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Regulations of *myo*-inositol homeostasis: Mechanisms, implications, and perspectives

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ABSTRACT

Phosphorylation is the most common module of cellular signalling pathways. The dynamic nature of phosphorylation, which is conferred by the balancing acts of kinases and phosphatases, allows this modification to finely control crucial cellular events such as growth, differentiation, and cell cycle progression. Although most research to date has focussed on protein phosphorylation, non-protein phosphorylation substrates also play vital roles in signal transduction. The most well-established substrate of non-protein phosphorylation is inositol, whose phosphorylation generates many important signalling molecules such as the second messenger IP_3, a key factor in calcium signalling.

A fundamental question to our understanding of inositol phosphorylation is how the levels of cellular inositol are controlled. While the availability of protein phosphorylation substrates is known to be readily controlled at the levels of transcription, translation, and/or protein degradation, the regulatory mechanisms that control the uptake, synthesis, and removal of inositol are underexplored. Potentially, such mechanisms serve as an important layer of regulation of cellular signal transduction pathways.

There are two ways in which mammalian cells acquire inositol. The historic use of radioactive ^{3}H-*myo*-inositol revealed that inositol is promptly imported from the extracellular environment by three specific symporters SMIT1/2, and HMIT, coupling sodium or proton entry, respectively. Inositol can also be synthesized *de novo* from glucose-6P, thanks to the enzymatic activity of ISYNA1. Intriguingly, emerging evidence suggests that in mammalian cells, *de novo myo*-inositol synthesis occurs irrespective of inositol availability in the environment, prompting the question of whether the two sources of inositol go through independent metabolic pathways, thus serving distinct functions. Furthermore, the metabolic stability of *myo*-inositol, coupled with the uptake and endogenous synthesis, determines that there must be exit pathways to remove this extraordinary sugar from the cells to maintain its homeostasis. This essay aims to review our current knowledge of *myo*-inositol homeostatic metabolism, since they are critical to the signalling events played by its phosphorylated forms.

1. Introduction

Protein phosphorylation cascades are perhaps the most recognizable signal transduction mechanisms where activities of specific protein-kinases are counterbalanced by protein-phosphatases. Controlling protein phosphorylation status will determine their

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functionality, by regulating their activity, localization, and fate (e.g. degradation). Phosphorylation events similarly control the signal transduction capability of *myo*-inositol (Michell, 2008). The combinatorial phosphorylation of this simple sugar could generate 64 different "signalling chips" of *myo*-inositol phosphates (IPs) (York, 2006). This already extraordinary complexity is enhanced by additional kinases phosphorylating a mono-phosphorylated positions generating the *myo*-inositol pyrophosphates (PP-IP) species (Nguyen Trung et al., 2022; Wilson et al., 2013). Furthermore, lipid-associated phosphatidylinositol (PI) is the substrate of specific kinases generating phosphoinositides (PIPs) (Hammond and Balla, 2015). Like protein phosphorylation cascades, the activities of inositol kinases are offset by phosphatases. Although many parallels can be drawn between protein phosphorylation and inositol phosphorylation mechanisms, a key difference exists. While proteins' promptness to phosphorylation could be controlled by their cellular abundance, which is determined by the relative levels of protein synthesis and degradation, *myo*-inositol degradation pathway is inactive in most mammalian cell types. In the absence of degradation, how is the cellular homeostasis of inositol controlled? In other words, does a regulated *myo*-inositol efflux mechanism exist to balance inositol influx and its endogenous synthesis? How does the 'abundance' of *myo*-inositol sustain its role as an osmolyte and influence the synthesis, metabolism and function of metabolites derived from phosphorylated *myo*-inositol? These thoughts underline the importance to appreciate the mechanisms governing *myo*-inositol homeostasis. This assay aims to review the current knowledge on *myo*-inositol homeostatic mechanisms, with the objective to draw attention to a process that potentially exerts the most basic layer of control over osmoregulation, as well as cellular levels of key signalling metabolites such as lipid-associated phosphatidylinositol and water-soluble inositol phosphates.

