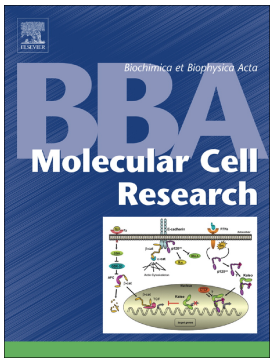

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P5B-ATPases in the mammalian polyamine transport system and their role in disease

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Abstract

Polyamines (PAs) are physiologically relevant molecules that are ubiquitous in all organisms. The vitality of PAs to the healthy functioning of a cell is due to their polycationic nature causing them to interact with a vast plethora of cellular players and partake in numerous cellular pathways. Naturally, the homeostasis of such essential molecules is tightly regulated in a strictly controlled interplay between intracellular synthesis and degradation, uptake from and secretion to the extracellular compartment, as well as intracellular trafficking. Not surprisingly, dysregulated PA homeostasis and signaling are implicated in multiple disorders, ranging from cancer to neurodegeneration; leading many to propose rectifying the PA balance as a potential therapeutic strategy. Despite being well characterized in bacteria, fungi and plants, the molecular identity and properties of the PA transporters in animals are poorly understood. This review brings together the current knowledge of the cellular function of the mammalian PA transport system (PTS). We will focus on the role of P5B-ATPases ATP13A2-5 which are PA transporters in the endosomal system that have emerged as key players in cellular PA uptake and organelle homeostasis. We will discuss recent breakthroughs on their biochemical and structural properties as well as their implications for disease and therapy.

1. Polyamines in mammalian cells

In the late 1600s, polyamines (PAs) made their debut in literature when crystalline structures were observed in human semen samples by Antoni van Leeuwenhoek. Years later, these crystals were identified and appropriately named as the PAs spermine (SPM) and spermidine (SPD) [1]. Once their chemical structures were elucidated, PAs were defined as small positively charged organic molecules, having two or more amino groups, that are found in all living organisms and are full protonated under physiological conditions [2]. Though the list of naturally occurring PAs is long, the most common ones are the tetramine SPM, triamine SPD and diamine putrescine (PUT) which happen to be the main PAs synthesized in mammalian cells [3]. Other PAs include agmatine (AGM) which, for many years, was assumed to only be synthesized in plants, insects, bacteria and invertebrates as these organisms express functional arginine decarboxylase for the formation of AGM from arginine. However, AGM was also detected in rat brains, followed by a later study clarifying that human cells do express active agmatinase to degrade AGM, but do not express arginine decarboxylase to synthesize it [4] [5]. Cadaverine is yet another naturally occurring PA that is well-characterized in bacteria and plants, but is scarcely found in other species [6].

Due to their positive charge, PAs bind to a diverse range of negatively charged molecules such as DNA, RNA, phospholipids, certain proteins (including ion channels) and ATP; resulting in the majority of PAs found in a bound rather than a free state [7]. Because PAs interact with numerous macromolecules in the cell, they are vital in regulating a vast range of cellular pathways involved in, amongst other, cell signaling, gene expression, protein translation, cell proliferation and differentiation, regulation of cell death as well the maintenance of the extracellular matrix [8]. For instance, PAs interact with DNA and cause its condensation by forming a bridge through electrostatic interaction between the DNA *via* the phosphate charges [9]. Different PAs have also been proposed to interact with DNA, whether the interaction occurs on the major or minor strand, inter or intra-strand; in their own specific ways [10]. Moreover, PAs can stabilize nucleosomes and affect chromatin remodeling most likely by affecting histone acetylation [11]. PAs also protect DNA from breaks in its double strand caused by other DNA-damaging agents such as hydroxyl ions [11,12]. At the RNA level, PAs bind to tRNA, mRNA and rRNA influencing their secondary structure and functionality. In fact, most of the PAs found in the cell are actually bound to RNA [13] [14].

Protein synthesis is also affected by PAs. A well-known example is SPD contributing to the formation of hypusine, a post-translational modification of the eIF5A translation factor which is necessary for its activation. Hypusinated eIF5A is important for mRNA turnover and to function as a translation initiation factor [15]. Most importantly, eIF5A plays a role in the translation of the mRNA of proteins that contain polyproline stretches, by facilitating the mRNA's interaction with ribosomes [16].

Modulating the functionality of ion channels also falls under the numerous effects of PAs. A wide range of channels, such as glutamate receptors *e.g.* N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, Transient Receptor Potential Canonical Channels (TRCP) and Inward Rectifier Potassium (Kir) channels are all influenced by PAs in one way or the other - often involving the direct binding of the PAs to the channels [15] [17,18].

The roles of PAs on cell physiology and signaling are very broad, ranging from regulation of autophagy, cell proliferation and differentiation to antioxidant effects and heavy metal quenching. This has been widely studied and covered in great detail in numerous works. For further exploration of the topic, one could read the Polyamine Cell Signaling book edited by Jian-Ying Wang and Robert A. Casero, Jr. [19].

PA homeostasis is tightly regulated and involves PA synthesis and degradation, as well as cellular uptake and secretion. Not surprisingly, PA homeostasis is disturbed and implicated in multiple diseases, such as cancer and neurodegeneration. While PA metabolism pathways are well characterized in mammalian cells, little is known about the molecular identity and physiological roles of the PA transporters involved in cellular uptake or secretion. In this review, we will focus on the role of the recently identified P5B-ATPase group of PA transporters that have emerged as key members of the long-sought-for and uncharacterized mammalian PA transport system (PTS), and are genetically implicated in cancer, cardiovascular and neurological diseases.

2. How do mammalian cells source and regulate their polyamines?

Evidently, since PAs partake and play a crucial role in an extensive gamut of biological processes, and have an impact on cell proliferation, their levels are tightly maintained. Mammalian cells get their supply of PAs through two main routes: **(a)** endogenous synthesis through the PA metabolic pathway and **(b)** PA uptake from exogenous sources such as food and the gut microbiome [20]. In rats, it was estimated that 47% of the PA body content is taken up from the diet and 10% from the intestinal microbiome, whereas 41% originates from *de novo* synthesis [21]. Once within the cell, PAs are subjected to the PA metabolic pathway where they can be interconverted, redistributed, secreted or degraded.

2.1. Polyamine synthesis and metabolism

As shown in Fig. 1, the endogenous synthesis of PAs in the cell begins with the generation of ornithine from the metabolism of arginine by the enzyme arginase in the urea cycle. Next, following a rate-limiting step, ornithine is decarboxylated by an enzyme called ornithine decarboxylase (ODC) to generate PUT. Following on, the synthesis of the larger SPD and SPM PAs necessitates the generation of aminopropyl moieties, which happens in the methionine salvage pathway by the decarboxylation of S-adenosylmethionine (SAM) involving the action of S-adenosylmethionine decarboxylase (AMD). These aminopropyl moieties are then used by SPD synthase and SPM synthase enzymes to generate SPD and SPM from PUT respectively [22]. Following synthesis, the PAs can be degraded and/or reconverted into smaller forms by a two-step process that first involves the formation of acetylated intermediates by the SPD/SPM N^1-acetyltransferase (SAT1) enzymes which reduces the positive charge of the PAs and thereby their potency to bind to other molecules like DNA or RNA [6]. The acetylation step is followed by consequent oxidation by PA oxidase (PAO), a flavin adenine dinucleotide-dependent enzyme, which re-converts the intermediates to SPD and PUT respectively. Moreover, the back-conversion of SPM to SPD can also occur by the SPM oxidase (SMOX) enzyme, without the formation of an acetylated

intermediate [2]. Though the interconversion by PAO yields a 3-acetamido propanal aldehyde as well as hydrogen peroxide (H_2O_2), the burden of the oxidative insult

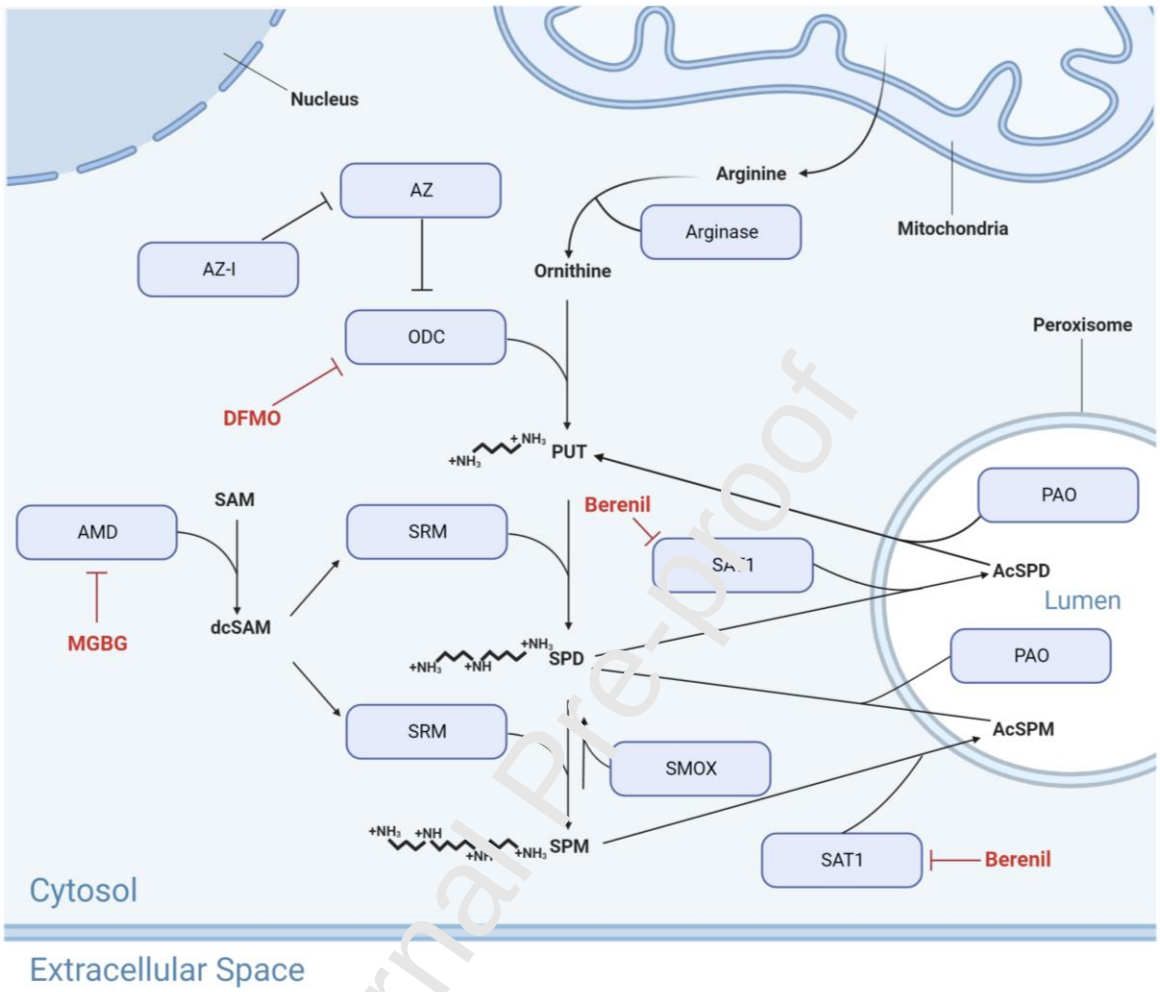

Figure 1. PA biosynthesis and metabolism. Enzymes involved in the synthesis and catabolism of PAs are shown in blue boxes. Chemical modulators used to inhibit different steps of PA biosynthesis are shown in red. Abbreviations: ODC - ornithine decarboxylase, AZ - Antizyme, AZ-I - antizyme inhibitor, AMD - S-adenosylmethionine decarboxylase, SRM - spermidine/spermine synthase, SMOX- spermine oxidase, SAM - S-adenosylmethionine, dcSAM - decarboxylated S-adenosylmethionine, AcSPD - acetyl-Spermidine, AcSPM - acetyl-Spermine, dcSAM - decarboxylated S-adenosylmethionine, SAT1 - SPD/SPM N^1-acetyltransferase, PAO - polyamine oxidase, PUT - putrescine, SPD - spermidine, SPM - spermine.

incurred upon the cell is reduced as this step takes place within the peroxisomes [3]. The synthetic and catabolic pathways in PA metabolism keep a delicate balance of cellular PA concentrations. Moreover, back-conversion between PA species not only modulates the relative PA availability, but the acetylated intermediates generated in the process are also more readily exported out of the cell making it yet another mechanism in maintaining overall intracellular PA pool [23].

