGF, 1 µg mL<sup>-1</sup> Hydrocortisone, 10 mM GlutaMAX supplement (Gibco: 35050061), 1% Penicillin-Streptomycin and 10% heat-inactivated Fetal Bovine Serum Standard, South America origin at 37 °C with 5% CO<sub>2</sub>. After lentiviral transduction, cells were selected with 1 µg/mL puromycin.

All cell lines were routinely tested for mycoplasma contamination using the Plasmotest Mycoplasma detection kit (Invivogen: rep-pt1).

### Western blotting

Cells were detached either by scrapping them in Dulbecco's phosphate buffered saline modified without calcium chloride and magnesium chloride (DPBS; Gibco: D8537) (SH-SY5Y) or, using 0.25% Trypsin-EDTA (Gibco: 25200056) (HMEC-1) for which the enzymatic reaction was stopped by addition of culture medium. Cell suspensions were centrifuged at 4°C for 5 minutes at 450 g (SH-SY5Y) and 2500

rpm (HMEC-1). Cell pellets were washed twice with DPBS and centrifuged again before being lysed in 138  
RIPA buffer (RIPA lysis and extraction buffer (Invitrogen: 89900) supplemented with protease cocktail 139  
inhibitors (SIGMAFAST Protease Inhibitor Cocktail Tablets, EDTA-Free (Sigma: S8830)). Lysis was 140  
done on ice for 30 minutes and a further centrifugation at 4°C for 30 minutes at 20,000 g. Supernatants 141  
were kept and protein concentration was estimated using the micro-BCA Protein Assay Kit (Pierce BCA 142  
Protein Assay Kit (Thermo Scientific: 23225)). 20 µg of protein were mixed with NuPAGE LDS sample 143  
buffer (Invitrogen: NP0007) with 5% β-mercaptoethanol. Samples were not boiled, separated on 4-12% 144  
Bis-Tris gels (Invitrogen: NP0321BOX) and transferred onto PVDF membranes (Thermo Scientific: 145  
88518) using a liquid transfer (1h15, 100V, 4°C). Membranes were then blocked in blocking buffer (5% 146  
milk powder in 1X TBS and 0.1% Tween20) for 1 h at room temperature, then incubated overnight at 147  
4°C with the primary antibodies 1% bovine serum albumin in 1X TBS-Tween20 (TBS-T) buffer and 148  
washed three times for 5 min in TBS-T. Membranes were then incubated with peroxidase-conjugated 149  
secondary goat anti-rabbit or goat anti-mouse antibodies in 1% milk powder in 1X TBS-T buffer for 1 150  
h at room temperature and later washed five times for 5 min in TBS-T. Signal was detected with 151  
chemiluminescence reagent (SuperSignal West Pico PLUS chemiluminescent Substrate, Thermo 152  
Scientific: 34095) using a Biorad camera (Vilber Lourmat) and its software (ImageLab). This specific 153  
protocol for ATP13A2 and ATP13A3 western blotting is also described in protocols.io 154  
([dx.doi.org/10.17504/protocols.io.81wgbyzqovpk/v1](https://doi.org/10.17504/protocols.io.81wgbyzqovpk/v1)). 155

Mouse monoclonal anti-GAPDH (G8795, lot #067M4785V, dilution 1:5,000), and rabbit anti- 156  
ATP13A2 antibodies (A3361, lot #0000102992, dilution 1:1,000) were purchased from Sigma. Goat 157  
anti-rabbit IgG (H+L) secondary antibody HRP conjugated (31460) and goat anti-mouse IgG (H+L) 158  
secondary antibody HRP conjugated (31430) were from Thermo Scientific and rabbit anti-ATP13A3 159  
antibody (HPA029471, lot # 000035781, dilution 1:2,000) was from Atlas Antibodies. 160

#### ATP/NADH-enzyme coupled ATPase assay.

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Purified human ATP13A2 protein was obtained as previously described in [13]. Protein concentration 162  
was determined using a Pierce 660 nm Protein Assay (Thermo Scientific: 22660) and the quality of the 163  
purification was assessed via SDS-PAGE followed by InstantBlue Coomassie protein stain (abcam: 164  
ab119211) staining and immunoblotting, as described in [11]. To measure ATPase activation of purified 165

ATP13A2, serial dilutions of the unlabeled, unclicked azide spermine, BODIPY-SPM, and unclicked 166  
alkyne BODIPY were prepared ranging from 0.01  $\mu\text{M}$  to 10 mM, in a final volume of 25  $\mu\text{L}$  per well in 167  
a 384-well clear polystyrene microplate. Then, 40  $\mu\text{L}$  of the reagent mix containing 50 mM MOPS- 168  
KOH (pH 7.0), 100 mM KCl, 30 mM  $\text{MgCl}_2$ , 0.6 mM NADH, 1.667 mM PEP, 2.4 U  $\mu\text{L}^{-1}$  pyruvate 169  
kinase, 2.4 U  $\mu\text{L}^{-1}$  lactate dehydrogenase, and 2 mM DTT, in the presence or absence of 1.25  $\mu\text{g}$  purified 170  
ATP13A2 protein, were added per well. Next, 5 mM ATP (pH 7.0) were added in each well to start the 171  
biochemical reaction. The plate was shaken for 30 seconds prior acquisition. Absorbance at 340 nm was 172  
measured at RT every 30 seconds for 1 h with a SpectraMax Plus 384 microplate reader (Molecular 173  
Devices). Data analysis was done using SoftMax Pro 7.0 and GraphPad Prism 9.3.1 software. This 174  
protocol is further described in protocols.io following 175  
[dx.doi.org/10.17504/protocols.io.6qpvr4do2gmk/v1](https://doi.org/10.17504/protocols.io.6qpvr4do2gmk/v1). 176

#### Chemical synthesis of fluorescently labeled polyamines

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The detailed synthesis of each compound is further described in **Supplementary Information, Figures** 178  
**S5-8**. 179

#### Acquisition of absorption and emission spectra

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Absorbance spectra were determined using the Cary 60 UV-Vis spectrophotometer with 1 nm spectral 181  
resolution. Attenuators were used to remove the effect of background and noise. Emission spectra were 182  
measured using an Edinburgh Instruments FLS 980 spectrometer at an excitation wavelength of 465 nm 183  
and emission data was collected at 1 nm intervals. For both emission and absorbance measurements, 184  
probe samples were dissolved in deionized water as to keep the optical density below 0.2. After this the 185  
samples were added to a quartz cuvette with 10 mm pathlength before being sealed and measured. 186

#### Cellular polyamine uptake and endocytosis assay.

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Cells were seeded in 12-well plates to reach 70% confluency the day of the experiment (approximately 188  
 $2.0 \times 10^5$  and  $1.5 \times 10^5$  cells per well, for SH-SY5Y and HMEC-1, respectively). To assess the polyamine 189  
uptake capacity, cells were incubated with 0.1  $\mu\text{M}$  to 100  $\mu\text{M}$  of fluorescently labeled polyamines in 190  
their respective medium for 0 to 16 h at 37°C and 5%  $\text{CO}_2$ . To assess endocytosis rate, cells were treated 191  
with a cocktail of endocytosis inhibitors containing 100  $\mu\text{M}$  Dynasore, 50  $\mu\text{M}$  Genistein, and 50  $\mu\text{M}$  192



Pitstop-2, dissolved in their respective culturing medium without supplementation of FBS, for 30 min 193  
at 37°C and 5% CO<sub>2</sub>, and kept at 4°C for 15 min. Then, cells were co-incubated with 50 µg/mL 194  
Alexa647-Transferrin (Invitrogen: T23366) for 20 min at 4°C, and further incubated for 20 min at 37°C 195  
and 5% CO<sub>2</sub>. After either treatment, cells were washed with DPBS or Versene Solution (Gibco: 15040), 196  
detached with either TrypLE or 0.25% Trypsin-EDTA, centrifuged and resuspended in DPBS containing 197  
1% Albumin Fraction V. Cell suspensions were filtered through a nylon filter to avoid clumps and kept 198  
on ice before acquisition using a BD FACS Canto II AIG or HTS Flow Cytometer (BD Biosciences). A 199  
488 nm 20 mW Solid State Blue (Coherent) laser with 530/30 BP detector, and a 633 nm 17 mW HeNe 200  
Red (JDS Uniphase) laser with 660/20 detector were used to record the mean fluorescent intensities 201  
(MFI) of 10,000 events per sample. These protocols are described in protocols.io following 202  
[dx.doi.org/10.17504/protocols.io.n92ldp8qx15b/v1](https://dx.doi.org/10.17504/protocols.io.n92ldp8qx15b/v1) and 203  
[dx.doi.org/10.17504/protocols.io.8epv5jjedl1b/v1](https://dx.doi.org/10.17504/protocols.io.8epv5jjedl1b/v1), respectively. Data analysis was done using Flowing 204  
Software 2 and GraphPad Prism 9.3.1. 205

#### Radiolabeled polyamine uptake assay. 206

Cells were seeded in 12-well plates to reach 70% confluency the day of the experiment. Cells were 207  
incubated with 0.5 to 5 µM of [<sup>14</sup>C]-radiolabeled polyamines ([<sup>14</sup>C]-PUT: ARC 0245-50 µCi; [<sup>14</sup>C]- 208  
SPD: ARC 3138-50 µCi; and [<sup>14</sup>C]-SPM: ARC 3139-50 µCi) in culture medium for 30 min at 37°C. 209  
Afterwards, cells were washed with cold DPBS, supplemented with 50 µM of the respective unlabeled 210  
polyamine. After two washing steps with cold DPBS, the cells were lysed using 0.1% SDS (Sigma, 211  
71725) in DPBS. After 10 min, the cell lysates were scraped off the wells and collected into scintillation 212  
vials containing 7 mL EcoLite Liquid Scintillation Cocktail (MP Biomedicals: 01882475-CF). [<sup>14</sup>C] 213  
radioactivity in counts-per-minute (CPM) was measured by liquid scintillation counting (TRI-CARB 214  
4910TR V Liquid Scintillation Counter, PerkinElmer). This protocol is further described in protocols.io 215  
following [dx.doi.org/10.17504/protocols.io.yxmvm2x85g3p/v1](https://dx.doi.org/10.17504/protocols.io.yxmvm2x85g3p/v1). 216