2. Inositol: a truly versatile molecule

myo-inositol is by far the most abundant inositol isomer present in mammalian cells. This isomer is characterised by having the 2-hydroxyl group (2-OH) in axial position, with the remaining five hydroxyl groups being parallel to the plane of the carbon ring (Fig. 1). Aside from *myo*-inositol, another eight enantiomeric isoforms of inositol exist and phosphorylated forms of four isomers (*muco*-, *neo*-, *scyllo*-, and D-*chiro*-inositol) have been identified, mostly in soil samples (L'Annunziata and Fuller, 1971; L'Annunziata et al., 1972; Turner et al., 2012). The origins and metabolisms of these other enantiomers of inositol are largely unknown and thus are interesting topics attracting growing attention (Thomas et al., 2016; Whitfield et al., 2018). We recommend readers interested in the other isoforms of inositol the following reviews (Gambioli et al., 2021; Thomas et al., 2016) and focus this assay on homeostatic control of *myo*-inositol (simplified as inositol thereafter), with a special emphasis on mammalian cells.

Inositol is an integral component of a diverse range of signalling molecules (Fig. 2). Other than forming inositol phosphates, inositol pyrophosphates and phosphatidylinositol phosphates, it is also an integral part of glycosylphosphatidylinositol (GPI) group which are attached/anchored to proteins (Kinoshita, 2020). In many eukaryotes, including yeast and plants, but with the exception of mammals, inositol is also a key component of sphingolipids such as ceramide phosphoinositol, the sphingolipid analogue of phosphatidylinositol (Gronnier et al., 2016). Archaea have exploited inositol to their benefit, by synthesizing the protective di-inositol-phosphate (DIP) under stress conditions (Wang et al., 2006) (Fig. 2).

In addition, free inositol, i.e. inositol that is not associated with other molecule(s) or chemical group(s), plays a critical cellular role as an osmolyte (Moeckel et al., 2003; Warskulat et al., 1997). The osmolytic function of inositol is evolutionarily conserved and is present in not just in mammalian cells but also in archaea (Rodionov et al., 2007). In mammals, inositol is essential for maintaining osmolarity in organs surrounded by a hyperosmolar environment, such as the kidney, the liver, and the brain (Fisher et al., 2002). Taking the brain as an example, the concentration of inositol in the brain is in the 2–10 mM range, which is far higher than its concentration in the cerebrospinal fluid (CSF) of ~100 μM (Fisher et al., 2002; Isaacks et al., 1994), thus allowing it to serve as an osmoregulator. It is worth remembering that the *SMIT1* Sodio-Myo-Inositol Transporter (see below) gene was cloned from the cDNA library of canine kidney cells (MDCK) treated with hypertonic stress. (Kwon et al., 1991, 1992). Human *SMIT1* is localised on chromosome 21 and is amplified in Down Syndrome patients, resulting in ~50% increase in inositol levels in the brain (Huang et al., 1999; Shetty et al., 1995). It was proposed that elevated levels of inositol might perturb osmotic equilibrium in the affected brains, thus misbalancing key electrolytes and contributing to the reduced intellectual ability phenotype (Shetty et al., 1995; Shonk and Ross, 1995).