2.2. Regulation of polyamine synthesis and metabolism

Adding to the complexity of the PA metabolic pathway, the enzymes involved in the synthesis and catabolism of PAs are themselves subject to heavy regulation. Firstly, the generation of PUT from ornithine by ODC is majorly regulated as it is the first rate-limiting

step in PA synthesis. ODC activity is regulated more by the amount of ODC protein present rather than modulation of ODC's catalytic activity. Active ODC, which has a very short half-life (up to 2 hours [24]) and high turnover, requires a homodimeric form as the active sites are located at the interaction sites between the two monomers. However, a protein called antizyme (AZ) binds to ODC monomers, which not only hinders the dimerization of ODC, but also causes ubiquitin-independent ODC degradation *via* the 26S proteasome reducing the half-life to a few minutes [3,24]. Interestingly, AZ translation is also affected by intracellular PA levels, which, when in high concentrations, cause a frameshift in AZ mRNA that skips an internal stop codon leading to more AZ expression and ODC inhibition [25]. AZ itself is also regulated by AZ-inhibitor (Fig. 1B), which binds to AZ with a higher affinity than that of ODC thereby preventing AZ from interacting with ODC, which is then prevented from being degraded [26]. Moreover, ODC's expression is regulated by the Myc/Max transcription factors, growth factors such as the epidermal growth factor, amino acids like glutamine and asparagine, oncogenes, hormones and even PAs [27] [28].

Another rate-limiting enzyme involved in the PA metabolic pathway that is heavily regulated is AMD which is required for the supply of the aminopropyl moieties necessary for the synthesis of SPD and SPM from PUT. In order for AMD to be activated, the pro-enzyme needs to undergo self-cleavage into two subunits – a process that is stimulated by PUT [29]. Moreover, AMD is also subject to high turnover *via* polyubiquitination and consequent degradation by the 26S proteasome, which is increased when SPD and SPM levels are high [29]. Additionally, high SPD and SPM levels negatively affect the AMD transcription through a poorly understood mechanism that may involve a PA-responsive element in the AMD gene [30].

Finally, the catabolic enzyme SAT1 is also regulated through several PA-dependent mechanisms. For example, the SAT1 gene has a PA-responsive element in the 5' region that, in some cell-types and in response to high PA levels, promotes the binding of certain transcription factors that promote SAT1 transcription [31]. Moreover, high PA levels improve SAT1 mRNA stability and can also promote alternate splicing of SAT1 RNA favoring splice variants that are less likely to be degraded *via* nonsense-mediated RNA decay thereby resulting in higher SAT1 activity [32]. High PA concentrations further increase SAT1 translation by removing mechanisms that inhibit its translation [6].

While it is evident that endogenous PA synthesis and metabolism is tightly controlled through feedback loops to keep the intracellular PA pool from fluctuating too much, synthetic chemical modulators (Fig. 1) can also affect different parts of the metabolic pathway by blocking different enzymes in PA synthesis (difluoromethylornithine (DFMO), methylglyoxal bis guanylhydrazone (MGBG)) and degradation (Berenil). Synthetic modulators are not only useful tools in understanding the PA metabolic pathway, but have also been proposed as useful therapeutic agents in disorders driven or exacerbated by a disturbed PA homeostasis [22]. However, cells can adapt to the loss of PA synthesis by stimulating the PA uptake routes [33] [34], which will be explained in the next section.

2.3. The mammalian polyamine transport system (PTS)

The endogenous PA synthesis is not the only mechanism of how mammalian cells source their PA supply. The other route is via PA transporters that govern PA uptake from the

extracellular milieu at the level of the plasma membrane as well as their local exchange at the organelle level. Counterintuitively, even though PAs are widely and ubiquitously present in all living organisms, having a common PTS is far from true. The pioneering studies regarding PA regulation and transport systems have been carried out by Tabor et al. (summarized in a special issue [35]) mainly in the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. Later on, several groups started to elucidate components of the mammalian PTS [36] [6] [37-41]. However, a comprehensive picture of the mammalian PTS and its molecular components is still lacking, but recent studies have led to some important breakthroughs. From countless studies attempting to characterize the mechanism of the mammalian PTS, various potential PA uptake routes, mentioned below, have been proposed but without a unifying narrative tying it all together. This makes a strong case for there not being one single PA uptake system, but more likely multiple transporters belonging to the mammalian PTS with all of them working in synergy. The main mechanisms of PA import into the cell that have been proposed are **(a)** transport through the plasma membrane followed by vesicular sequestration (section 2.3.1.); or **(b)** endocytic routes that are mediated by glypicans and/or caveolae (section 2.3.2.) [39]. In the next sections, we will summarize the status in the field regarding putative and biochemically confirmed PA transporters that may be part of the mammalian PTS. So far, transporters belonging to the Solute Carrier (SLC), ATP-Binding Cassette (ABC) and P5B-ATPase transport families have been highlighted as candidate PA transporters, but only a few of them have been biochemically or structurally validated (Table 1)

2.3.1. Polyamine Transport *via* the plasma membrane

One candidate PA transporter is an ABC transporter, whereas most of the proposed PA transporters fall into the SLC superfamily, which is the second largest group of membrane proteins consisting of 458 transporters [42]. The members of the SLC family transport a wide spectrum of ligands ranging from amino acids, ions and sugar molecules, and up to eight *SLC* genes have so far been implicated in PA transport [43] [36]. A short description of the transporters is provided below and summarized in Table 1.

ABCB1, a member of the ATP-binding cassette transporter superfamily, encodes for the multidrug resistance transporter 1 (MDR1) [44]. Members of this superfamily have been shown to mediate the transport of a broad range of substrates across membranes such as amino acids, peptides and metal ions [44]. More precisely, MDR1 belongs to the B subfamily and is so far the only mammalian ABC transporter linked to PA transport. Using Chinese Hamster Ovary (CHO) cells and the PA transport deficient CHO-MG cells transfected with mouse MDR1, Aziz et *al.* highlight that the multidrug resistance activity of MDR1 requires active PA transport [45]. It is worth noting that CHO-MG cells are a commonly used cell model with PA transport deficiency [40]. These cells were generated through random mutagenesis to become resistant to the toxic AMD inhibitor MGBG, which is also taken up in cells by the PA transporters [46].

Three of the candidate SLC PA transporters are the organic cation transporters (OCT) – 1-3 that are encoded for by the ***SLC22A1-3*** genes respectively [47]. While OCT1 and 2 are mainly expressed in the liver and kidneys, OCT3 has a broader expression profile [36]. ^{3}[H]-AGM uptake was enhanced in Human Embryonic Kidney (HEK) cells overexpressing OCT2 and OCT3, but not OCT1, as compared to non-transfected cells, while ^{3}[H]-PUT uptake

remained unchanged [48]. OCT2 and OCT3 were proposed as bidirectional AGM transporters at the plasma membrane, which was partially confirmed by Winter *et al.*, since OCT1 and OCT2 were described as both AGM and PUT importers [49]. Using mouse or rat isoforms for the *SLC22A1-3* genes, SPD and SPM were added to the list of transported ligands of OCT1-3, with SPM being the PA with the lowest rate of transport [50] [47]. However, other studies in multiple cell lines overexpressing OCT1-3, including HEK cells, yielded contradictory results [51] [36]. Therefore, no clear involvement of these SLC members in the mammalian PTS can be ascertained.

SLC3A2 has been proposed as a plasma membrane diamine transporter (DAX) mediating PUT export and arginine uptake in human colorectal carcinoma (HCT116) cells, suggesting a PUT/arginine exchange function [52], however, direct biochemical evidence on purified SLC3A2 is lacking. SLC3A2 knockdown leads to a decreased export of PUT and monoacetylated SPD [52]. Interestingly, in DFMO treated HCT116 cells, SLC3A2 may mediate the uptake of extracellular PUT indicating its ability to work in reverse mode according to the intracellular PA requirements [37]. Since SLC3A2 regulates the uptake of PAs in human neuroblastoma [53] and diffuse intrinsic pontine glioma, SLC3A2 is considered as an interesting therapeutic target [34], possibly in combination with DFMO. Moreover, arginine is metabolized to form ornithine (*via* arginase), which is then used to make PUT (Fig. 1); or to produce nitric oxide (NO) (*via* NO synthase), which is an important regulator of PA uptake as will be described further below (see section 2.3.2.) [38]. Thereby, by modulating arginine uptake, SLC3A2 may regulate the intracellular PA pool by not just promoting PA synthesis, but also by promoting PA uptake through the NO pathway. Since SLC3A2 may form a complex with SAT1 at the plasma membrane in HCT116 cells, SLC3A2 is also thought to facilitate the export of acetylated PAs [52]. However, it is worth noting that SLC3A2 is part of the heterodimeric amino acid transporters (HATs) by binding to at least six L-type amino acid transporters (LATs, SLC7A5-8/10/11) (reviewed in [54]). HATs are described as plasma membrane exchangers with a broad range of substrates from neutral, aromatic, cationic and neutral amino acids, depending on the heterodimer formed. HATs formed with SLC3A2 may therefore not only transport PAs directly, but also amino acids that indirectly affect PA homeostasis.

SLC12A8A, encoding for the cation-Cl^- cotransporter 9, isoform a (CCC9a) is widely expressed in mammals and located in intracellular compartments, but some splice variants manage to reach the plasma membrane [55]. Over-expression of SLC12A8A in HEK cells was shown to increase uptake of PAs (mainly SPD) as well as amino acids [55]. The uptake of PAs was enhanced by the acidic amino acids aspartic and glutamic acids, but was Na^+, Cl^- and K^+ independent [55].