#### Statistics and data analysis. 217

Experiments on the different cell models were executed by different researchers, which provided 218  
consistent results that independently confirmed the major conclusions. Data are expressed as the mean 219  
± SD, or with individual data points (replicates of multiple independent experiments). GraphPad Prism 220

9.3.1 software was used to plot all graphs and to perform all of the required statistical assessments. 221

Statistical tests for each graph are described in the legend together with the number of independent 222

biological experiments. For the quantification of immunoblots, ImageJ was used. 223

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## Results

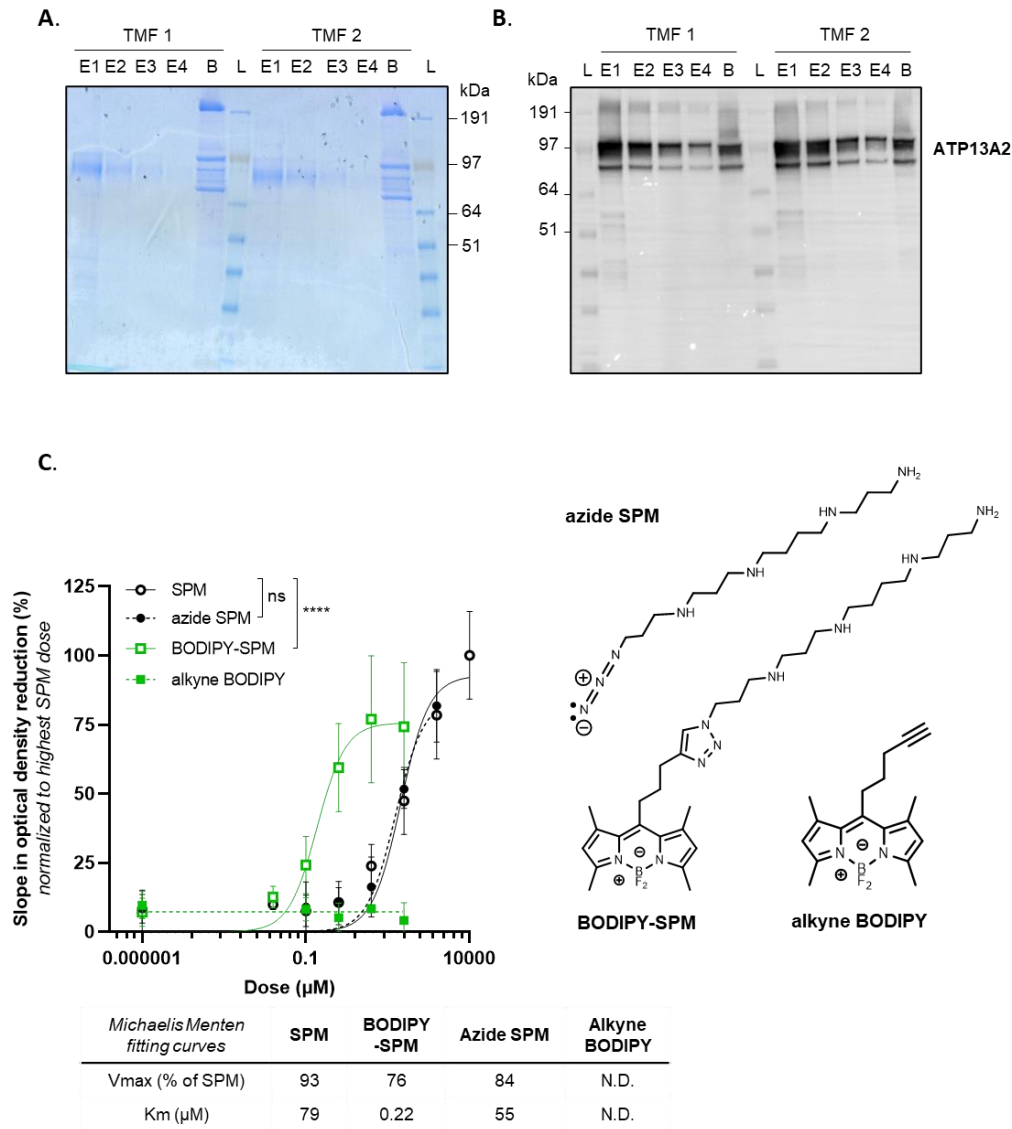
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### 1. BODIPY-conjugated SPM is a genuine transport substrate of ATP13A2

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hATP13A2 exhibits the highest affinity for SPM [13], which binds to a narrow channel-like substrate binding site at the luminal side of the protein [16-20]. Whether the polyamine pocket may also accommodate the bulkier BODIPY-SPM for subsequent transport remains unclear. Since SPM transport by ATP13A2 is coupled to the hydrolysis of ATP, we examined whether BODIPY-SPM also stimulates ATP turnover, and hence represents a genuine substrate. To this end, we purified human WT ATP13A2 (**Figure 1A, B**) and performed an ATP/NADH enzyme-coupled ATPase assay with SPM or BODIPY-SPM (**Figure 1C**). ATP13A2's ATPase activity is stimulated with either SPM, BODIPY-SPM or clickable azide SPM (an intermediate in the coupling strategy), but not with clickable BODIPY-alkyne (**Figure 1C**). Hence, ATP13A2 recognizes the BODIPY-labeled or clickable SPM as genuine substrates. Surprisingly, ATP13A2 displays a higher apparent affinity, and slightly lower maximal velocity ( $V_{max}$ ), for BODIPY-SPM ( $K_m$  0.22  $\mu$ M) than SPM ( $K_m$  79  $\mu$ M).

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**Figure 1. Purification and ATPase activity of hATP13A2.** **A.** Coomassie staining showing the purified human ATP13A2 protein present in the different elution fractions (E1 to E4) from total membrane fraction (TMF) and onto the beads (B). **B.** Immunoblot analysis of ATP13A2 purified protein from the fractions depicted in **A.** **C.** ATPase activity of purified ATP13A2 (1.25 µg) measured with increasing concentrations of unlabeled spermine (SPM), clickable spermine (azide SPM), free BODIPY (alkyne BODIPY) and BODIPY-SPM, which structures are presented on the right ( $N = 2$  to  $7$ , technical duplicates, two-way ANOVA with Tukey's multiple comparisons test). The Y axis depicts the slope in optical density at 340 nm reflecting NADH consumption and further ATP consumption, normalized to the highest dose of SPM. N.D., not determined.

We previously studied ATP13A2-dependent BODIPY-SPM uptake in human neuroblastoma SH-SY5Y cells, a frequently used cell line in Parkinson's disease research [13,23]. Here, we characterized the time- and dose-dependency of BODIPY-SPM uptake in SH-SY5Y cells overexpressing ATP13A2 wild type (A2 WT-OE) or a transport-dead mutant (D508N-OE). Both cell models exhibit comparable