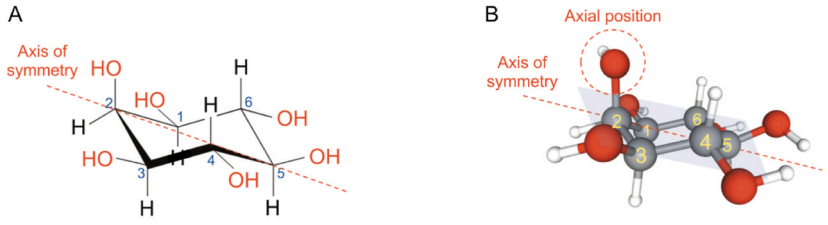

Fig. 1. The structure of *myo*-inositol
The chair conformation of *myo*-inositol (A) highlights the axial hydroxyl position at carbon 2 (2OH), namely that 2OH is perpendicular to the plane of the inositol ring. The other hydroxyl groups at positions 1, 3, 4, 5 and 6 are alternating equatorial moieties with minimized steric hindrance. *myo*-inositol has an axis of symmetry passing through carbons 2 and 5, and therefore carbon 1 and 3, and carbon 4 and 6 are enantiomeric. 3D-conformer of *myo*-inositol (B) is showing the relative size of each atom. Carbons in grey, oxygen in red and hydrogens in white.

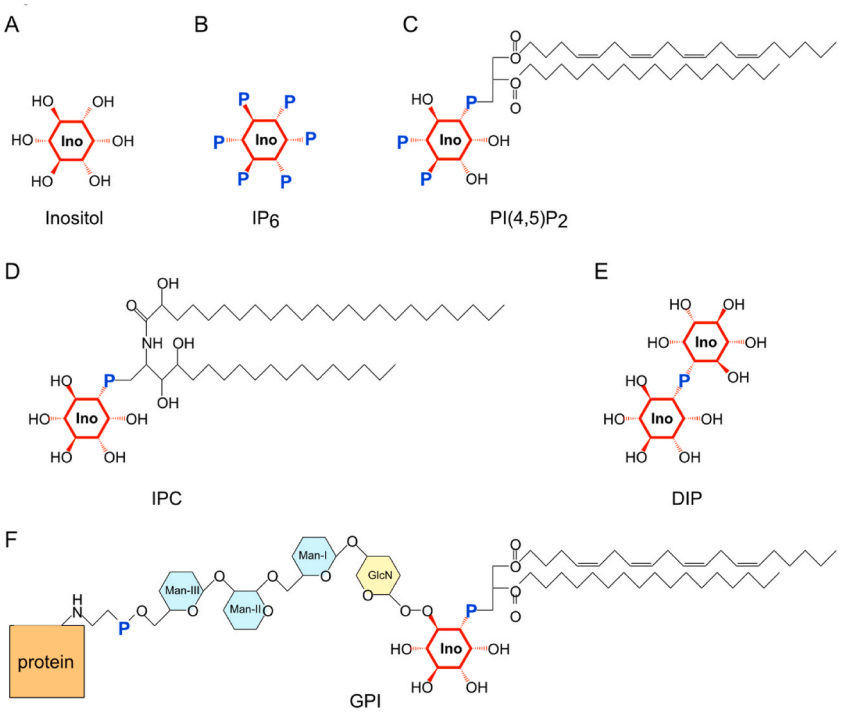

Fig. 2. Inositol: a truly versatile molecule
Mill conformations of *myo*-inositol (A) and molecules derived from *myo*-inositol. Inositol hexakisphosphate (IP_6) or phytic acid (B). Inositide lipid phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) (C). Sphingolipid glycosylphosphatidylinositol (GPI) (D). Archean metabolite di-myo-Inositol-Phosphate (DIP) (E). Glycophosphatidylinositol (GPI) of GPI-anchored proteins (F). Inositol carbon backbones are represented in red. Phosphate groups are depicted in blue.

3. Inositol synthesis and degradation

A few rare cases of organisms (e.g. *Schizosaccharomyces pombe*) are natural inositol auxotrophs and consequently rely on importing inositol from the environment to survive. Most other species are capable of synthesizing inositol endogenously by the sequential actions of two ubiquitously present enzymes (Fig. 3): L-*myo*-inositol-3-phosphate synthase (MIPS, or simply IPS, EC 5.5.1.4) and *myo*-inositol-monophosphatases (IMPA1-2 or IMPases1-2 EC3.1.3.25). MIPS uses glucose-6-phosphate (glucose-6P) as a substrate and NAD^+ as a co-factor to produce inositol-3-phosphate (inositol-3P). The resulting inositol-3P has two fates. In one, it can be dephosphorylated by IMPases to form free inositol, which is then used for phosphoinositide biosynthesis (Fig. 3). Alternatively, it can be phosphorylated by inositol-tetrakisphosphate 1-kinase (ITPK1) to initiate the synthesis of inositol phosphates (Desfougeres et al., 2019) (Fig. 3). The model organism *Saccharomyces cerevisiae* played a pivotal role in the discovery and characterisation of MIPS activity (Culbertson and Henry, 1975; Dean-Johnson and Henry, 1989; Donahue and Henry, 1981). The gene encoding MIPS in yeast, *INO1*, is