SLC47A1, encoding for the multidrug and toxins extrusion transporter 1 (MATE1), is a H^+/cation antiporter located mainly in the kidneys and liver [49]. Over-expression in HEK cells showed a pH-dependent increase in AGM transport [49]. Interestingly, though ^{3}[H]-PUT was not considered as a substrate of MATE1, PUT, SPD and SPM supplementation partially inhibited MATE1-mediated AGM uptake. Additional results from this study further propose a model in which MATE1 would act in concert with OCT2 to regulate intracellular levels of AGM [49].

SLC22A16 encodes for the carnitine transporter 2 (CT2) which is strictly expressed in adult human testes [56], bone marrow, and fetal liver [57]. Initially, CT2 was characterized in *Xenopus laevis* oocytes as a bidirectional transporter of ^{14}[C]-L-carnitine [56]. Besides a higher uptake of ^{14}[C]-L-carnitine in HTC116 cells overexpressing human CT2, uptake of ^{14}[C]-SPD and bleomycin-A5 (an antineoplastic agent holding a SPD moiety) has also been observed, broadening the spectrum of substrates for CT2 to potentially include PAs [58].

While all the above-mentioned transporters are putative PA transporters, so far only one member of the SLC superfamily, **SLC18B1**, has been biochemically validated as such [59]. SLC18B1 is a vesicular PA transporter (VPAT) with a widespread expression in humans with its mRNA levels being highest in the lung, placenta and adrenal gland [60]. Using purified human SLC18B1 protein reconstituted in proteoliposomes, Hiasa *et. al* biochemically demonstrated that SLC18B1 actively transported SPD and SPM, which bind most likely in two different binding sites, using H^+ ions as counter transport ions [59]. Alongside the two PAs, SLC18B1 is also hypothesized to transport serotonin, histamine and AGM [59,60]. Interestingly, SLC18B1 is highly expressed in the brain in vesicles of astrocytes located in the hippocampus, where it regulates cellular PA uptake and availability for subsequent release as potential gliotransmitters. Astrocytes have a central role in PA storage in the brain and in controlling synaptic plasticity and regulation of neuronal activity [59]. Not surprisingly, SLC18B1 KO in mice present a reduction in brain PA levels, impaired cognition and functioning of postsynaptic glutamate receptors [61].

2.3.2. Polyamine uptake through endocytosis and P5B-ATPases

Aside from the above-mentioned transporters potentially playing a role in PA uptake, PAs are thought to enter the cell through caveolin-mediated endocytosis or glypican-mediated endocytosis [39]. Caveolae are cholesterol and sphingolipid rich invaginations in the plasma membrane and are one of the players involved in endocytosis [62]. **Caveolin-1** (Cav1) binds to cholesterol in caveolae and has been shown to be a negative regulator of caveolin-mediated endocytosis by stabilizing the plasma membrane association of caveolae and thereby preventing caveolae internalization [62]. Interestingly, when Cav-1 is knocked-out (KO) in mice, the V_{max}, but not the K_m of PA uptake was increased - demonstrating that the uptake frequency, but not the binding affinity, was increased [37].

PA uptake from the extracellular milieu also relies on NO as well as on glypicans, which are a family of cell-surface proteoglycans that are embedded in the outer leaflet of the plasma membrane thanks to a glycosylphosphatidylinositol anchor [63]. Glypicans with heparan sulfate (HS) chains capture PAs – with the highest affinity for SPM (Fig. 2) [64]. Once PAs bind to HS chains on glypican-1, the complex becomes internalized, resembling receptor-mediated endocytosis. When glypicans reach the later compartments of the endosomal system, the HS chains are then cleaved by NO, which releases the PAs from the glypican complex [38]. Several observations have placed NO as a vital player in PA uptake. For instance, PA depletion increases the NO-sensitive regions in HS chains of glypicans, induction of NO increases PA uptake and cells deficient in NO release are also deficient in PA uptake [38]. Interestingly, in Cav-1 KO mice, the expression of NO synthase increases, which may compensate for the negative impact of Cav-1 KO on endocytosis [37].

In the midst of all the proposed routes of PA uptake outlined above, a new family of transporters have emerged as key players in the mammalian PA uptake pathway that could work in conjunction with the endosomal PA uptake pathways previously proposed. Two ubiquitous transporters called **ATP13A2** and **ATP13A3** belonging to the family of **P5B-ATPases**, have been shown to be involved in PA uptake [41] [40]. P5B-ATPases came into the spotlight as putative PA transporters by screening for genes that prevent norspermidine toxicity as well as norspermidine mediated suppression of RNA interference in *Caenorhabditis elegans* [65]. In the screen *catp-5*, one of the three *C. elegans* P5B-ATPase genes (see section 3.) which modulates PA uptake and genetically interacts with ODC, was identified [65]. Also, in *S. cerevisiae*, negative genetic interactions were found between the ATP13A2 ortholog YPK9, SPE1 (ODC), SPE2 (AMD) and SPE3 (SPD synthase) (www.thebiogrid.org). In mammalian cells, it was later demonstrated that overexpression of the human ATP13A2 isoform promotes paraquat toxicity (a herbicide and PA analog), as well as cellular PA uptake [66,67]. With a combination of ATPase and transport assays involving purified human protein, ATP13A2 has since been biochemically validated as a PA transporter with highest affinity to SPM and SPD. ATP13A2 exerts PA transport from the luminal to cytosolic environment [41]. Its presence in late endo-/lysosomes promotes sequestration of endocytosed PAs to the cytosol, thereby contributing to cellular PA uptake and overall PA content. Conversely, blocking endocytosis prevents the cellular PA uptake through ATP13A2. The export of PAs from the lysosome to the cytosol ensures the healthy functioning of the lysosomes by preventing lysosomal PA accumulation. In the absence of ATP13A2, lysosomes alkalize and rupture, leading to lysosomal dependent cell death. In addition, ATP13A2 loss lowers the availability of PAs in the mitochondria, leading to mitochondrial dysfunction and elevated oxidative stress pointing to a dual function of ATP13A2 in cellular PA uptake on the one hand *versus* organelle homeostasis and PA distribution on the other hand [41]. This discovery further outlined the endo-/lysosomal system, preceded by endocytosis, as a key route for PA uptake in mammalian cells.

ATP13A3 and other mammalian members of the P5B family of ATPases, **ATP13A4** and **ATP13A5**, are predicted to be structurally similar to ATP13A2 (Fig. 4) and also share the conserved sequence within the substrate binding region hinting that all mammalian P5B-ATPases may be involved in PA transport [68]. While cellular or biochemical evidence confirming ATP13A4 and ATP13A5 as members of the PTS is currently unavailable, ATP13A3, on the other hand, has been implicated in the PTS. Indeed, genetic mutations in ATP13A3, which led to a disturbed mRNA and protein expression of ATP13A3, are responsible for the impaired PA uptake in CHO-MG cells [40]. Interestingly, overexpression of ATP13A3 or ATP13A2 complements the defective PTS in CHO-MG cells, pointing to overlap in PA transport function, and clearly establishing ATP13A3 as part of the mammalian PA uptake pathway [40]. However, the low MGBG sensitivity of CHO-MG cells was partially restored by ATP13A3 expression, but not ATP13A2, indicating that P5B-ATPases also present unique properties [40]. Indeed, as will be discussed further in the review, the different P5B-ATPases reside in different intracellular compartments of the cell and therefore may fulfill different roles based on the local PA homeostasis and also the presence of regulatory players.

3. What are P5B-ATPases?

The P5B-ATPases ATP13A2-5 are members of the P-type class of ATPases, which consists of five subfamilies (P1-5), which can then be further divided into subgroups (A, B, C *etc.*). These phylogenetic divisions correlate to the specific substrates of the different P-type ATPases and carry characteristic and highly conserved signature motifs in the fourth transmembrane (M4) region [69-71]. P-type ATPases are found in all living organisms, and are called as such since they undergo reversible auto-phosphorylation during the ATP-coupled transport of molecules or solutes against the thermodynamically favored electrochemical gradient across membranes [69]. To do this, all P-type ATPases, including P5B-ATPases, undergo a series of conformational changes, following the Post-Albers mechanism (Fig. 3), which serves to prevent the back-flow of their substrates [72].

The major subfamilies of P-type ATPases are:

- P1: Heavy metal transporters in eukaryotes
- P2: Transporters of inorganic ions such as Ca^{2+}, Na^{+}, K^{+} and H^{+}
- P3: Proton pumps in plants and fungi
- P4: Lipid flippases transporting lipids from the exoplasmic to cytoplasmic membrane leaflet
- P5: Transported substrates remained elusive until recently.

P5-ATPases are found in all eukaryotic genomes, but are absent in bacterial genomes [73] [74], and are grouped in two well-separated clusters corresponding to the P5A and P5B-ATPases [70] [74]. The separation of P5A and P5B-ATPases from a common P-ATPase ancestor must have occurred early in eukaryotic evolution. Exactly one P5A (ATP13A1 in humans) and at least one P5B isoform are found in all eukaryotic genomes, except for excavates, entamoebas and land plants, which have lost the P5B genes [73]. Phylogenetic analysis revealed that the invertebrate P5B-ATPases cluster in a different clade than the vertebrate isoforms, strongly suggesting that their functions may have diverged [68]. While most invertebrates only contain a single P5B isoform, which shares features with ATP13A2 and ATP13A3, multiple P5B isoforms exist in higher vertebrates (four in humans, ATP13A2 to 5) and in some invertebrate lineages where independent gene duplications occurred (*e.g.* three genes in *C. elegans*, *catp-5 to 7*) [68]. ATP13A2 and ATP13A3 duplicated in early vertebrates from a common P5B ancestor gene in the animal deuterostome lineage [68]. The long evolutionary separation introduced a significant sequence diversity, which may point to a more diverged function between ATP13A2 and ATP13A3 [68]. The mammalian ATP13A4-5 isoforms emerged from two relatively recent and successive gene duplications from ATP13A3 in higher vertebrates with the rise of lobe-finned fish and amphibiae, indicating that the cellular functions of ATP13A3-5 may be closely related [68].

While the phylogenetic analysis clearly shows that the P5-ATPases are subdivided into P5A and P5B groups, members of P5A and P5B-ATPases exhibit similar biochemical properties with spontaneous auto-phosphorylation apparently in the absence of a transported substrate. However, the P5A and P5B topology strikingly differs (respectively twelve *vs* ten transmembrane regions), as well as the organization of the N-terminal domain (NTD; membrane spanning hair-pin *versus* membrane embedded) and intracellular localization (ER *versus* endosomal targeting), pointing to clearly distinct functions. Moreover, the P5A M4

region contains a PP(E/D)xPx(E/D) motif holding two highly conserved negatively charged amino acid residues, whereas the M4 region in the P5B sequences are characterized by a PP(A/V)xP(A/V)x motif with two conserved hydrophobic residues [73,74]. The presence of negative charges in the M4 helix of P5A compared to the corresponding hydrophobic residues in P5B already points to different substrate specificities of both subgroups, which have recently been revealed by biochemical and structural studies, *i.e.* polypeptides for P5A-ATPases and PAs for P5B-ATPases [75] [41].