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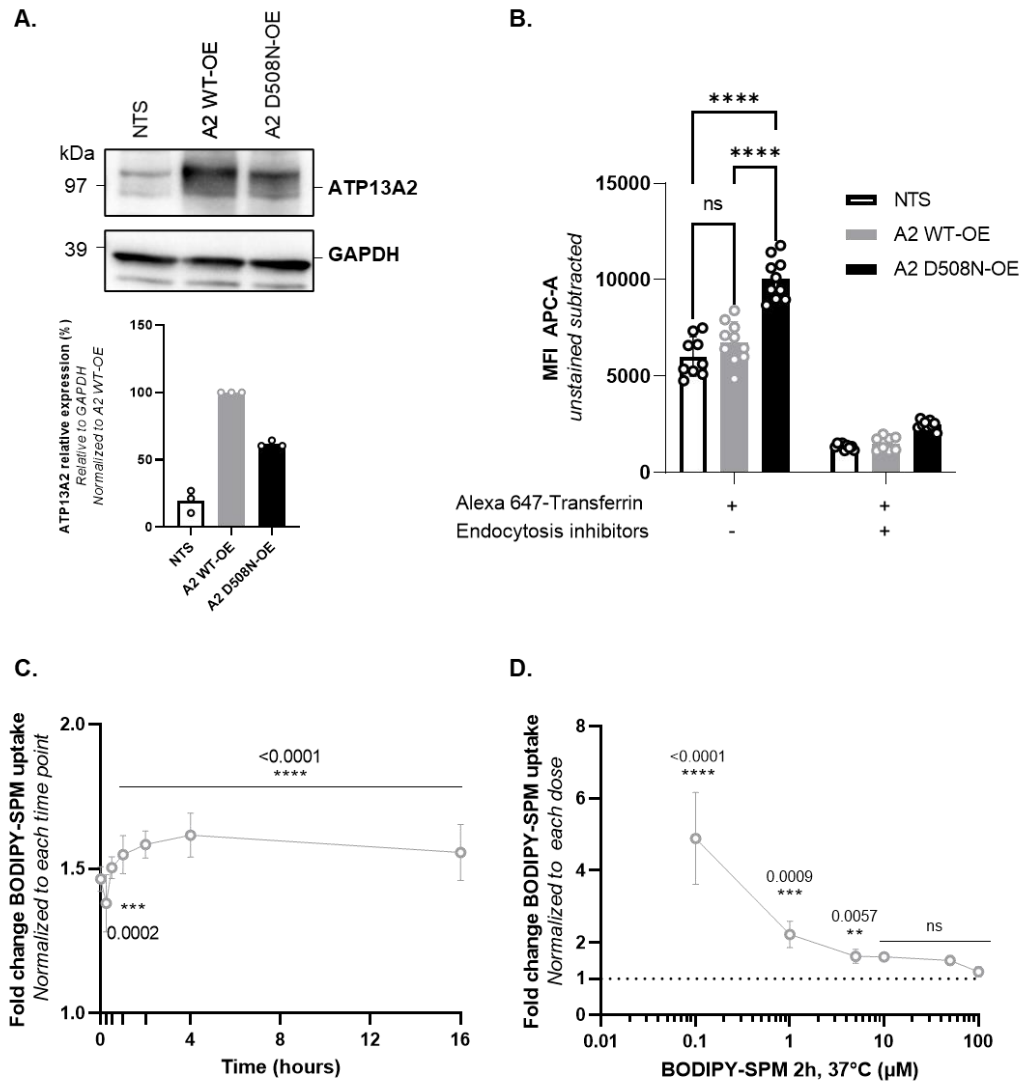
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ATP13A2 protein expression levels (**Figure S1A**) and interestingly, a significantly higher endocytosis 251  
rate in A2 D508N-OE cells compared with A2 WT-OE and NTS cells (**Figure S1B**), suggesting that the 252  
difference in BODIPY-SPM uptake between A2 WT-OE and A2 D508N-OE cells can be attributed to 253  
ATP13A2 transport activity. The time-dependency was evaluated by incubating the cells with 5  $\mu$ M 254  
BODIPY-SPM (a concentration that was used before; [13]) up to 16 h (**Figure S1C**). The maximal fold 255  
difference in BODIPY-SPM uptake between A2 WT-OE and A2 D508N-OE cells was reached at 4 h 256  
(1.6-fold) (**Figure S1C**), whereas maximal cellular uptake capacities were still not achieved at 16 h 257  
(**Figure S1C**). We selected a 2 h incubation time, which falls within the linear phase of the BODIPY- 258  
SPM uptake (**Figure 2A**), to examine the dose-dependency of BODIPY-SPM uptake in ATP13A2 cell 259  
models (**Figure 2B** and **Figure S1C**). We observed a 5-fold higher BODIPY-SPM uptake in A2 WT- 260  
OE *versus* A2 D508N-OE cells at the lowest BODIPY-SPM concentration (0.1  $\mu$ M, **Figure S1D**), but 261  
the fold difference decreases at higher concentrations. Nevertheless, a significant and reproducible 1.6- 262  
fold higher uptake was observed at 5  $\mu$ M BODIPY-SPM (**Figure 2C**), which was used in previous 263  
studies [13]. Finally, we compared BODIPY-SPM with radiolabeled spermine ( $^{14}$ C-SPM) uptake in A2 264  
WT-OE cells to assess the impact of the fluorescent tag on cellular uptake capacities (**Figure 2D**). After 265  
incubating A2 WT-OE and D508N-OE cells for 30 min with either 5  $\mu$ M of BODIPY-SPM or  $^{14}$ C-SPM, 266  
we observed a significant 1.33-fold higher uptake for  $^{14}$ C-SPM (**Figure 2D**). The effect of the 267  
fluorescent tag on SPM uptake reflects the higher apparent affinity, but lower  $V_{\max}$  of ATP13A2 for 268  
BODIPY-labeled *versus* native spermine (**Figure 1C**). Overall, our data demonstrate that the BODIPY- 269  
SPM probe is a valuable tool to evaluate the ATP13A2 transport activity in cells. 270



**Figure S1. Characterization of ATP13A2-expressing SH-SY5Y cell models.** **A.** ATP13A2 protein expression level in SH-SY5Y cells transduced with lentiviral particles to overexpress ATP13A2 wild type (A2 WT-OE) or the catalytically dead mutant ATP13A2 D508N (A2 D508N-OE). Immunoblots showing ATP13A2 expression levels and quantification (bar graph) relative to GAPDH signal (N=3). **B.** The endocytosis rate in the ATP13A2 cell models was assessed by an Alexa 647-transferrin assay. A2 WT-OE, A2 D508N-OE and non-transduced (NTS) cells were incubated with Alexa 647-transferrin in the presence or absence of a cocktail of endocytosis inhibitors: dynasore (100 μM), genistein (50 μM) and pitstop-2 (50 μM). The mean fluorescence intensity (MFI) of 10,000 events was recorded on the Canto II HTS flow cytometer (N=3, two to four technical replicates, two-way ANOVA with Tukey's multiple comparisons test). In both panels (**A** and **B**), the NTS cell line was used as a control. **C.** and **D.** Graph depicts the fold change in BODIPY-SPM uptake between A2 WT-OE and A2 D508N-OE cells, normalized to each time point considered (**C**) or each dose considered (**D**) (N=3 to 10, technical duplicates, two-way ANOVA with Sidak's multiple comparisons test).

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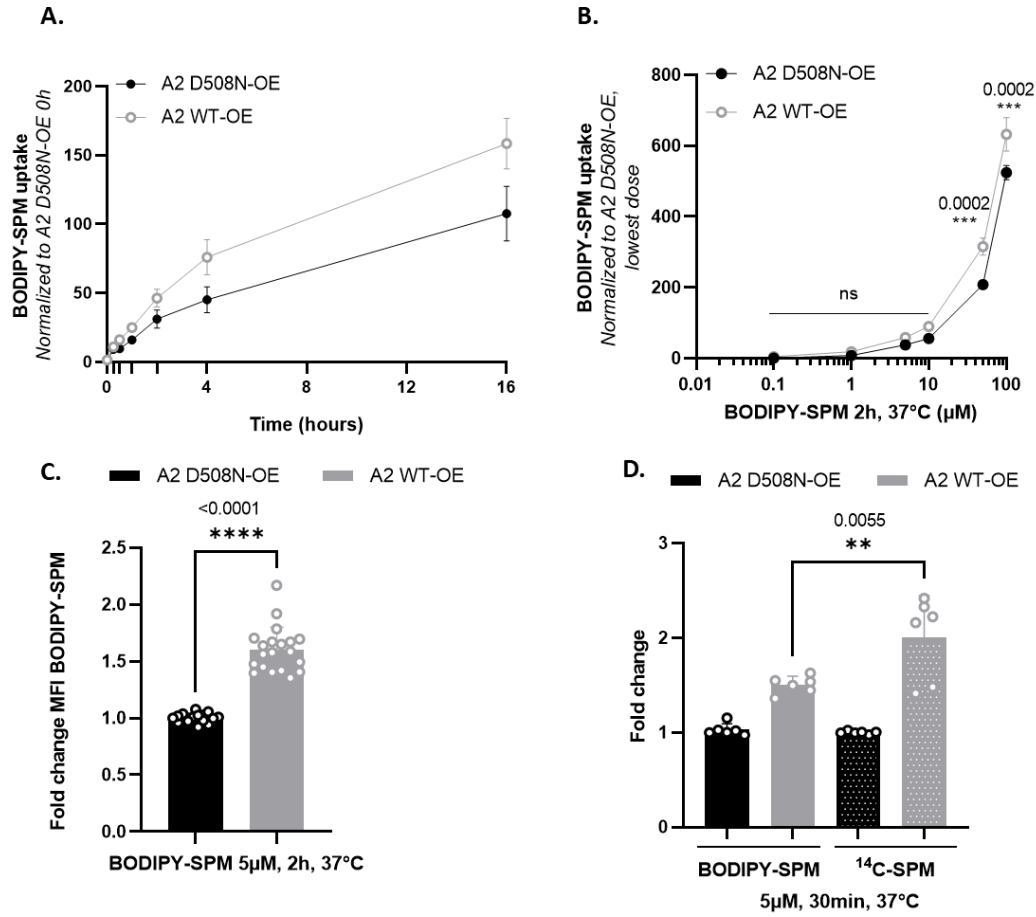
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**Figure 2. Time- and dose-dependency of BODIPY-SPM uptake in ATP13A2-expressing SH-SY5Y cell models.** **A-B.** SH-SY5Y cells were incubated with 5  $\mu\text{M}$  BODIPY-SPM for the indicated times (**A**, time dependency) or with increasing concentrations of BODIPY-SPM for 2 h (**B**, dose dependency). The mean fluorescence intensity (MFI) of 10,000 events was recorded using a BD Canto II HTS flow cytometer. Graphs represent the MFI recorded in ATP13A2 WT OE (A2 WT-OE) and ATP13A2 D508N-OE (A2 D508N-OE) cells, normalized to the 0 h incubation time in A2 D508N-OE cells (in **A**) or to the lowest concentration of BODIPY-SPM (*i.e.* 100  $\mu\text{M}$ ) in A2 D508N-OE cells (in **B**). **C.** Graph depicts the fold change in BODIPY-SPM uptake (5  $\mu\text{M}$ ; 2 h) between A2 WT-OE and A2 D508N-OE cells (N=10, technical duplicates, unpaired t-test). **D.** Comparison of the uptake of 5  $\mu\text{M}$  BODIPY-conjugated (BDP-SPM) *versus* 5  $\mu\text{M}$  radiolabeled spermine ( $^{14}\text{C}$ -SPM) in SH-SY5Y A2 WT-OE and D508N-OE cells for 30 minutes (N=3, technical duplicates, one-way ANOVA with Tukey's multiple comparisons test).