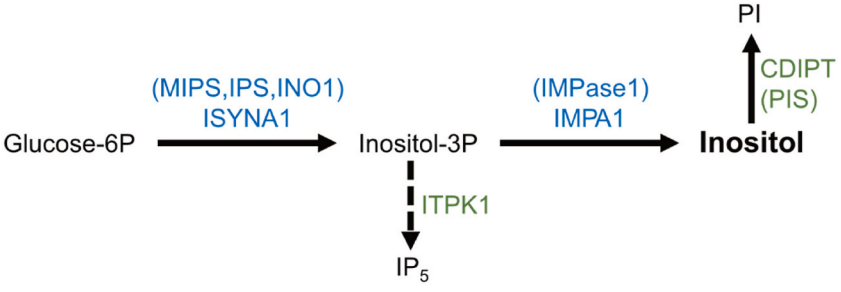

Fig. 3. Inositol's biochemical synthesis
Inositol is synthesized from glucose-6P by the sequential actions of two enzymes. ISYNA1 (Inositol SYNthase A1 (EC 5.5.1.4)), or IPS (Inositol Phosphate Synthase), also known as Ino1 in budding yeast, converts glucose-6P to inositol-3P. IMPA1 (Inositol Monophosphatase 1 (EC 3.1.3.25) or IMPase 1) dephosphorylates inositol-3P to form inositol. Inositol-3P can be used for the synthesis of inositol pentakisphosphate (IP_5) (dashed line) by ITPK1 (Inositol-Tetrakisphosphate 1-Kinase (EC 2.7.1.134)), while CDIPT (CDP-Diacylglycerol–Inositol 3-Phosphatidyltransferase (EC 2.7.8.11)) or PIS (Phosphatidylinositol Synthase) can use inositol to synthesize lipid phosphatidylinositol (PI).

essential for budding yeast's viability in the absence of external inositol (Culbertson and Henry, 1975). Knocking out the mammalian homolog of MIPS, *ISYNA1*, similarly prevented human cell proliferation after a few cell divisions in inositol-free media (Qiu et al., 2020). Consistent with the essential functions of inositol in osmoregulation and synthesis of signalling molecules, *ISYNA1*$^{-/-}$ knockout in HEK293T cells exhibited drastic changes in lipidome and global gene expression profile (Suliman et al., 2022), and *ISYNA1*$^{-/-}$ knockout mice generated by the International Mouse Phenotyping Consortium demonstrated embryonic lethality (Dickinson et al., 2016). Further works will be required to complete the characterization of *ISYNA1*$^{-/-}$ mice and to test whether dietary supplement of inositol in heterozygous mothers could rescue early lethality as it occurred in *IMPA1*$^{-/-}$ or *SMIT1*$^{-/-}$ knockout mice (see below). MIPS homologs are tightly regulated at the level of transcription by the cellular metabolic status. In yeast, *INO1* gene expression is fine-tuned by both transcriptional activators and repressors. It is activated by the transcriptional complex Ino2/Ino4, which senses cellular inositol depletion (Ambroziak and Henry, 1994). Therefore, *INO1* expression is tightly controlled by the availability of inositol (Graves and Henry, 2000). Additionally, *INO1* expression is repressed by Opi1 (White et al., 1991), whose upstream regulator is cAMP-dependent protein kinase A (Sreenivas and Carman, 2003). In higher eukaryotes, MIPS shows developmental stage- and tissue-specific patterns of expression. In adult mice, expression of *ISYNA1* is most prominent in Sertoli cells in the testis and is expressed at lower levels in other organs tested, such as the brain, kidney, lung and ovary (Chauvin and Griswold, 2004). In Sertoli cells, *ISYNA1* expression is induced by hypertonic medium (Chauvin and Griswold, 2004). *ISYNA1* expression is also controlled by the p53 oncogene through a p53 response element located in the seventh exon (Koguchi et al., 2016). Importantly, and in contrary to the yeast *INO1*, mammalian *ISYNA1* transcription is not controlled by extracellular inositol levels (Guan et al., 2003). Furthermore, a recent study suggested that *ISYNA1* expression is upregulated by basic metabolisms, specifically by glucose levels and phosphatidic acid, through their impacts on *ISYNA1* promoter methylation (Lazcano et al., 2022).