Cryo-EM studies revealed that P5A-ATPases are most likely transmembrane protein dislocases that are part of a quality control system that removes wrongly-inserted and/or wrongly-targeted tail-anchored proteins from the ER membrane [75]. This general ER quality control function explains the plethora of phenotypes related to the secretory pathway that have previously been reported in models with P5A dysfunction [76]. Conversely, all P5B-ATPases most likely function as PA transporters within the PTS, which will be further discussed in this review. Striking similarities, but also unique differences have been reported by comparing the P5A and P5B-ATPase structures (section 3.2.1.3.), confirming their common evolutionary relationship, yet also allowing for functional diversification.

3.1. Expression profiles and subcellular localization of P5B-ATPases

Mammalian cells seem to express multiple PA transporters that present differences in tissue localization, subcellular targeting, substrate specificity and/or regulation pointing to a cell-type specific PTS (Table 1). P5B-ATPases are targeted to various, but overlapping compartments of the endo-/lysosomes. Furthermore, while some P5B isoforms are ubiquitously expressed, others display a restricted tissue distribution. Differences in (sub)cellular and tissue distribution indicate that the mammalian P5B isoforms, although they may have evolved similarly, are more dedicated to specialized tissue and/or compartment specific functions. Note that the P5B protein expression has not been extensively mapped due to the lack of good quality antibodies and little information from MS-based methods, most likely related to their low abundant expression.

3.1.2. Tissue distribution

Since ATP13A2 and ATP13A3 are both expressed in most human tissues they probably fulfill housekeeping functions. However, tissue differences in expression levels indicate that they may serve organ-specific functions as well [68,77]. Highest RNA expression levels of ATP13A2 and ATP13A3 are found in the brain and liver, respectively [68,77] (https://www.proteinatlas.org). On the other hand, ATP13A4 and ATP13A5 show a more limited tissue distribution suggesting these isoforms serve tissue-specific functions [68,77]. ATP13A4 and ATP13A5 are expressed in the brain and epithelial glandular cells, which are mainly associated with the lung (ATP13A4/ATP13A5), mouth (ATP13A4), nose (ATP13A5), epididymis (ATP13A4) or breast (ATP13A5) [68] (https://www.proteinatlas.org).

During development in mice, no differential expression is observed for ATP13A2, but ATP13A3-5 are developmentally regulated with high expression of ATP13A3 during the early embryonic stages, whereas ATP13A4 and ATP13A5 mainly express postnatally [68]. Moreover, in the human brain, ATP13A4 levels peak postnatally at late neurogenesis

whereas ATP13A3 expression is highest prenatally [78]. Expression of ATP13A2 and ATP13A5 in the human brain appears more constant over development [78].

3.1.3. Cell type specific expression

To compare expression of P5B-ATPases among cell types of different tissues, we consulted Tabula Muris, a compendium of single cell transcriptome data from mouse that comprises more than 100,000 cells from 20 organs and tissues [79]. According to Tabula Muris, most cells express Atp13a2 and Atp13a3. The cell types with highest expression of Atp13a2 are found in bone marrow and include macrophages, multipotent progenitor cells and megakaryocytes. Atp13a3 is most highly expressed in the lung (ciliated columnar cells of tracheobronchial tree and epithelial cells), bladder urothelial cells, pancreatic ductal cells and large intestine (goblet cells and epithelial cells). Corroborating previous expression analyses, Tabula Muris indicates a more cell type-specific expression of Atp13a4 and Atp13a5. Atp13a4 is most highly expressed in Bergman glia and astrocytes. Although we found little/no expression of Atp13a5 in the Tabula Muris database, consultation of another transcriptomics dataset [80] on mouse brain revealed enrichment of Atp13a5 in (vascular) endothelial cells. Of interest, the latter dataset also indicated a broad expression of Atp13a2 and Atp13a3 in the brain as well as a strong enrichment of Atp13a4 in astrocytes which the authors confirmed by RT-qPCR [80].

3.1.4. Intracellular localization

P5B isoforms are present in partially overlapping endosomal sub-compartments (Fig. 2), further indicating that P5B-ATPases may fulfill redundant as well as complementary functions [68]. The generally accepted view is that ATP13A2 localizes to intracellular acidic vesicular compartments, *i.e.* the late endosomes and lysosomes, based on several studies that observed co-localization of overexpressed (tagged) ATP13A2 with LAMP1/2a, CD63, Rab7 and Lysotracker [68,81–87]. However, in both differentiated SH-SY5Y cells and rat primary neurons, endogenous ATP13A2 associates closely together with LC3, a marker of the autophagosomes, and CD63, a marker of the late endosomes [87]. Using home-made antibodies, the authors concluded that endogenous ATP13A2 mainly localizes to multivesicular bodies, a morphologically distinctive late endosome compartment. Unfortunately, the lack of high-quality antibodies detecting endogenous ATP13A2, makes it difficult to confirm these results, but also overexpression of tagged ATP13A2 localizes to multivesicular bodies in human neuroglioma H4 cells [88].

Currently, limited information is available regarding the endogenous subcellular localization of ATP13A3-5. Using tagged constructs overexpressed in HeLa cells, ATP13A3 was shown to mainly localize to the early/recycling endosomes overlapping with Rab5 and Rab11 localization [68]. Moreover, ATP13A3's subcellular localization may change in response to different stimuli such as PAs or DFMO, demonstrating a new regulatory aspect of ATP13A3 in controlling the PA homeostasis [89]. ATP13A4 displayed a broader endosomal distribution involving early/late endosomes and possibly lysosomes [68], although one study reported that ATP13A4 may localize to the ER, but this may possibly represent an overexpression artifact [78]. So far, the subcellular localization of ATP13A5 has not yet been unambiguously determined due to issues with expression stability in various cell lines [68].

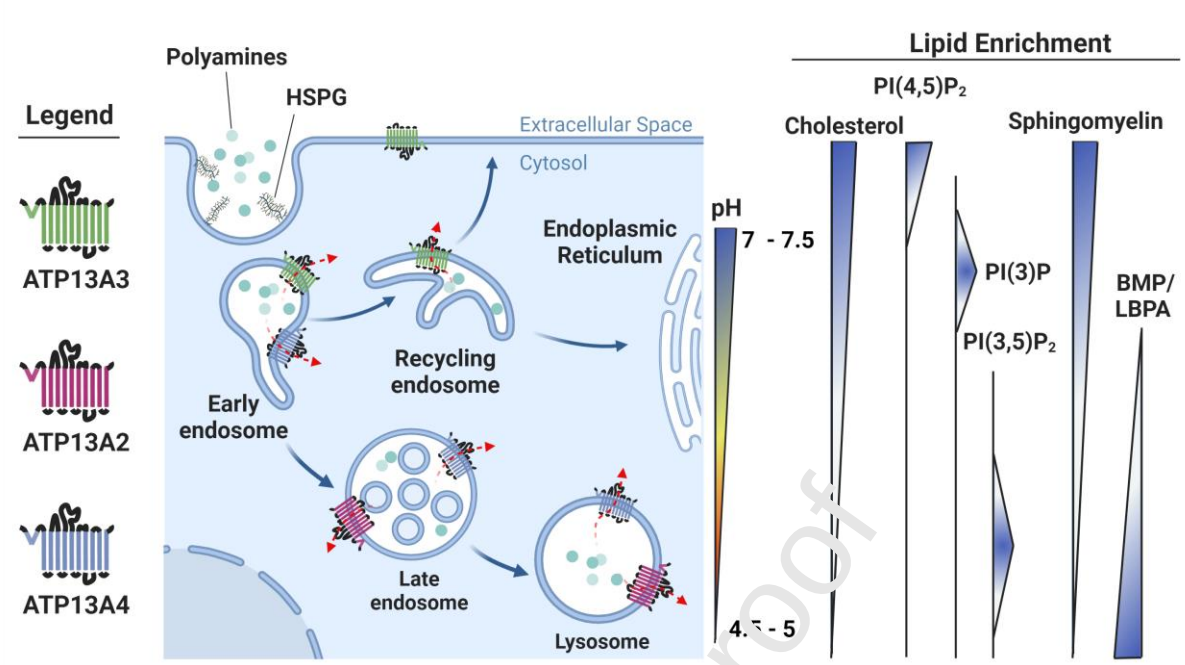

Figure 2. Distribution of P5B-ATPases in the endosomal compartment. Abbreviations: HSPG - heparan sulfate proteoglycans. $PI(4,5)P_2$, PI(3)P and $PI(3,5)P_2$ are phosphoinositides. BMP - bis(monoacylglycero)phosphate. LBPA - lysobisphosphatidic acid. Figure adapted from Hullin-Matsuda *et. al* [168].

3.2. Biochemical and structural properties of ATP13A2, the P5B prototype

Of the mammalian P5B-ATPases, only ATP13A2 has been biochemically and structurally characterized in-depth offering insights into its molecular architecture, biochemical properties, substrate specificity and regulation.

3.2.1. Breakthrough insights into the structure and transport mechanism of P5B-ATPases

3.2.1.1. The general architecture of P-type ATPases

The overall physical structure of the P-type ATPases is made up of multiple catalytic domains that are each defined by their function in the Post-Albers mechanism (Fig. 3) [69]. Consisting of, on average, ten membrane spanning hydrophobic helices, P-type ATPases contain three main cytosolic-facing domains called the actuator [A], nucleotide binding [N] and the phosphorylation [P] domains [70].

At the [P] domain the ATPases get auto-phosphorylated by a built-in kinase in the [N] domain to eventually get de-phosphorylated again by the built-in protein phosphatase within the [A] domain. The phosphorylation is reversible and takes place on the aspartate in the highly conserved **D**KTGTLT sequence, a signature motif found in all P-type ATPases, leading to an acid-stable phospho-aspartate residue [70]. The [A] domain is attached to the transmembrane parts of the protein by long flexible linkers, which enable the rotation of the [A] domain to alternate between presenting or hiding the phosphatase from the auto-

phosphorylation site [70]. A general description of the P-type ATPase transport mechanism is provided in Fig. 3.