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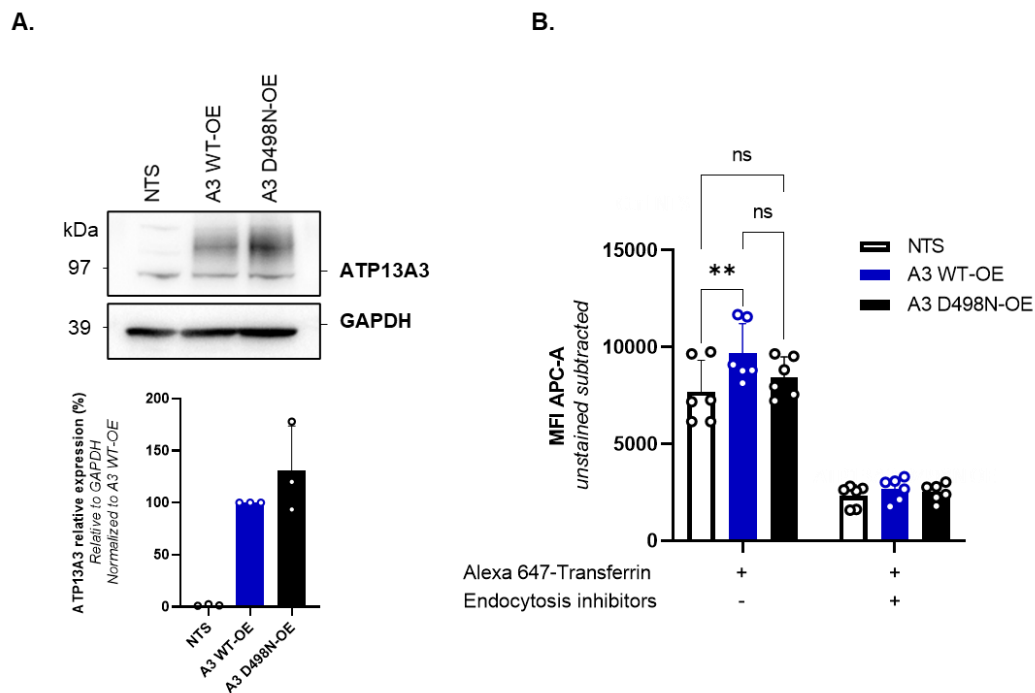
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## 2. Evaluation of BODIPY-PUT and BODIPY-SPD uptake in a new human ATP13A3 cell model. 297 model. 298

Besides ATP13A2, we previously reported that ATP13A3 also modulates cellular BODIPY-polyamine 299 uptake in CHO cells, with the strongest impact on BODIPY-PUT uptake [11]. Here, we decided to 300 evaluate the uptake of the BODIPY-PUT and BODIPY-SPD probes in the context of human ATP13A3, 301 and turned to immortalized human dermal microvascular endothelial cells (HMEC-1). HMEC-1 cells 302 are frequently used in the context of pulmonary arterial hypertension, a disease that is associated with 303 ATP13A3 mutations [24-27] and do express ATP13A3. We generated HMEC-1 cells with stable 304 lentiviral mediated over-expression of ATP13A3 WT (A3 WT-OE) or a catalytic dead mutant D498N 305 (A3 D498N-OE), and confirmed that both the ATP13A3 expression level (**Figure S2A**) and endocytosis 306 rate (**Figure S2B**) of A3 WT-OE and A3 D498N-OE HMEC-1 cells were comparable. Hence, 307 differences in BODIPY-polyamine uptake between A3 WT-OE and A3 D498N-OE cells can be 308 attributed to a functional involvement of the transport ATPase. 309

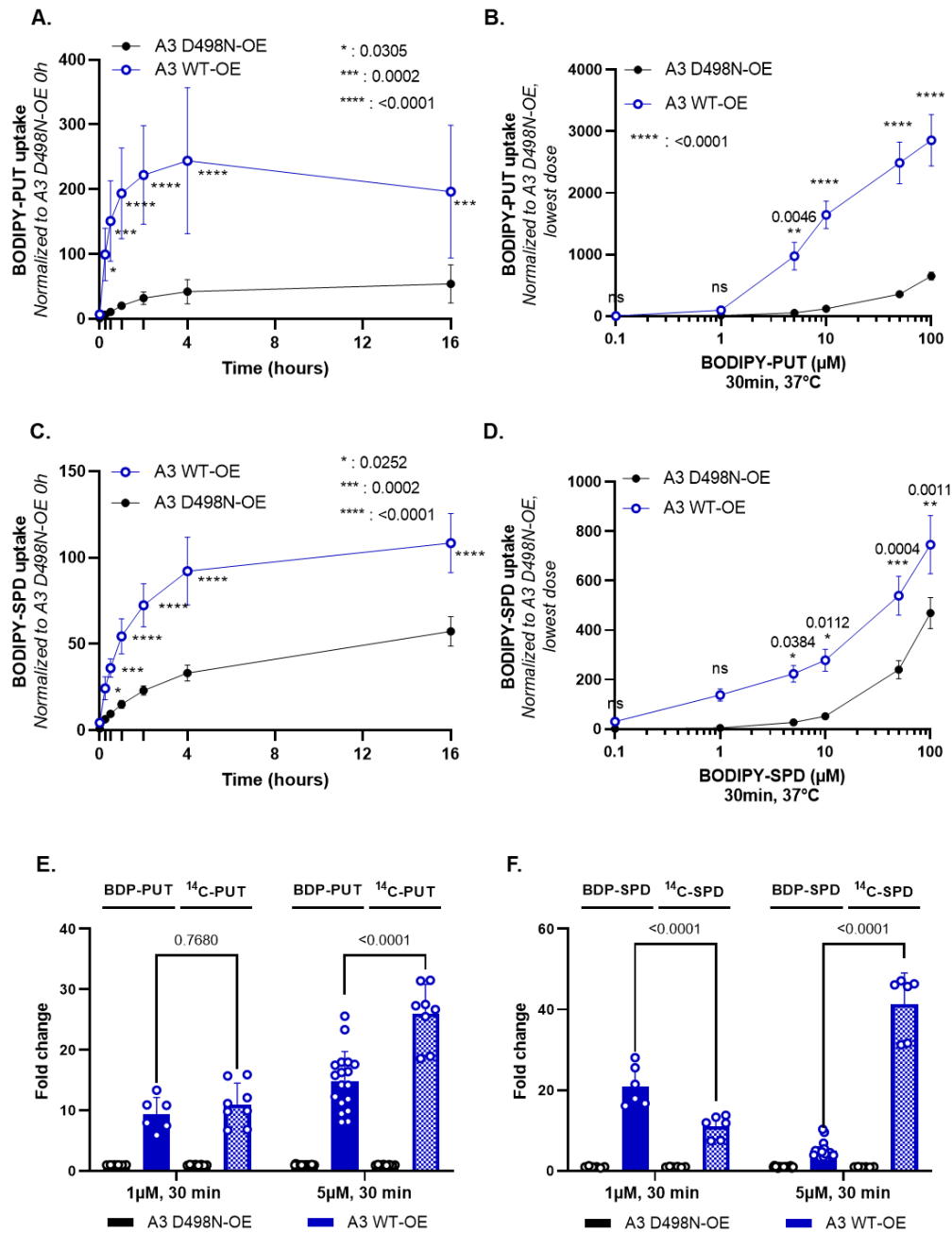


**Figure S2. Characterization of ATP13A3-expressing HMEC-1 cell models.** A. HMEC-1 cells were transduced 311 with lentiviral particles to overexpress ATP13A3 wild type (A3 WT-OE) or the catalytically dead mutant 312 ATP13A3 D498N (A3 D498N-OE). Immunoblots showing ATP13A3 expression levels and quantification (bar 313 graph) relative to GAPDH signal (N=3). B. Evaluation of the endocytosis rate in A3 WT-OE and A3 D498N-OE 314 HMEC-1 cells. Cells were incubated with Alexa 647-transferrin in the presence or absence of a cocktail of 315



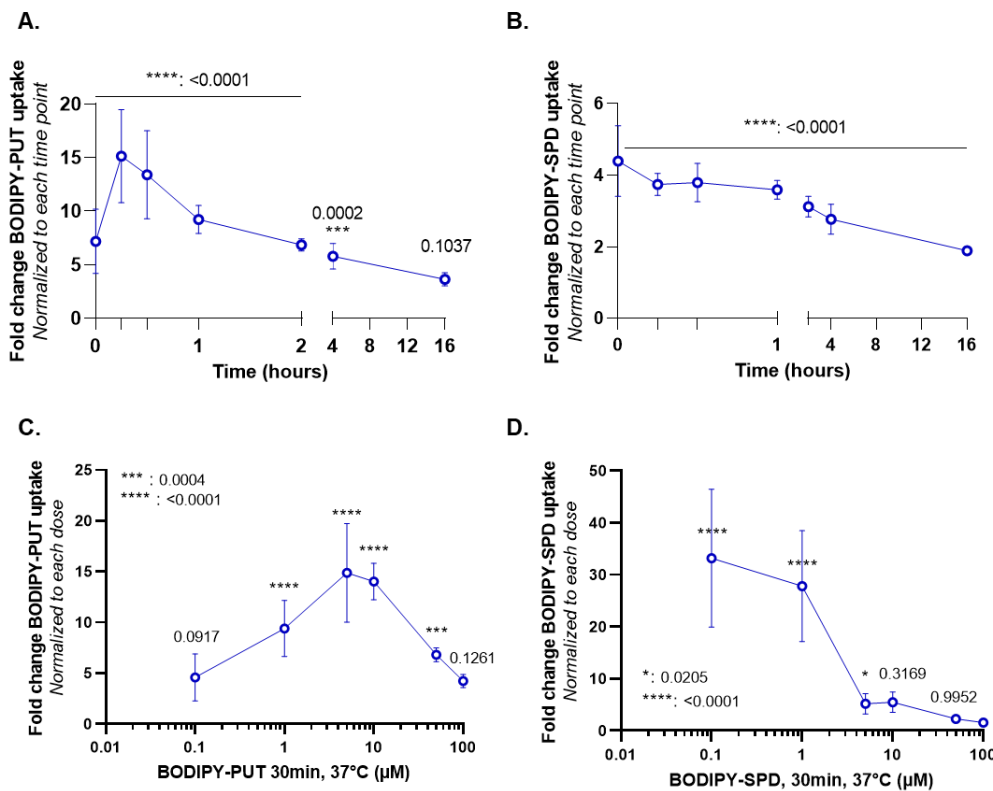
endocytosis inhibitors: dynasore (100  $\mu$ M), genistein (50  $\mu$ M) and pitstop-2 (50  $\mu$ M). The mean fluorescence intensity (MFI) of 10,000 events was recorded on the Canto II HTS flow cytometer. N=3 with technical duplicates, two-way ANOVA with Tukey's multiple comparisons test. In both panels, the non-transduced cell line (NTS) was used as a control.