To become inositol, the inositol-3P synthesized by ISYNA1 must be dephosphorylated by IMPases such as IMPA1 (Fig. 3). IMPA1 is best known for being a key pharmacological target of lithium, which is commonly used for the treatment of bipolar disorder. Lithium treatment by blocking IMPA1 activity drastically increased inositol-3P level and decreased the ratio of inositol to inositol-3P in both yeast and humans cells (Lopez et al., 1999; Sherman et al., 1985), indicating that lithium-sensitivity is a common feature of IMPases. Nonetheless, analysis of primary cortical neurons showed that lithium treatment increased inositol mono-phosphate (IP) levels but had only a minor impact on inositol levels, prompting a reinterpretation of the so-called 'inositol depletion hypothesis' of lithium treatment (Berridge et al., 1989; Saiardi and Mudge, 2018).

Mammalian cells have two IMPase homologs, IMPA1 and IMPA2. Although several works suggested genetic associations between *IMPA2* and neuropsychiatric diseases, *IMPA2*$^{-/-}$ knockout mice exhibited only mild behavioural changes (Cryns et al., 2007). *IMPA1*$^{-/-}$ knockout mice, on the other hand, were embryonically lethal and these embryos produced significantly less inositol. The embryonic lethality could be rescued by feeding the heterozygous mothers with inositol throughout pregnancy and lactation (Cryns et al., 2008), again suggesting that endogenously synthesized inositol plays a critical role in early embryonic development. Surviving *IMPA1*$^{-/-}$ adult mice showed hyperactive behaviours and increased sensitivity to pilocarpine-induced seizures, indicating that inositol homeostasis is required for adult mice neurophysiology. IMPases are inhibited by lithium in at least two ways. Since IMPases use magnesium as a co-factor, lithium is a noncompetitive inhibitor of IMPases *in vitro* (Hallcher and Sherman, 1980). Lithium also represses IMPases gene expression in both yeast and humans (Murray and Greenberg, 2000; Seelan et al., 2004). In yeast, expression of IMPase *INM1* is activated by inositol and repressed by alternative carbon sources (Murray and Greenberg, 2000), but similar findings have not been discovered in higher eukaryotes.

The drastic phenotypes exhibited by *ISYNA1* and *IMPA1* knockout mice models suggest that endogenous inositol synthesis is key to embryonic development. Given such importance, it is imperative to address several questions regarding the basic biology of endogenous inositol synthesis. First, why does *ISYNA1* show tissue-specific patterns of expression and why are early embryonic development and adult brain particularly sensitive to the absence of ISYNA1 and IMPase? Could the demonstrated physical filtering blockades, of the placenta and of the blood-brain barrier (Spector, 1988; Staat et al., 2012) be restricting the accessibility to exogenous inositol? More importantly, could the endogenously synthesized inositol and exogenously acquired inositol be functionally different, such as belonging to different subcellular pools/compartments (see below)? In addition, do they preferentially enter the lipid synthesis route or the cytosolic route of inositol phosphate synthesis? These important questions have not been fully addressed because of limitations in previously used techniques. Earlier studies on inositol cellular metabolism were exclusively reliant on the use of exogenously added, radiolabelled tritium inositol (^{3}H-Inositol) and subsequent Sax-HPLC analysis (Azevedo and Saiardi, 2006). This approach suffers from a key weakness, which is that it is entirely blind to any metabolites originated from ISYNA1-mediated synthesis of non-radiolabelled inositol-3P. Hence, ISYNA1's relative contributions towards inositol homeostasis in key contexts such as embryonic development and normal functioning of the brain is a virtually unexplored area of research. Therefore, we should now take advantage of recent technical breakthroughs, such as HILIC-MS/MS, and CE-MS to properly address these questions (Ito et al., 2018; Qiu et al., 2020).