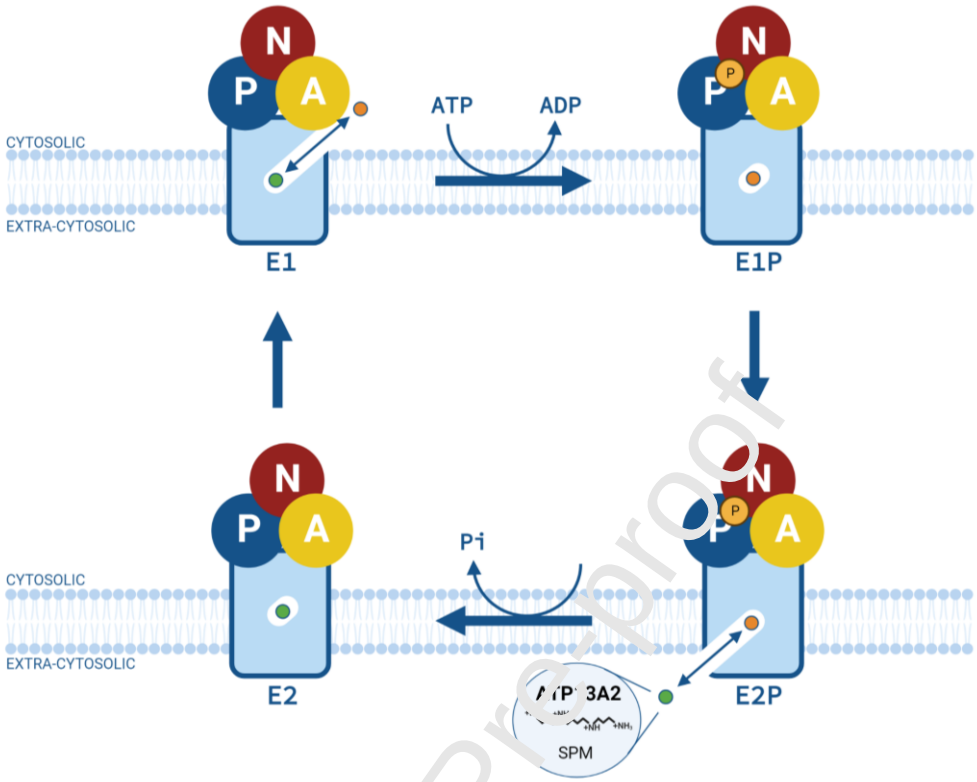

Figure 3. Post-Albers model of the catalytic cycle of P–type ATPases - The cycle starts when the P-type ATPase is in the E1 conformation and the ligand-binding sites for the first ligand (orange) face the cytosolic side where, in the case of P2-type ion transporters, the first ligand binds to a site with high affinity [70]. This causes the α helices within the core of the ATPase to shift their position, which triggers ATP binding and hydrolysis leading to auto-phosphorylation of the P-type ATPase, resulting in the E1P conformation [70]. In this E1P state the ligand is occluded within the transmembrane region as its exit is barred from either side [70]. Following a slow rate-limiting step, the transporter undergoes a conformational change to the E2P state where the binding sites for the first ligand switch from high to low affinity causing the ligand to be released from the enzyme into the extra-cytosolic side. The P-type ATPase may now display binding sites with a high affinity for a second ligand (green) that, upon binding, causes the ATPase to dephosphorylate and enter the E2 conformation where the second ligand is occluded within the transmembrane region [70,72]. In the final step where the enzyme reverts back to the E1 conformation, the binding sites switch from a high luminal to a low cytosolic affinity for the second ligand, thereby releasing it into the cytoplasmic side while restoring the high cytosolic affinity for the first ligand. Being back in the E1 state, the enzymes are ready to transport the first ligand to the extra-cytosolic side and the catalytic cycle is restarted again [70,72]. Note that the transport can be uni-directional if a single substrate is transported in one direction (from cytosolic to extra-cytosolic, *e.g.* Ca^{2+} in SPCA1 or Cu^{2+} in ATP7A; or from extra-cytosolic to cytosolic, *e.g.* PAs in P5B-ATPases and lipids in P4 lipid flippases); or can be bi-directional if counter-transport of multiple ligands takes place (*e.g.* exchange of Ca^{2+}/H^{+} in SERCA1a and Na^{+}/K^{+} in Na^{+}/K^{+}-ATPase).

3.2.1.2. Structures and architecture of P5B-ATPases

Insights into the molecular architecture of P5B-ATPases are just emerging based on the released structures of human ATP13A2 [90-93] and its ortholog Ypk9 from the thermophilic yeast *Chaetomium thermophilum* [94]. All structures were determined by cryo-electron microscopy (cryo-EM) yielding so far six different conformations of human ATP13A2 (WT and mutant enzyme), covering almost the entire transport cycle, which advanced our understanding of the PA transport mechanism: 1. The E1 state [91-93], 2. The ATP-bound

state E1-ATP [93] (or E1-AMPPCP [90] and E1-AMPPNP [92]); 3. E1P-ADP (with ATP and Mg^{2+} [91] or AlF_4^--ADP [90,93]); 4. The E1P state (in the presence of the phosphate analogue AlF_4^- [93]); 5. The open SPM-bound E2P state (with the phosphate mimetic BeF_3^- [90,92,93]); 6. The SPM-bound E2-P_i state (with AlF_4^- and SPM [90,93]; MgF_4^{2-} and SPM [92]; or SPM, and the regulatory lipids phosphatidic acid and phosphatidylinositol(3,5)bisphosphate ($PI(3,5)P_2$) [91]). So far, ATP13A2 structures in the E2 state have not yet been resolved. For Ypk9, cryo-EM structures were reported of the E2P state (in the presence of BeF_3^-), the SPM-bound E2P state (in the presence of BeF_3^- and SPM) and the SPM-bound E2-P_i state (in the presence of AlF_4^- and SPM, or $MgCl_2$ and SPM) [94].

The ATP13A2 and Ypk9 structures depict a typical P-type ATPase architecture, including the three conserved cytosolic [A], [P], and [N] domains, and ten transmembrane spanning helices (TM1–10) [90-94]. Furthermore, ATP13A2 and Ypk9 contain additional NTD and C-terminal domains (CTD) [90-94]. The NTD has a spade-like structure with three helices that penetrate the membrane, without spanning it, which represents a unique feature of P5B-ATPases as compared to other P-type ATPases. The CTD forms a short cytosolic α helix that interacts with the [P] domain [90-94]. Unfortunately, the unique NTD is not fully resolved, possibly due to a high flexibility of the region, and its underlying functional role remains incompletely understood (see section 3.2.2.1.). Of note, Ypk9 contains a long extreme N-terminus that holds an N-terminal loop interacting with the cytosolic domains in the E2P state, representing a possible auto-regulatory element [94], whereas the ATP13A2 extreme N-terminus is much shorter [90-93].

3.2.1.3. Notable structural differences between P5A and P5B-ATPases

The new structural insights into the structure of ATP13A2 and the predictions made by AlphaFold for the other P5B-ATPases offer further clarity on the similarities and differences between P5B and P5A-ATPases and further underscore the rationale behind the evolutionary split between the two (section 3). Recently, the crystal structure of Spf1, the yeast P5A-ATPase, was reported [75]. Though all the main domains forming the typical P-type ATPase architecture were confirmed in Spf1, a few key differences were observed in the substrate pockets of P5A and P5B-ATPases. In Spf1, the substrate-binding pocket is formed by TM2 and TM6, which contact each other on the ER luminal side to form a surprisingly larger and V-shaped pocket to fit peptides that is also open laterally to expose the pocket to the lipid phase of the cytosolic leaflet of the ER membrane. The inside of the pocket presents a negative electrostatic potential and is lined with both hydrophilic and hydrophobic amino-acid side-chains that are highly conserved in P5A-ATPases across species, but that differ from P5B-ATPases [75]. Contrastingly, P5B-ATPases form a narrow and elongated substrate-binding pocket, suitable to accommodate PAs, which is separated from the lipid phase and is much smaller than that of Spf1 [93] [90].

Another unique difference between P5A and P5B-ATPases is in the NTD, which in the case of P5A-ATPases, is made up of a seven-stranded β barrel embedded in the cytosol that contains two extra helices [75].

Lastly, Spf1 also contains an extension between TM5 and the [P] domain that forms an elongated “arm-like” domain that is absent in P5B-ATPases [75]. Though the exact function

of the arm extension is unknown, Grenon *et. al* demonstrated that its deletion in Spf1 leads to impaired phosphoenzyme formation proportionate to the ATPase activity suggesting that the arm domain is not essential for, but facilitates, ATP hydrolysis [95].

3.2.1.4. The polyamine transport mechanism in P5B-ATPases

A similar PA transport mechanism was proposed based on the structures of human ATP13A2 and yeast Ypk9 [90-94], indicating that it may be highly conserved among P5B-ATPase isoforms and orthologs.

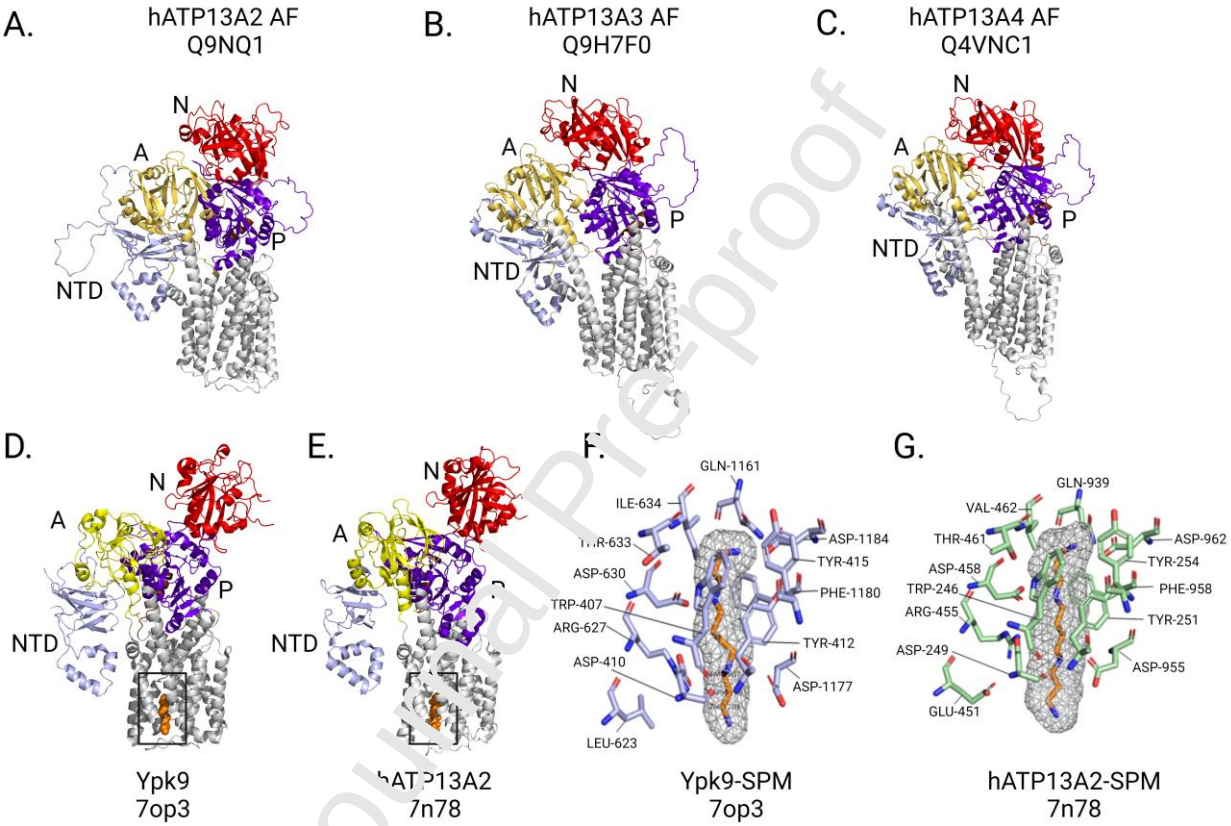

Figure 4. (A) - (C) AlphaFold predictions of hATP13A2, hATP13A3 and hATP13A4 respectively [96]. **(D) - (E):** Cryo-EM structures showing SPM-bound Ypk9 and hATP13A2. **(F) - (G):** Depiction of the SPM-binding pocket in hATP13A2 and YPK9.