Next, we analyzed the time- and dose-dependency of BODIPY-SPD and BODIPY-PUT uptake in the ATP13A3 cell models (**Figure 3** and **Figure S3**). First, we evaluated the ATP13A3-dependent BODIPY-PUT and BODIPY-SPD uptake (5 $\mu$ M) in HMEC-1 over time (**Figure S3A, C** and **Figure 3A, C**). At 4 h, the cellular uptake of both probes reached saturation. Interestingly, at 5  $\mu$ M BODIPY-polyamine, A3 WT-OE cells exhibit a more pronounced uptake than A2 WT-OE cells, already at early time points. Therefore, a shorter incubation time of 30 min is required to remain in the linear uptake phase in A3 WT-OE cells (**Figure 3A, C**) in comparison to the 2 h incubation in A2 WT-OE cells (**Figure 2A**). This may reflect the differences in (i) endosomal localization between ATP13A3 (early/recycling endosomes) and ATP13A2 (late endo/lysosomes) [11-13], (ii) cell type properties (endothelial versus neuroblastoma cells) and/or (iii) relative expression levels of ATP13A3 *versus* ATP13A2.



**Figure 3. Time- and dose-dependency of BODIPY-PUT and BODIPY-SPD uptake in ATP13A3-expressing HMEC-1 cell models.** **A.** and **C.** Time dependent BODIPY-polyamine uptake in HMEC-1 cells incubated with 5 μM BODIPY-PUT (**A**) or BODIPY-SPD (**C**). The mean fluorescence intensity (MFI) of 10,000 events was recorded using a BD Canto II HTS flow cytometer. Graphs represent the fold change between the MFI recorded in A3 WT-OE versus A3 D498N-OE cells, normalized to the 0 h incubation time in A3 D498N-OE cells. (N=3 to 9, with technical duplicates, two-way ANOVA with Sidak's multiple comparisons test **B.** and **D.** Dose-response of BODIPY-PUT (**B**) or BODIPY-SPD (**D**) uptake in HMEC-1 cells at 30 min. The MFI of 10,000 events was recorded using a BD Canto II HTS flow cytometer. Graphs represent the fold change difference between the MFI recorded in A3 WT-OE and A3 D498N-OE cells, normalized to the highest concentration of BODIPY-polyamine (i.e. 100 μM) in A3 D498N-OE cells (N=3 to 9, with technical duplicates, two-way ANOVA with Sidak's multiple comparison test). **E.** and **F.** Comparison of the uptake of 1 and 5 μM BODIPY-conjugated (BDP-PUT and BDP-

SPD) *versus* radiolabeled putrescine and spermidine ( $^{14}\text{C}$ -PUT and  $^{14}\text{C}$ -SPD) in HMEC-1 A3 WT-OE and D498N-OE cells for 30 min (N=3 to 9, technical duplicates, two-way ANOVA with Tukey's multiple comparisons test).



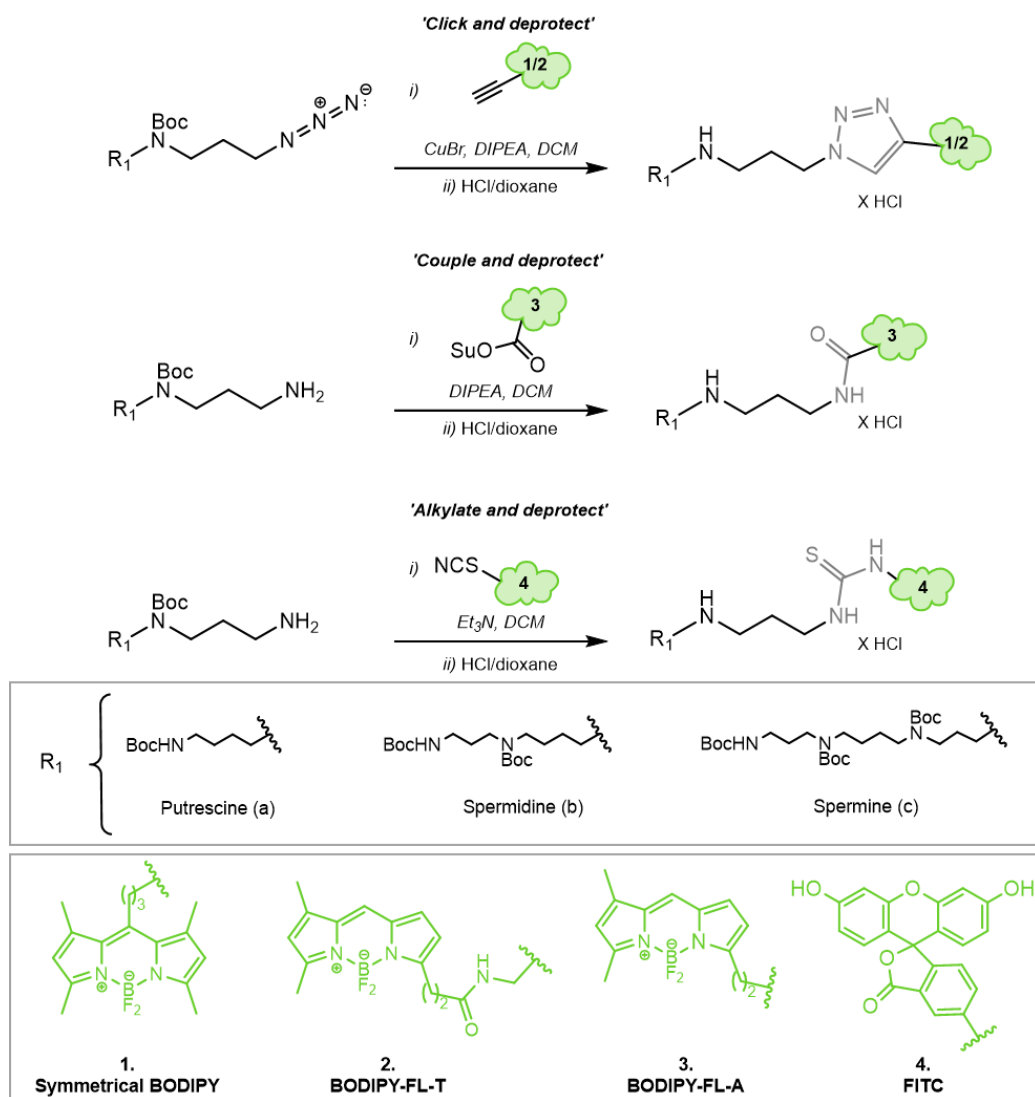
**Figure S3. BODIPY-PUT and BODIPY-SPD uptake in HMEC-1 cells overexpressing ATP13A3 WT or D498N.** Fold change in BODIPY-PUT (A, C) and BODIPY-SPD (B, D) uptake between A3 WT-OE and A3 D498N-OE cells, normalized to each time point considered (A, B) or each dose considered (C, D). N=3 to 9, technical duplicates, two-way ANOVA with Sidak's multiple comparisons test.

Second, we performed a dose-response experiment with increasing concentrations of BODIPY-PUT or -SPD (0 - 100 μM) after 30 min incubation (Figure 3B, D). At 1 μM, ATP13A3-mediated BODIPY-SPD uptake is more pronounced than BODIPY-PUT, indicating that ATP13A3 present a higher apparent affinity and/or  $V_{\max}$  for BODIPY-SPD. A higher maximal fold increase is also observed with BODIPY-SPD (33-fold at 0.1 μM, Figure S3D) than for BODIPY-PUT (15-fold at 5 μM, Figure S3C). The higher fold uptake with 5 μM  $^{14}\text{C}$ -SPD (41-fold) (Figure 3E) than with 5 μM  $^{14}\text{C}$ -PUT (26-fold) (Figure 3F) indicates that ATP13A3 presents a higher transport activity with SPD than with PUT. To investigate the impact of the fluorescent headgroup, we compared the uptake of BODIPY-labeled with  $^{14}\text{C}$ -radiolabeled SPD and PUT in A3 WT-OE cells at two different concentrations (1 μM and 5 μM) (Figure 3D, E). At 1 μM, a comparable fold change in uptake can be observed between BODIPY-PUT

(9.4-fold) *versus*  $^{14}\text{C}$ -PUT (11-fold), whereas the uptake of BODIPY-SPD is significantly higher (21- 361 fold) than  $^{14}\text{C}$ -SPD (11.6-fold). At 5  $\mu\text{M}$ , the uptake of the BODIPY probes is lower compared with  $^{14}\text{C}$ - 362 labeled polyamines, especially for BODIPY-SPD. Our results therefore suggest that ATP13A3 presents 363 a higher apparent affinity and/or reduced maximal uptake activity towards BODIPY- as compared to 364  $^{14}\text{C}$ -labeled probes, similar to what we have observed for ATP13A2. 365

### 3. Chemical synthesis of novel green fluorescent polyamine conjugates. 366

Our results so far demonstrated that both radiolabeled and BODIPY-polyamines are taken up in cells 367 *via* ATP13A2 and ATP13A3. Next, we explored whether the coupling strategy or the headgroup 368 fluorophore of the fluorescent conjugates may influence the transport by ATP13A2 and/or ATP13A3 369 (**Figure 4**). The synthesis of the click-chemistry derived BODIPY-conjugated polyamines (**compounds** 370 **1a-c** in **Figure 4**) was performed as previously described [11,21] with small modifications indicated in 371 **Supplementary Information**. Here, we report the synthesis of various additional polyamines 372 conjugated to green fluorophore headgroups. First, BODIPY-FL-T-polyamines were synthesized *via* a 373 similar click-chemistry reaction as for the original BODIPY probes [21], but with the difference that 374 azido-polyamines (-PUT, -SPD or -SPM) were clicked onto the alkyne-BODIPY-FL fluorophore. This 375 resulted in the formation of a triazole ring (T) between the polyamine and the BODIPY-FL groups 376 (referred to as FL-T probe, **compounds 2a-c**, **Figure 4**). Second, a succinimidyl ester-amine coupling 377 strategy yielded the synthesis of BODIPY-FL-A conjugated polyamines in which an amide bond (A) is 378 created between the polyamine and BODIPY-FL groups (referred to as FL-A probe, **compounds 3a-c**, 379 **Figure 4**). Lastly, an isothiocyanate-amine coupling strategy was used to generate FITC-conjugated 380 polyamines with a thiourea bond between the polyamine and the fluorophore (referred as FITC probes, 381 **compounds 4a-c**, **Figure 4**). The detailed chemical synthesis of each polyamine-conjugate is outlined 382 in **Supplementary Information** and the complete chemical structures are drawn in **Figure S4A-C**. As 383 expected, the fluorescent properties of the different fluorophore-polyamine conjugates (i.e. the 384 wavelengths of maximum absorption and emission  $\lambda_{\text{Abs}}$  (max) and  $\lambda_{\text{Em}}$  (max) as well as the Stokes' shift) 385 are very similar (**Figure S4E**). 386

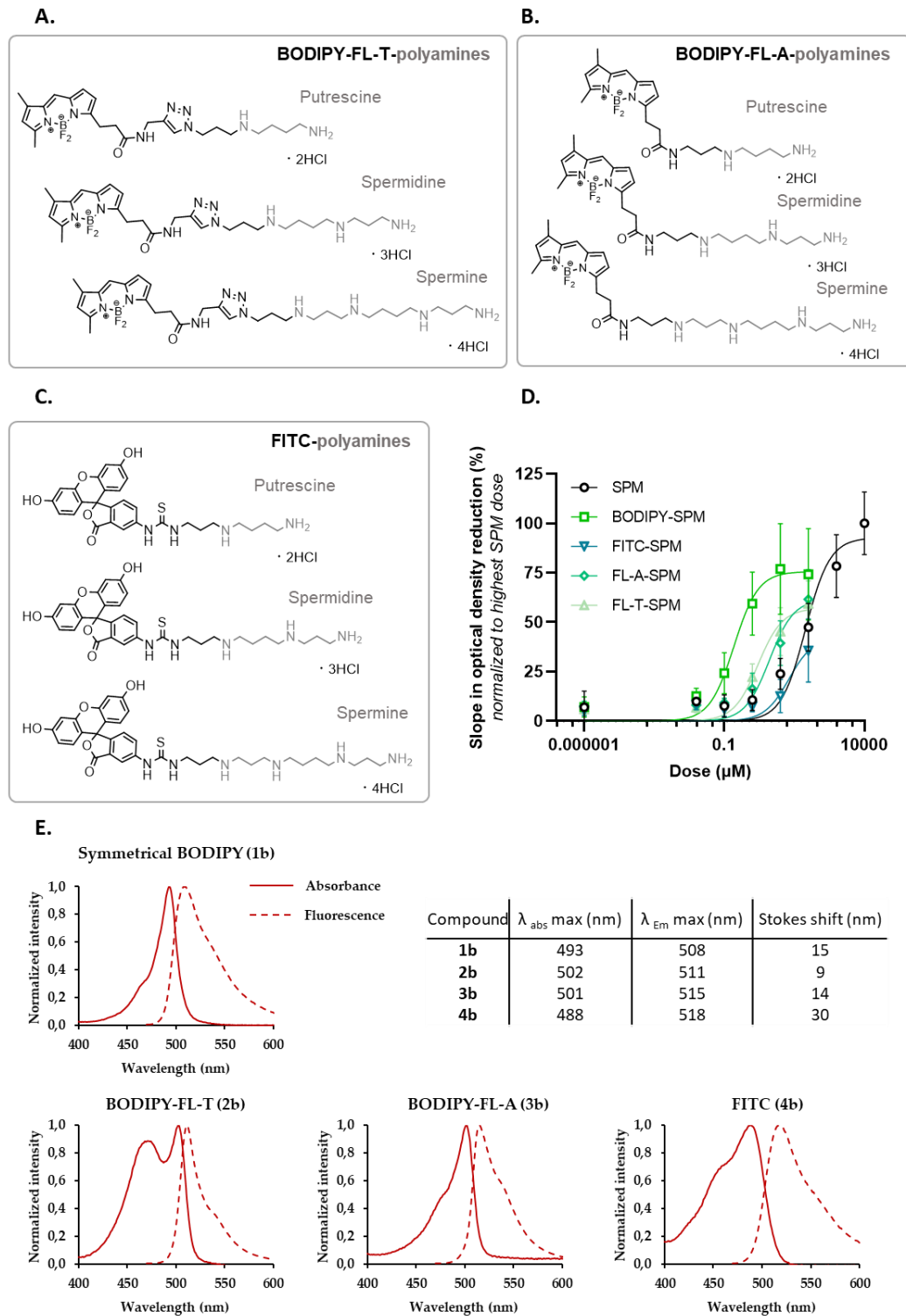


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**Figure 4. Simplified scheme representing the chemical synthesis of the different green fluorescent polyamine conjugates used in the study.** A detailed description of the synthesis is given in **Supplementary Information**.

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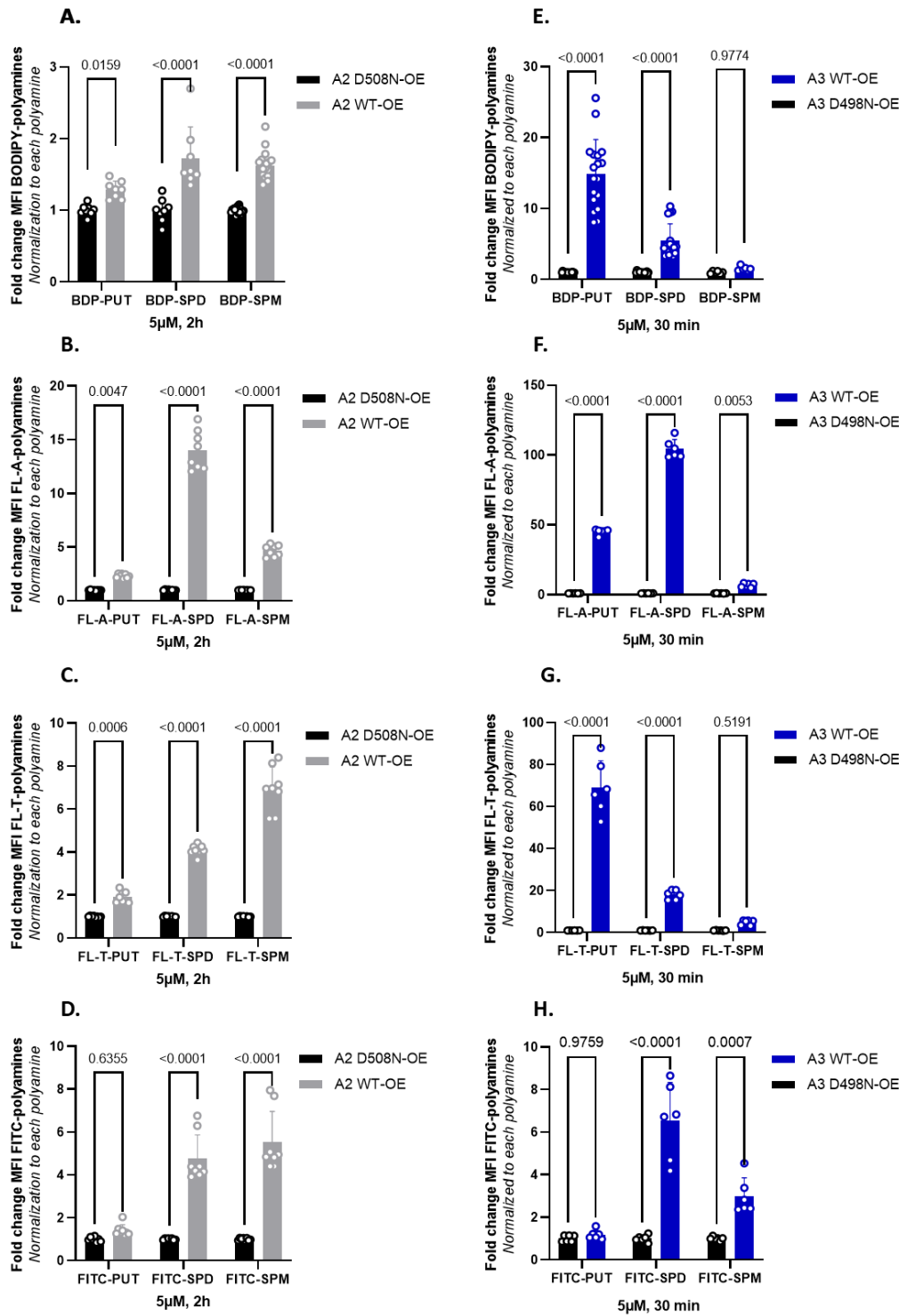
**Figure S4. Full structures of the different green fluorescent polyamine conjugates used in the study and biochemical characterization of spermine conjugates.** **A.** FL-T-polyamine structures. **B.** FL-T-polyamine structures. **C.** FITC-polyamine structures. **D.** ATP/NADH-enzyme coupled ATPase assay. ATPase activity of purified ATP13A2 (1.25  $\mu\text{g}$ ) measured in the presence of increasing concentration of the following substrates: unlabeled spermine (SPM), BODIPY-SPM, FITC-SPM, FL-A-SPM and FL-T-SPM (N=3 to 9, technical duplicates). The Y axis depicts the slope in optical density at 340 nm reflecting NADH consumption and further