In the presence of ATP and Mg^{2+}, P5B-ATPases undergo spontaneous auto-phosphorylation, which occurs independently of PA binding [41,68,84]. This leads to the transition from the E1 to the E1-ATP state and subsequent E1P-ADP state, which is accompanied by a tilt of the [N] domain to lay closer to the [A] and [P] domain [91,93]. Following ADP release, the transporter shifts to the E2P state creating an electronegative luminal PA-binding pocket [93] [91] [92]. There is a consensus that the spatial matching between the PA molecule and the substrate-binding cavity formed by helices TM2, TM4 and TM6, is dictated by the interaction between the positively-charged amine groups of the PAs and the aromatic and acidic side chains positioned strategically along the cavity (Fig. 4) [90-94]. More specifically, the PA molecule is stabilized by hydrogen bonds and cation-**π** interactions involving several acidic and aromatic amino acid residues lining the PA-binding pocket, respectively [90] [91]. In addition, the architecture of the PA-binding site explains

ATP13A2's preference for SPM as a transported substrate, with the residues shaping the pocket almost completely conserved between Ypk9 and ATP13A2 (Fig. 4). The narrow channel design enables linear PAs to migrate through the protein, while cyclic PAs may be too large to enter [92]. Furthermore, the molecular symmetry in SPM and the unique spacing of amine and aliphatic groups allow this PA molecule to bind the PA-binding site with the highest probability and affinity [92].

SPM binding induces dephosphorylation of the pump, resulting in the transition from the E2P to E2-P_i state. Cytosolic release of SPM is expected to occur during the transition from the E2-P_i state to the E2 state. Interestingly, while E2-P_i structures of other P-type ATPases display a substrate-bound/occluded conformation, the E2-P_i structures of ATP13A2 are in an outward-facing state [92] [93]. Unfortunately, structural information concerning the E2 state is currently lacking, which hampers our understanding of exactly how PAs are embedded, translocated and subsequently released into the cytosol. One study hypothesized that SPM translocation to the cytosol occurs through a cavity towards the cytosol [91], while others suggested an atypical transport path in which SPM may exit within the cytosolic leaflet of membrane [93]. Alternatively, it was proposed that cytosolic SPM release is achieved by sliding of the cytoplasmic M4b segment that restricts the exit pathway in the E2P and E2-P_i states [90]. On the other hand, since a transport intermediate with fully occluded SPM has not been observed and SPM occupies a linear cavity, a pump-channel model was speculated, in which P_i release leads to a pump-channel intermediate with both luminal and cytosolic gates open allowing the SPM to flow through the transmembrane region possibly using the electrochemical gradient [92]. In the case of Ypk9, a transport mechanism similar to what is observed for P4- and P5A-ATPases was proposed, in which the cytosolic exit pathway is formed by TM2, TM4, and TM6 whereby the lack of electronegative residues in this cleft may lead to direct release of SPM into the cytosol [94]. Additional structures capturing conformations between the E2 and E1 transitions will be required to fully resolve the SPM translocation path.

3.2.2. Regulation of P5B-ATPases

P-type ATPases are often subject to autoregulation via their N- or C-termini, sometimes also called the regulatory domains [R] [70]. They can lead to auto-inhibition by folding over the catalytic domains or promoting multimerization [97-101], whereas interaction with external regulators such as lipids or proteins as well as post-translational modifications may unlock the activity [84].

3.2.2.1. The P5B N-terminus

The observation that SPM-dependent ATPase activity of purified Ypk9 is stimulated when the first 100 amino acids are removed, supports the hypothesis that the P5B N-terminus might play an auto-inhibitory role [94]. However, a direct interaction of the NTD with the catalytic domains has only been seen with the longer extreme N-terminus of Ypk9, but not with the shorter N-terminus of ATP13A2. In the case of ATP13A2, the N-terminus has been suggested to serve an auto-inhibitory function that is regulated by lipids [84,102]. The signaling lipids phosphatidic acid and PI(3,5)P_2 specifically interact with the N-terminus of ATP13A2 [84,91] and three putative lipid binding sites (LBS) with conserved positively charged residues (LBS1, 65FRWKP; LBS2, 74RLRLR; LBS3, 155KRVLR) were proposed

based on protein-lipid overlays [84]. Regulation by $PI(3,5)P_2$ and phosphatidic acid as well as the LBS2 and LBS3 were found to be crucial for ATP13A2-mediated protection against heavy metal and mitochondrial toxicity within cells [102]. Regarding the implications of these lipid interactions on ATP13A2 activity, some discrepancies exist in the literature. Addition of both phosphatidic acid and $PI(3,5)P_2$ were critical to induce maximal SPM-dependent ATPase activity of purified human ATP13A2 from *S. cerevisiae* [41]. The phosphatidic acid-induced activation of ATP13A2 was confirmed in another study, which was abolished following mutagenesis of the first two positively charged residues in LBS3 [91], again highlighting the importance of phosphatidic acid interaction at LBS3 in ATP13A2 activation. Furthermore, the interaction with $PI(3,5)P_2$ was confirmed (amongst other phosphatidylinositols) but its regulatory role was not investigated [91], while another study reported that $PI(3,5)P_2$, but not phosphatidic acid, stimulated the SPM-dependent ATPase activity of ATP13A2 [92]. Differences in protein expression systems, purification and solubilization methods could explain the observed variability [92].

The precise function of the peculiar membrane-embedded NTD remains unclear. Deletion of the NTD of purified ATP13A2 leads to either a decreased SPM-induced ATPase activity (Δ1-183) [92] or does not influence activity at all (Δ2-179) [93]. Moreover, impaired phospho-enzyme formation was reported for two N-terminal deletion mutants of ATP13A2 [90,92]. Partial deletion of the NTD (Δ29-49) in ATP13A3 leads to impaired cellular SPD and SPM uptake, most likely related to protein dysfunction [89]. However, an in-depth biochemical characterization is lacking and therefore, no firm conclusions can be made.

Besides a role in auto-regulation, structural studies revealed that anchoring the NTD to the lipid membrane may stabilize the E2P conformation [90,94]. The interaction of phosphatidic acid and $PI(3,5)P_2$ may stabilize the NTD to facilitate the transition from the E1P state to the E2P state upon phosphorylation [90,94]. Additionally, a belt with a strong positively charged surface was identified at the cytosol-membrane interface, which may recruit the negatively charged lipids phosphatidic acid and $PI(3,5)P_2$ leading to their accumulation around ATP13A2, indicating that lipid regulation may not solely rely on the LBS1-3 regions [91,93]. This might be a unique P5B-ATPase feature since a positively charged surface was observed in other P5B isoforms but not in SERCA, P4 lipid flippases or P5A-ATPases [93].

3.2.2.2. The P5B C-terminus

The C-terminus of ATP13A2 and Ypk9 forms a short helix, which interacts with the [P] domain mainly by hydrophobic interactions [90-94], but its functional role is not yet resolved. The C-terminus of ATP13A2 seems to be critical for its catalytic activity, as C-terminal deletion mutants showed abolished SPM-dependent ATPase activity [92,93]. On the other hand, truncation of the C-terminus only moderately affected EP formation [90]. The CTD was also suggested to be important for protein stability [91,93]. C-terminal deletion mutants tended to form high-molecular-weight aggregates [93] or demonstrated impaired protein expression [91]. One structural study identified a putative $PI(3,5)P_2$ binding pocket formed by the CTD highlighting a putative role in regulating activity [92]. An overview of the different insights into ATP13A2's structure and regulation from the independent studies can be found in Table 2.

3.2.3. Implications for other mammalian P5B-ATPases

The biochemical and structural insights on ATP13A2 also offer clues about the transport function of the other P5B-type isoforms. ATP13A2 and the other human-P5B ATPases share less than 40% amino acid identity [91]. However, other P5B-ATPases most likely present similar topology as ATP13A2 involving ten transmembrane helices and a membrane-associated, but not membrane-spanning NTD [68]. Structural predictions of AlphaFold confirm that all ATP13A2 orthologs (invertebrates and vertebrates) and homologs (ATP13A3-5) may adopt a highly conserved domain organization (Fig. 4) with a spade-like structure in the NTD pointing to conserved functional roles. Also, the acidic and aromatic side chains that line the PA-binding cavity in the ATP13A2 transmembrane region are strongly conserved among P5B-ATPases, suggesting that P5B-ATPases may transport the same or closely related PAs [93]. This has been confirmed in the yeast Ypk9 structures that have been resolved with a bound SPM (Fig. 4). However, the signature motif of the mammalian ATP13A5 in TM4 (PP**V**LP) slightly deviates from that of the other P5B-ATPases (PP**A**LP in ATP13A2-4). Mutating the PPALP sequence of ATP13A2 into PPVLP impaired the SPM-induced ATPase activity and hampered PA uptake, suggesting that substrate recognition or specificity are altered [41]. While both alanine and valine are hydrophobic amino acids, valine is bulkier than alanine and therefore, the substitution might reduce the diameter of the substrate binding pocket causing steric hindrance, disfavoring transport of SPD or SPM, but the substitution may possibly favor the shorter PUT or other small amine-containing molecules. Substituting the alanine to valine in the ATP13A2 substrate binding site reduces SPM affinity and maximal turnover [41]. Similar to ATP13A2, ATP13A3 and ATP13A4 also undergo spontaneous auto-phosphorylation highlighting that besides the structure, the transport mechanism and biochemical properties are conserved [68,84]. This may explain why ATP13A2 is able to, at least partially, complement the loss of ATP13A3 in the CHO-MG model [40].

4. P5B-ATPases in human diseases

Disturbances in the PA cellular content due to defective metabolism and/or transport have been associated with several diseases, ranging from cardiovascular and neurological disorders to cancer. This involves genetic mutations, as well as variations in the expression or activity of PA metabolic enzymes as well as PA transporters.

Being newly established PA transporters, it comes of no surprise that most members of the P5B-ATPase family have been implicated in human diseases where a disrupted PA homeostasis has been observed (Table 3) [40,41]. At the time of writing, no links between ATP13A5 and disease have yet been reported.

4.1. P5B-ATPases in neurological diseases

4.1.1. ATP13A2 in neurodegeneration

Genetic mutations in *ATP13A2* have been associated with a spectrum of several related neurodegenerative disorders including early onset Parkinson's disease [81,83,103-108] and dementia with Lewy bodies [81,103,104].

ATP13A2 has been also linked to other neurodegenerative disorders including Kufor-Rakeb syndrome (a severe juvenile-onset parkinsonian disorder with dementia) [83,109], hereditary spastic paraplegia [82,110-113] (described by lower limb spasticity which in some cases is also accompanied by neurological characteristics) [111], neuronal ceroid lipofuscinosis (manifested as motor deterioration, dementia, seizures and visual impairment along with lysosomal accumulation of the autofluorescent lipopigment ceroid lipofuscin) [114,115], amyotrophic lateral sclerosis (characterized by degeneration of the motor neurons causing muscle weakness) [116] and multiple system atrophy (a neurodegenerative movement disorder) [117]. A disturbed ATP13A2-related PA transport function most likely is an underlying mechanism to the pathogenesis and/or progression of these disorders. Truncation or frameshift mutations have been identified pointing to loss of ATP13A2 function as disease-causing, but other factors may contribute such as ER stress, which has been reported for truncation mutants in Kufor-Rakeb syndrome [118]. Biochemical studies demonstrated that disease-associated point mutations in ATP13A2 also disrupted the PA transport activity with the degree of dysfunction correlating with the disease phenotype [41]. A complete loss-of-function was observed with Kufor-Rakeb syndrome mutations, whereas a milder defect was found for early onset Parkinson's disease mutations. On the other hand, the same mutations (*e.g.* T512I) can be found in both Kufor-Rakeb syndrome and in complicated hereditary spastic paraplegia, indicating that also other factors are involved in determining the disease phenotype. But why the clinical consequences of *ATP13A2* mutations diverge remains unclear.