ATP consumption, normalized to the highest dose of SPM. **E.** Normalized absorption and emission spectra of 397  
symmetrical BODIPY-SPD (1b), BODIPY-FL-T-SPD (2b), BODIPY-FL-A-SPD (3B) and FITC-SPD (4b) 398  
together with a table providing the spectral properties of these compounds. 399

To evaluate whether the new probes are substrates for ATP13A2, we performed ATP/NADH enzyme- 400  
coupled ATPase assays on purified ATP13A2 with FL-A-SPM, FL-T-SPM and FITC-SPM (**Figure** 401  
**S4D**) using SPM and BODIPY-SPM as references. ATP13A2's activity is not only stimulated by SPM 402  
and BODIPY-SPM, but also by FL-A-, FL-T- and FITC-SPM showing that the different fluorophores 403  
or coupling strategies do not prevent the recognition of the polyamine core as a substrate. Interestingly, 404  
only BODIPY-SPM, but not the other fluorophore-SPM conjugates display a higher apparent affinity 405  
for ATP13A2, since the new probes stimulate ATP13A2 ATPase activity to the same extent as the 406  
unlabeled SPM up to a concentration of 10  $\mu$ M (**Figure S4D**). 407

#### 4. Fluorescent polyamine probes present different structure-function relationships towards 408 ATP13A2 and ATP13A3. 409

In a next step, we compared the uptake of the new green fluorescent polyamines in both ATP13A2 and 410  
ATP13A3 cell models using the original BODIPY-polyamines as a reference (**Figure 5A, E**). ATP13A3 411  
and ATP13A2 cell models were exposed to a fixed concentration of 5  $\mu$ M fluorescent polyamine for 30 412  
min or 2 h, respectively (**Figure 5**). In general, we observed a larger fold-change of uptake with the new 413  
FL-A and FL-T probes than with the reference BODIPY polyamines. The large window between uptake 414  
in cells expressing WT *versus* the catalytic dead mutant shows that all probes are mainly transported *via* 415  
the catalytic activity of ATP13A2 or ATP13A3 (**Figure 5**), in line with the ATPase data for ATP13A2 416  
(**Figure 4**). Strikingly, the uptake windows obtained with the new BODIPY-FL-probes (FL-A and FL- 417  
T) are much larger than with the reference BODIPY polyamines. The structural difference lies not only 418  
in the substitution pattern on the fluorophore core, but also on the position of the connection towards 419  
the polyamine. Both of these factors may affect the cellular uptake *via* ATP13A2 and/or ATP13A3. 420  
Despite their lower apparent affinity for the transporter compared with the original BODIPY analogs, 421  
the signal to noise ratio for the uptake was highest with the FL-A probes, followed by the FL-T probes. 422



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**Figure 5. Comparison of the cellular uptake of BODIPY-conjugated versus BODIPY-FL-A, BODIPY-FL-T and FITC-conjugated polyamines in ATP13A2 and ATP13A3 cell models.** A.-D. SH-SY5Y ATP13A2 WT-OE and D508N-OE cells were incubated for 2 h with 5 µM of each fluorescently labeled polyamine. E.-H. HMEC-1 ATP13A3 WT-OE and D498N-OE cells were incubated for 30 min with 5 µM of each fluorescently labeled polyamine. The mean fluorescence intensity (MFI) was recorded using a BD Canto II flow cytometer based on the settings used for BODIPY-polyamines and normalized as indicated on the Y axis. Experiments were done four independent times (N=4) in ATP13A2 cell model and three independent times (N=3) in ATP13A3 cell model, 424  
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with technical duplicates. Statistical analysis was done using GraphPad Prism and a two-way ANOVA test with Sidak's multiple comparison test. 431  
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In line with the biochemically confirmed substrate specificity, A2 WT-OE cells present a significantly higher uptake of all SPD and SPM conjugates. A more modest, but significant uptake of the BODIPY-PUT analogs was observed, whereas uptake of FITC-PUT was not significantly different from A2 D508N-OE cells (**Figure 5D, H**). Conversely, A3 WT-OE cells exhibit a higher uptake of BODIPY-labeled PUT and SPD probes than of the fluorescent SPM conjugates (significantly different only for FITC-SPM and FL-A-SPM). The fluorophore FITC seems to prevent PUT recognition in ATP13A3 cells (**Figure 5H**). This is remarkable, because ATP13A3 accepts all other fluorophore-PUT conjugates reported here (**Figure 3E-F, Figure 5E-G**), as well as <sup>14</sup>C-PUT (**Figure 3E, F**). Notably, FL-A-SPD offers the best uptake window for both ATP13A2 (14-fold) or ATP13A3 (105-fold), and therefore emerges as the best probe to follow either ATP13A2 or ATP13A3 transport activity in cells. Conversely, FL-T-PUT exhibits the highest specificity for ATP13A3 (35-fold more uptake in ATP13A3 *versus* ATP13A2 cells), whereas FITC-SPM presents the highest specificity for ATP13A2 (1.8-fold more uptake in ATP13A2 *versus* ATP13A3 cells). Together, our data show that ATP13A2 and ATP13A3 exhibit overlap in substrate specificity, whereas a probe specific structure-activity relationship (SAR) towards ATP13A2 and ATP13A3 has been observed (**Figure 5F, G**). 433  
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## Discussion

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Fluorescently labeled polyamine conjugates represent powerful tools to assess polyamine transport at the cellular level and dissect the mPTS that remains poorly characterized. In this study, we compared the properties of different green fluorescently labeled polyamines to analyze the transport activity in cells of the two housekeeping P<sub>5B</sub>-type ATPases, ATP13A2 and ATP13A3, which emerge as major determinants of cellular polyamine uptake [11,13].

### 1. Comparison of ATP13A2 and ATP13A3 substrate specificity

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We provided the first biochemical evidence that fluorescently labeled polyamines stimulate the catalytic turnover of ATP13A2 indicating that they are genuine substrates of ATP13A2. We previously documented that fluorescent polyamine conjugates enter cells *via* endocytosis [13] and are subsequently transported *via* the activity of ATP13A2 from late endo/lysosomes to the cytosol and further into other compartments, including mitochondria [13,23]. We also showed that ATP13A2 activity responds to a range of fluorescent polyamine analogs indicating flexibility of the substrate binding pocket. We observed the best correlation between the cellular uptake levels of BODIPY-FL-T labeled polyamines (BODIPY-FL-T SPM > SPD > PUT) and the reported apparent affinity of ATP13A2 for unlabeled polyamines (SPM > SPD > PUT; [13]). This correlation is weaker for other polyamine conjugates, indicating that BODIPY-FL-T probes may accommodate best to the substrate binding pockets amongst the here tested fluorophore polyamine analogs.

The radio- and fluorescently-labeled polyamine uptake data for ATP13A3 point to PUT and SPD as preferred substrates of the protein, but not all polyamine probes provide the same results. All fluorescent SPD analogs and the radiolabeled SPD probe are taken up *via* ATP13A3, indicating that SPD truly represents a transported substrate. ATP13A3 also promotes the uptake of radiolabeled PUT, which is in line with the strong uptake of PUT coupled to BODIPY, BODIPY-FL-A or BODIPY-FL-T. These data are in good agreement with our previous study in CHO-MG cells where ATP13A3 complements PUT uptake deficiency [11]. However, no ATP13A3-dependent uptake was observed with FITC-PUT suggesting that the FITC-label disrupts PUT recognition by ATP13A3. Conversely, only FITC-SPM, but not other SPM analogs, are taken up in cells *via* ATP13A3, making it less likely that SPM represents a transported substrate of ATP13A3. This contrasts with a recent study that highlighted both SPD and

SPM, but not PUT, as likely ATP13A3 substrates based on radiolabeled uptake experiments in 477  
pancreatic cancer cells [15]. On the other hand, in CHO-MG cell models, we previously observed that 478  
unlabeled PUT, SPD and SPM competed with BODIPY-PUT uptake *via* ATP13A3, indicating that 479  
PUT, SPD and SPM may all be transported by ATP13A3 [11]. The apparent discrepancy between these 480  
studies may point to cell-type specific properties of ATP13A3 or may reflect differences in the 481  
experimental conditions, such as exposure time, fluorescent polyamine concentrations or cell culture 482  
conditions. Based on the uptake data it remains challenging to pinpoint the precise endogenous 483  
substrates of ATP13A3, and biochemical confirmation on purified ATP13A3 will be required to 484  
establish the relative affinities of ATP13A3 towards the endogenous polyamine species. 485

The overlapping substrate specificities suggest that ATP13A2 and ATP13A3 fulfill - at least in part - 486  
redundant functions in cellular polyamine uptake. This has been confirmed in CHO-MG cells with loss 487  
of ATP13A3 functionality that can be fully rescued not only with ATP13A3, but also with ATP13A2 488  
complementation [11]. It remains unclear whether the redundancy of the isoforms would play a 489  
compensatory or maladaptive role in diseases associated with ATP13A2 or ATP13A3 mutations. 490

Overall, our series of green fluorescent probes are useful tools to assess ATP13A2 and ATP13A3 491  
activity in cells, but not to deduce the endogenous substrate specificities. While both isoforms promote 492  
the uptake of all SPD labeled analogs, FL-A-SPD offers the best uptake window. FL-A-SPD therefore 493  
emerges as an excellent probe to follow the combined cellular activity of ATP13A2 and ATP13A3 with 494  
high sensitivity and uptake potential. 495

## 2. Towards the design of ATP13A2 and ATP13A3 specific polyamine probes 496

ATP13A2 and ATP13A3 both transport SPD pointing to an overlap in substrate specificity. However, 497  
the P<sub>5B</sub> isoforms present differences in their activity towards PUT or SPM analogs, since the fluorophore 498  
and coupling strategies differently affect the uptake of the probes in ATP13A2 *versus* ATP13A3 cell 499  
models. So far, FL-T-PUT emerges as the best polyamine analog to probe ATP13A3 activity selectively, 500  
whereas FITC-SPM may be considered to more selectively follow ATP13A2 activity. Previously, we 501  
described that ATP13A3, but not ATP13A2, mediates cellular toxicity to methylglyoxal bis- 502  
(guanyldiazone) (MGBG), a toxic SPD analog and an inhibitor of polyamine biosynthesis [11]. The 503

overlapping, as well as isoform-specific substrate recognition of ATP13A2 *versus* ATP13A3 is 504  
compatible with a strong, but not complete, conservation of the substrate binding pocket between P<sub>5B</sub> 505  
isoforms. Indeed, most critical residues for polyamine binding and recognition are conserved between 506  
ATP13A2 and ATP13A3 [19], allowing that the polyamine part of the fluorescent probe may enter the 507  
channel-like polyamine binding pocket regardless of the linker or fluorophore. Polyamines bind in their 508  
linear form and the polyamine affinity depends on the number of amine groups and the spacing between 509  
them [19]. The polyamine part of the fluorescent probes may subsequently translocate within the 510  
membrane domain of the transporter following an unresolved path. Since the polyamine binding site 511  
may be too narrow to accommodate a bulky fluorescent headgroup, we hypothesize that the fluorescent 512  
tag may instead move along/in between transmembrane helices or – given its hydrophobic nature- may 513  
stick out within the membrane bilayer. The translocation mechanism of fluorescent polyamines may 514  
resemble the ‘credit card swipe’ mechanism described in lipid flippases of the P<sub>4</sub>-type ATPase family. 515  
An entire phospholipid substrate does not fit within the central transmembrane domain of the flippase, 516  
hence only the polar headgroup region of the phospholipid interacts with the protein, while the fatty acyl 517  
tails slide through the hydrophobic region of the lipid bilayer membrane surrounding the protein [28]. 518  
A similar translocation mechanism for the fluorescently labeled polyamines may explain why ATP13A2 519  
and ATP13A3 translocate fluorescent polyamine analogs with different fluorophores and linkers. The 520  
isoform-specific amino-acid differences in the proximity of the polyamine binding site may contribute 521  
to the SAR by influencing the passage of the bulky fluorescent label. The higher apparent affinity of 522  
ATP13A2 for BODIPY-SPM as compared to SPM or other fluorescent SPM probes may therefore be 523  
possibly explained by specific interactions between BODIPY and surrounding residues in the 524  
transmembrane domain. Besides BODIPY- and FITC-labeled polyamines, also nitrobenzoxadiazolyl 525  
(NBD)- [29], anthracene- [30] and indotricarbocyanine-labeled polyamines [31] have previously been 526  
developed to monitor the activity of the mPTS. Also the NBD-labeled spermine is most likely taken up 527  
*via* ATP13A3 [15], but it remains to be tested whether all these probes are indeed transported *via* 528  
ATP13A2 and/or ATP13A3. 529

### 3. Distinct polyamine uptake kinetics in ATP13A2 and ATP13A3 cells 530

Although cell type specific differences may play a role, we found that ATP13A3 mediates stronger and 531  
faster uptake of radio- and fluorescently labeled polyamines than ATP13A2. ATP13A3 contributes 532  
much more to cellular PUT and SPD uptake than ATP13A2. ATP13A3 may also contribute more to 533  
cellular SPM uptake, since the uptake of fluorescent SPM analogs in ATP13A2 and ATP13A3 cells is 534  
comparable despite the shorter treatment time in ATP13A3 models. The expression of ATP13A3 in 535  
earlier compartments of the endosomal system as well as specific biochemical properties ( $K_m$ ,  $V_{max}$ ) 536  
may explain the more dominant and faster polyamine uptake via ATP13A3 over ATP13A2. 537

Our results show that with a proper choice of the polyamine probe, concentration and short treatment 538  
time, it is possible to exclusively measure ATP13A3 dependent uptake in cells expressing both 539  
ATP13A2 and ATP13A3. However, it remains much more challenging to dissect the ATP13A2 activity 540  
in the background of ATP13A3, since ATP13A2-specific probes have not yet been identified. In 541  
addition, endocytosed probes first meet ATP13A3 in the earlier endosomal compartments from which 542  
they can be exported by ATP13A3 before reaching ATP13A2 in the late endo/lysosomal compartments. 543

Importantly, the distinct substrate specificities and uptake kinetics of ATP13A2 as well as the 544  
contribution of the fluorophore and linker to substrate recognition and affinity indicate that designing 545  
ATP13A2-specific probes may be feasible. With isoform specific probes we would be able to follow 546  
transporter specific uptake in various cell types and physiological conditions. In disease models we may 547  
pick up isoform-specific transport deficiencies and map possible compensatory changes in the activity 548  
of other  $P_{5B}$  ATPase isoforms. Moreover, the broad range of fluorescent polyamines that enter cells *via* 549  
ATP13A2 and/or ATP13A3, suggest that polyamines may be considered as vehicles to take up drugs or 550  
other molecular cargo [32]. For instance, polyamine-antracene conjugates have previously been 551  
designed to target cancer cells with an increased polyamine uptake system [33] or *Plasmodium* 552  
*falciparum* parasites [34]. Structural studies of  $P_{5B}$ -ATPases with bound polyamine conjugates as well 553  
as functional analysis of other polyamine conjugates will lead to a better understanding of the SAR, 554  
which will facilitate the design of isoform-specific strategies. 555

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#### 4. Green fluorescent probes with improved synthesis and properties

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The novel green fluorescent polyamine probes described here follow two different synthetic strategies. 559  
Our original synthesis encompassed a click chemistry-based approach between an azide-functionalized 560  
polyamine and an alkyne-labeled BODIPY fluorophore. Here, we also explored amide and 561  
isothiocyanate coupling to a properly protected polyamine analog with one single free amino group. 562  
Conveniently, such protected polyamines are intermediates in our original probe synthesis, and therefore 563  
shorten the route by one step. We found that the amide coupling strategy gave rise to the most promising 564  
fluorescent polyamine series (BODIPY-FL-A series; **3a-c**). As many more fluorophore oxysuccinimide 565  
esters are available from commercial sources, we expect that other analogs can be readily synthesized, 566  
opening the way to further improvement of the fluorescent polyamine properties. 567

Here, we found that the new probes, despite having a lower affinity than our previously reported 568  
fluorescent polyamine probes, display a higher cellular uptake at 5  $\mu$ M. It indicates that the new probes 569  
(except for FITC-PUT, **4c**) are superior substrates for ATP13A2 and ATP13A3, and underlines the 570  
importance of the nature of the fluorophore and the coupling strategy between the fluorophore and the 571  
polyamine. 572

In conclusion, the here reported fluorescent polyamine probes are attractive for the study of polyamine 573  
uptake by ATP13A2 and APT13A3, and the BODIPY-FL-A series emerged as the most efficient of the 574  
tools described here. 575

576

**Author Contributions:** “Conceptualization, P.V. and S.V.; methodology, M.H., N.J., J.C., M.A. and 577  
C.VdH; validation, M.H., N.J. and J.C.; formal analysis, M.H.; resources, C.VdH, J.C., R.V. and S.V.; 578  
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**Supplementary Materials:** Figure S1: Characterization of ATP13A2-expressing SH-SY5Y cell 588  
models; Figure S2: Characterization of ATP13A3-expressing HMEC-1 cell models; Figure S3: 589  
BODIPY-PUT and BODIPY-SPD uptake in HMEC-1 cells overexpressing ATP13A3 WT or D498N; 590  
Figure S4. Full structures of the different green fluorescent polyamine conjugates used in the study and 591  
biochemical characterization of spermine conjugates; Figure S5: Synthesis of putrescine precursors, 592  
reagents and conditions; Figure S6: Synthesis of spermine and spermidine precursors, reagents and 593  
conditions; Figure S7: Conjugation of the fluorophore to the relevant protected polyamine; Figure S8: 594  
Boc-deprotection of the relevant Boc-protected probe; as well as detailed synthesis procedures, 595  
<sup>1</sup>H-NMR, <sup>13</sup>C-NMR, HRMS and LC-purity data. 596

**Data Availability Statement:** All datasets generated or analyzed in this study can be found through the 597  
Zenodo depository, reserved doi: 10.5281/zenodo.7434965. All experimental protocols can be found on 598  
protocols.io. 599

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Millipore Corporation an exclusive license for the commercialization of BODIPY-PUT, BODIPY-SPD 607  
and BODIPY-SPM described in this study. 608

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