All diseases where *ATP13A2* mutations have been identified, are marked by an impaired lysosomal and mitochondrial axis. At the cellular level, ATP13A2 dysfunction hinders the export of PAs out of the lysosomes resulting in their accumulation in the lysosomal lumen. As a consequence, there is an impairment of the lysosomal degradative capacity and pH, which leads to lysosomal rupture and cathepsin B-mediated lysosomal cell death. Alongside maintaining healthy lysosomal functionality, ATP13A2 also plays a role in maintaining mitochondrial function. ATP13A2-deficient models exhibit mitochondrial dysfunction such as reduced membrane potential and ATP production, increased mitochondrial mass, fragmentation and reactive oxygen species (ROS) production [119]. Rotenone, a pesticide and mitochondrial complex I inhibitor that is an environmental Parkinson's disease risk factor, leads to mitochondrial ROS and toxicity causing cell death that can be attenuated upon ATP13A2-mediated PA transport [120]. PAs like SPM present antioxidant properties, and function as free-radical scavengers, which is well documented [121,122]. PAs are known to bind to the mitochondrial membrane through which they transported, *via* currently-unidentified transporters, where they modulate mitochondrial processes such as the mitochondrial permeability transition, Ca^{2+} cycling, regulation of mitochondrial protein synthesis and respiration [123-127]. The transport function of ATP13A2 also provides cellular protection against other environmental risk factors, such as heavy metal toxicity from manganese, zinc or iron exposure, which may relate to the combined metal chelating and antioxidant effects of PAs [87,128-130]. Parallel to that, a lack of ATP13A2-mediated PA transport has been shown to enhance α-synuclein membrane association and multimerization, which is promoted by SPM and lysosomal membrane integrity [131]. Thus, ATP13A2 provides protection to both environmental (pesticides, heavy metals) and genetic (α-synuclein) risk factors of Parkinson's disease.

4.1.2. ATP13A4 in neurodevelopmental diseases

ATP13A4 has been implicated in a spectrum of neurodevelopmental diseases such as autism spectrum disorder, schizophrenia, specific language impairment and childhood apraxia of speech [78,132-134]. A chromosomal inversion disrupted the *ATP13A4* gene in two specific language impairment patients characterized by delayed expressive and receptive language, without further cognitive deficiencies [132]. Moreover, whole-exome sequencing identified a heterozygous *ATP13A4* E646D variant in three study participants with childhood apraxia of speech, a rare and severe motor speech disorder [133]. An autism susceptibility locus was mapped on chromosome 3q25-27, near to the location of *ATP13A4*, in a genome-wide screen for ASD [135]. Subsequent DNA sequencing of thirty patients revealed an *ATP13A4*-E646D sequence variant in six study participants and an A356V substitution in another patient [132]. In addition, increased *ATP13A4* mRNA expression was observed in the cortex (Brodmann area 9 [136] and Broca's area [134]) of patients with schizophrenia, whereas earlier genetic studies reported ATP13A4 gene deletions in patients with schizophrenia [137]. Finally, a link with epilepsy has been suggested following the discovery of inherited duplications at 3q28-q29, encompassing *ATP13A4*, in patients with epileptic encephalopathies [138]. Currently, there is no further biochemical or mechanistic information regarding the putative role of ATP13A4 in neurodevelopmental disorders, however, the genetics of the P5B-ATPases ATP13A2 and ATP13A4 point to a fundamental role in brain physiology and disease.

4.2. P5B-ATPases in vascular disease

4.2.1. ATP13A3 in pulmonary arterial hypertension

Recently, the discovery of a range of heterozygous mutations in ATP13A3 found in a cohort of patients suffering from the rare and fatal disease Pulmonary Arterial Hypertension (PAH), has positioned dysregulated PA homeostasis as a potential contributor to the pathogenesis of the disease [139-142]. Though the cases with the ATP13A3 mutations were adult-onset and heterozygous, a homozygous mutation was also identified in a child with a severe and early-onset form of PAH leading to death at 4 years of age [142,143]. While the pathogenicity of the ATP13A3 missense mutations in PAH has yet to be elucidated, the protein truncation mutants are predicted to be degraded *via* nonsense-mediated decay most likely causing loss-of-function through haploinsufficiency [143]. Interestingly enough, the ATP13A3 mutations cluster in regions that are important for the ATPase's function such as the catalytic, regulatory or substrate binding sites [141,142]. Despite needing further study, it is postulated that PAH mutations in ATP13A3 contribute to the pathogenesis of the disease by disrupting the tightly regulated PA homeostasis.

PAH, a pan-vasculopathy, has a complex pathogenesis of which the overall effect is vascular remodeling caused by endothelial dysfunction, hyperproliferation and apoptosis-resistance of pulmonary arterial smooth muscle cells, thrombosis and inflammation leading to vasoconstriction and the obstruction of the arterial lumen resulting in an increased arterial hypertension [144,145]. Since PAH is essentially characterized by neoplastic growth, the role of PA homeostasis and its (dys)regulation becomes very important as PAs play vital roles in cancers and other hyperproliferative diseases [146]. Not surprisingly, PA levels are increased in PAH patient lung tissue as well as plasma samples, and targeting PA synthesis

improved outcomes both *in vitro* and in rat models [147,148]. Pulmonary hypertension can be induced in rats by monocrotaline, which is the most classical and widely used *in vivo* model of PAH. Here, an increase in PA synthetic enzymes ODC and AMD was observed, which correlated with increased PA levels in lung tissue [149]. Upon treatment with DFMO both the increase in lung PA content as well as PH development were attenuated [149]. However, how PAs could be linked to the most well-studied pathways of PAH still remains unknown. The majority of familial PAH cases present themselves with mutations in the bone morphogenetic protein receptor type II (BMPR2) of the transforming growth factor-beta signaling family pathway. However, with a sizable percentage of PAH patients suffering from idiopathic PAH, where the etiology of the disease is unknown, and the disease penetrance being as low as 20%, other genetic factors are expected to contribute to the disease [150].

4.3. Polyamines and P5B-ATPases in cancer

Given the central role of PAs' effects on a DNA, RNA and protein level; the cell cycle is also affected by PA levels. When cells are deficient in PAs, the cell cycle is arrested at the G1/S transition during which DNA synthesis is decreased and the inhibitors of cyclin-dependent protein kinases CDK2 and CDK4, $p27^{Kip1}$ and $p21^{Cip1/WAF1}$, are increased [151]. When it comes to proliferation, PAs have been shown to exert their effect through different signaling cascades. For instance, overexpressing ODC increases MAPK and tyrosine kinase activity in the Ras/MAPK pathway [152,153]. Moreover, SPD preferentially stimulates the phosphorylation of p42 and p44 of the MAPK pathway as well as the transcription of the proto-oncogene *c-myc*, whereas PUT promotes the transcription of *c-fos* and *c-jun* in cultured rat epithelial cells. This demonstrates that different PAs may have specific effects on nuclear proto-oncogenes [154]. Many cancers show higher levels of hypusinated eIF5A2, which is associated with metastasis and poorer prognosis [155]. The elongation factor may facilitate the expression of several oncogenic proteins containing polyproline stretches, such as the oncogenic *c-Abl* tyrosine kinase [155].

Reducing PA levels by inhibiting ODC with DFMO, led to halted cell proliferation in rat hepatoma cells, which could be rescued with exogenous PA supplementation [156]. Similarly, the over-expression of SAT1, effectively caused a reduction in PA levels and resulted in decreased proliferation, viability and an increase in apoptosis [157]. SAT1 is also directly activated by the tumor suppressor p53, which is a master regulator of the cell cycle and plays a vital role in preventing cancers from becoming malignant [158]. SAT1 further modulates the p53-mediated response to ROS and ferroptosis. Conversely, SAT1 expression is decreased in a wide variety of cancers demonstrating that cancer cells may reduce PA degradation to increase their PA pool and sustain their hyperproliferative phenotypes [158]. Thus, targeting the PA metabolic pathway has emerged as an appealing therapeutic strategy for cancer treatment [158]. However, ODC targeting has been examined in clinical trials for various cancers albeit with limited success, most likely because of an upregulation of the PA uptake system. Therefore, combination therapies that block PA synthesis (with DFMO) and uptake (with AMXT-1501) simultaneously have shown great promise for neuroblastoma and diffuse intrinsic pontine glioma therapy, and are being tested in clinical trials (ClinicalTrials.gov Identifier: NCT03536728) [34]. So far, the molecular identity of the targets for AMXT-1501 or other known PTS inhibitors remains unknown. But since P5B-ATPases emerge as major determinants of PA uptake *via* endocytosis, they may

also play a role in cancer, and may be considered as therapeutic targets for cancer. The role of P5B-ATPases in cancer is therefore an interesting avenue for future studies to explore.

Differences in ATP13A2 expression levels have been associated with some types of cancer with an increase or decrease depending on the type of cancer [159]. Similarly, ATP13A3 has also been implicated in a range of cancers such as pancreatic, head and neck and cervical cancers where patients having higher ATP13A3 expression show a poorer overall survival rate [160-163]. When performing transcript level comparisons on publicly-available human cancer datasets, ATP13A3 was invariantly and significantly over-expressed in pancreatic cancer as well other cancer types when compared to normal tissue samples [162]. Interestingly, comparing six different human pancreatic cancer cell lines to an immortalized normal human pancreatic duct cell line as a control, Madan *et. al* observed that the cells with higher PA transport and ATP13A3 expression required lower doses of DFMO as they were more reliant on PA uptake for their viability. Similarly, the same cells responded better to combination therapy involving DFMO with a PA transport inhibitor when compared to other pancreatic cancer cells that had lower ATP13A3 expression [162]. ATP13A3 has even been proposed as a biomarker for these cancers [160,162,163].

ATP13A4 has been associated with lung adenocarcinoma (LUAD). In order to build a gene interaction network to reveal changes in gene expression during carcinogenesis, Zhou *et. al* used weighted gene co-expression network analysis on the expression profile of LUAD from the cancer genome atlas to determine differential correlations between cancer and adjacent normal tissues as a control [164]. Interestingly, ATP13A4-AS1, an antisense long noncoding RNA (lncRNA) originating from ATP13A4 was identified and predicted to interact with genes such as advanced glycosylation end-product specific receptor (AGER) and angiopoietin-like 7 (ANGPTL7) whose lower expression has been strongly linked to carcinogenesis [164]. Kaplan-Meier analysis further revealed a similar result for ATP13A4-AS1 which correlated with worse patient outcome due to its lower expression in LUAD when compared to controls – an observation that the authors further confirmed using RT-qPCR [164]. In another case report, Next Generation Sequencing on a patient suffering from non-small cell lung cancer (NSCLC) revealed several baseline fusions between anaplastic lymphoma kinase (ALK) protein and other genes – amongst which *ATP13A4-ALK* was also included [165]. ALK rearrangements and fusions are one of the most common genetic aberrations found in NSCLC and though patients harboring such aberrations initially benefit from tyrosine-kinase inhibitor treatment, resistance to therapy eventually develops owing to new ALK mutations and/or other fusion partners [165].

Finally, ATP13A4 has also been linked to ovarian cancer where, in a study on high-grade serous ovarian cancer patients in the Ovarian Tumor Tissue Analysis cohort, ATP13A4 was amongst 11 copy number amplified genes that impacted the clinical outcome of patients [166].

5. Open Questions and Future Perspectives

The discovery of new members of the mammalian PTS is a major breakthrough in the PA field as it significantly expands our understanding of PA homeostasis in health and disease. Naturally, the recent advances in identifying P5B-ATPases as part of the mammalian PTS

raise as many questions as have been solved and have laid the groundwork for further research.

5.1. Transport mechanism and regulation of P5B-ATPases

Despite the advent of structural information of P5B-ATPases, several questions regarding the PA translocation pathway and regulation by the NTD and CTD remain. Moreover, the electrochemical gradient within the endo-/lysosomal system may favor the luminal to cytosolic transport of PAs (although the precise free luminal and cytosolic concentrations are unknown). Thus, the question emerges whether P5B-ATPases represent genuine primary active transporters of PAs, or whether PA transport merely follows the electrochemical gradient. It is possible that P5B-ATPases exert active counter-transport (cytosol to lumen) of other ions, such as heavy metals or protons, for which ATP hydrolysis may be required.

5.2. Specific roles of P5B-ATPase isoforms in PA homeostasis and disease

The mammalian PTS has long stayed an enigma and advances in the field have been few. This is most likely related to the complexity of the PTS with not one single PA transporter, but multiple parallel PA uptake systems and transporters that operate together – also explaining why all the different models and transporters have been proposed. Furthermore, the PTS tool box may differ amongst various cell types as a consequence of regulated gene expression, making it difficult to quantitatively compare data obtained from various cell types [167]. Within the PTS, P5B-ATPases clearly emerge as key PA transporters expressed in overlapping compartments of the endosomal pathway. P5B-ATPases present overlap in substrate specificity and biochemical properties, which may explain the functional redundancy and complementation between P5B isoforms. Despite this redundancy, it is unclear why P5B-ATPase members are genetically linked to very different diseases. This may relate to isoform-specific tissue expression patterns as well as regulatory control mechanisms *via e.g.* the NTD and CTD. For instance, the different compartments of the endo-/lysosomal pathway that the different P5B-ATPases find themselves in have different lipid composition (Fig. 2) and therefore the termini may interact with different lipids in a manner unique to each isoform [168]. It will therefore be important to map the P5B interaction partners, post-translational modifications and cell-type specific expression patterns. Comparing the relative expression of PA transporters and genes of PA metabolism in various cell-types will provide insights into the relative importance and cross-talk of PA synthesis and uptake. By comparing the phenotypes of P5B isoform specific KO mouse models as well as map cell-type specific changes in P5B isoforms in a disease context, we will take closer steps towards understanding their (patho)physiological roles.

5.3. P5B-ATPases as therapeutic targets

Targeting the PA metabolic and uptake pathway has long been suggested as an important therapeutic strategy for several disorders [34,169-172]. The identification of P5B-ATPases as novel members of the mammalian PTS may present new therapeutic opportunities. Towards drug discovery, biochemical assays for screening and methods to solve structures have already been developed, which will be suitable for the identification and development of

P5B agonists (*e.g.* for Parkinson's disease and PAH) and antagonists (*e.g.* for cancer). However, treatments targeting PA transporters may not be without risk, since the PA homeostasis needs to be critically controlled to prevent either PA deprivation or overload, which are both detrimental for cells. So far, DFMO has been used for trypanosomiasis and is under clinical evaluation for cancer therapy, possibly in combination with PTS inhibitors like AMXT-1501, which holds promise. This suggests that modulating the PA homeostasis in patients by targeting the PTS may be feasible and safe.

6. Conclusion

Our understanding of how PAs partake in a wide range of physiological processes has expanded by leaps since the discovery of PAs just a few centuries ago. PAs are present in all living organisms where their numerous functions are vital for the healthy functioning of the organism. As summarized in this review, deviation from the tightly-controlled PA homeostasis, which elegantly balances synthesis, uptake and metabolism, can lead to a spectrum of diseases. Yet in mammals, a big aspect of PA homeostasis, the PTS, has remained poorly characterized. Identifying P5B-ATPases as members of the mammalian PTS represents a major breakthrough in the field allowing us to investigate their role in PA homeostasis, organelle function, as well as (patho)physiological importance. P5B-ATPases emerge as therapeutic targets, and the recent advances in elucidating the structure of ATP13A2 entails the potential of structure-based drug design. Today the field stands one step closer to elucidating the mechanisms involved in PA homeostasis in mammalian cells.

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8. List of the Tables

Table 1. Overview of the confirmed and putative mammalian polyamine transporters. Genes and proteins indicated by an asterix (*) represents the confirmed (biochemically and/or structurally) PA transporters. Acronyms used for protein names referred to the one used in section 2.3.1

Gene	Protein	Tissue distribution (mRNA levels)	Link with PA transport
P-type ATPases			
*ATP13A2**	ATP13A2*	Ubiquitous, highest in brain especially the dopaminergic neurons of the *substantia nigra*	SPD and SPM export from the lysosomal lumen
ATP13A3	ATP13A3	Ubiquitous, highest in liver	PUT, SPD and SPM uptake
ATP13A4	ATP13A4	Restrictive to brain and glandular epithelial cells especially lungs, mouth, epididymis (ATP13A4)	Sequence similarities with ATP13A2/3
ATP13A5	ATP13A5	Glandular epithelial cells especially nose (ATP13A5) and breast (ATP13A5)	Sequence similarities with ATP13A2/3
SLC			
SLC3A2	DAX	Ubiquitous, highest in brain, endocrine tissues and kidneys	PUT and monoacetylated SPD export
SLC12A8A	CCC 9a	Ubiquitous	PUT, SPD and SPM uptake
*SLC18B1**	VPAT*	Ubiquitous, highest in lungs and placenta in human; highest in lungs and brain regions (hippocampus, cortex and cerebellum) in mice	Antiporter H^+/SPD and SPM Additional substrates: AGM, serotonin, histamine
SLC22A1-3	OCT1-3	Highest in liver (OCT1 and OCT3), skeletal muscles, heart and placenta (OCT3) and kidneys (OCT2) specifically in the luminal membrane of the distal tubules	Bidirectional AGM transport but controversial
SLC22A16	CT2	Restrictive to bone marrow, fetal liver and adult testes	L-carnitine and SPD uptake
SLC47A1	MATE1	Highest expression in kidneys, especially in the apical membrane of the proximal and distal convoluted tubules	AGM uptake

ATP-Binding cassette			
ABCB1	MDR1	Highest in the gastrointestinal tract and endocrine tissues	Indirect link. MDR1 function depends on PA import.

Table 2. Summary of available ATP13A2 structures. n-dodecyl-β-D-maltopyranoside (DDM), cholesteryl hemisuccinate (CHS), *N*-acetylglucosaminyltransferase I–negative (GnTI-), lauryl maltose neopentyl glycol (LMNG), phosphatidylinositol(3,5)bisphosphate ($PI(3,5)P_2$), N-terminal domain (NTD), C-terminal domain (CTD), phospho-enzyme (EP), transmembrane helix (TM), spermine (SPM) and lipid binding site (LBS).

	Sim S. *et al.* [93]	**Tillinghast J. *et al.*** [92]	**Tomita A. *et al.*** [90]	**Chen X. *et al.*** [91]
Expression hosts	Sf9 cells	HEK293S GnTI- cells	HEK293S GnTI- cells	HEK293F cells
Solubilization conditions	DDM, CHS	LMNG, CHS	DDM, CHS	Digitonin
Lipid interaction	None	$PI(3,5)P_2$ proximal to the CTD	None	None Mutagenesis of LBS at the NTD resulted in reduced ATPase activity
Substrate binding pocket	Interface of TMs 1–2, 3–4, and 5–10	Formed by TM1, TM2, TM4a, TM5, and TM6	Long tunnel between TM1-TM2 and TM4-TM5	Cavity formed by TM1b, TM2, TM4a, and TM6
Proposed SPM exit	Through a cytosolic leaflet of the membrane	Pump-channel with both luminal and cytosolic gates open	Sliding of the TM4b segment	Cavity towards cytosol
Role of NTD	No effect of NTD deletion	NTD deletion reduces EP formation and ATPase activity	NTD deletion reduces EP formation	-
Role of CTD	CTD deletion abrogates ATPase activity	CTD deletion abrogates ATPase activity	CTD deletion moderately affects EP formation	CTD deletion impaired protein expression

Table 3. List of diseases linked to P5B-ATPases. *l'**Genetic**' refers to experimental evidence based on identifying gene mutations, while '**expression**' refers to experimental evidence showing changes in the mRNA and/or protein expression in a given disease. '**Contradictory**' points to the lack of consistent correlations between studies reporting the gene-disease relation, *i.e.*, one study shows a positive correlation, while another shows the opposite.

P5B-ATPase	Related Diseases	Type of Evidence *
ATP13A2	Amyotrophic lateral sclerosis [116]	Genetic mutations
	Cancer [159]	Expression (higher/lower based on the type of cancer)
	Dementia with Lewy bodies [81,103,104]	Expression (contradictory)
	Early onset Parkinson's disease [81,83,103-108]	Genetic and expression (contradictory)
	Hereditary spastic paraplegia [82,110-113]	Genetic
	Kufor-Rakeb syndrome 67–76 [83,173-181]	Genetic
	Multiple system atrophy [117]	Genetic
	Neuronal ceroid lipofuscinosis [114,115]	Genetic (in dogs and human)
ATP13A3	Cervical cancer [160]	Genetic and expression

	Head and neck cancer [161]	Expression
	Pancreatic cancers [162] [89]	Expression
	Pulmonary arterial hypertension [139-141]	Genetic
ATP13A4	Autism spectrum disorders [78,132,137]	Genetic
	Childhood apraxia of speech [133]	Genetic
	Language delay [132,137]	Genetic and expression
	Schizophrenia [134,137]	Genetic (contradictory)
	Epileptic Encephalopathies [138]	Genetic

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P5B-ATPases in the mammalian polyamine transport system and their role in disease

Highlights:

1. Cellular polyamine homeostasis relies on a balance between uptake and metabolism.
2. Polyamine transport remains poorly understood, but is disturbed in disease.
3. P5B-ATPases are novel members of the enigmatic polyamine transport in mammals.
4. P5B-ATPases are implicated in major human diseases and emerge as drug targets.
5. Structural insights unveil the P5B-ATPase polyamine transport mechanism.