Furthermore, it is worth noticing that some inositol was still synthesized in *ISYNA1*$^{-/-}$ knockout cell lines before they stopped dividing in inositol-free media (Qiu et al., 2020). This is an intriguing finding, as it has been believed for decades that ISYNA1 is the sole enzyme responsible for endogenous inositol synthesis. Whether this is an indication of an alternative biosynthetic pathway will require further investigation.

Inositol degradation is less studied than inositol synthesis because the former is largely inactive in most mammalian cell types. Inositol degradation is initiated by *myo*-inositol oxygenase (MIOX, EC 1.13.99.1), which carries out oxidative cleavage of inositol to form D-glucuronic acid (Reddy et al., 1981). MIOX activity was initially detected in rat kidney homogenates (Charalampous, 1959; Charalampous and Lyras, 1957), and was subsequently found to be a gene broadly conserved from bacteria to humans. In many species of bacteria such as *Bacillus subtilis*, the function of MIOX is to break down inositol to use it as a carbon source (Ramaley et al., 1979). Although higher eukaryotes carry *MIOX* genes, the physiological significance of MIOX activities appears limited. For instance,

knocking down all four *MIOX* homologs in *Arabidopsis* increased inositol concentrations but did not cause any defects in plant growth or development (Endres and Tenhaken, 2011). It was hypothesized that MIOX activity may be utilized for L-ascorbic acid biosynthesis in plants (Lorence et al., 2004) but the hypothesis was not supported by radioactive tracing experiment using ^{3}H-myo-inositol (Ivanov Kavkova et al., 2019). In mammals, tracing $^{14}C_2$-inositol in different organs demonstrated that MIOX activity, as manifested by the release of $^{14}CO_2$ (D-glucuronic acid undergoing oxidative phosphorylation will release CO_2), was only detected in the cortex and medulla regions of the kidney (Howard and Anderson, 1967). Decades later, Sax-HPLC experiment using cell lines and ^{14}C-inositol labelled inositol showed that labelled atoms only ended up in phosphatidylinositol phosphate or inositol phosphates and not in other organic molecules that could derive from D-glucuronic acid metabolism, supporting the idea that inositol degradation does not occur in most cell types other than the kidney. While it is tempting to hypothesize that MIOX is required for the clearance of inositol from the urine, the presence of inositol in urine samples of healthy individuals (An et al., 2019) suggests that MIOX activity has limited importance under normal circumstances. Consistently, a recent study showed that *MIOX* knockout mice did not show any noticeable phenotypes, although *MIOX* overexpression did exacerbate renal dysfunction in streptozotocin-induced diabetic mice (Sharma et al., 2020). As *MIOX* expression was shown to be upregulated by hyperosmolarity (Prabhu et al., 2005), it is possible that MIOX activity acts as a fail-safe mechanism to support renal functions under certain circumstances, perhaps by regulating the availability of inositol as an osmolyte.

To summarize, the consensus in the field is that although inositol degradation is required by microorganisms, mainly bacteria, for carbon assimilation, the pathway is only active in limited cell types of the kidney and may only become important under certain disease conditions (An et al., 2019).

4. Inositol cellular import and export

Inositol enters mammalian cells using two types of transporters, differentiated by the nature of the co-transported cations: sodium or proton. Mammals carry two sodium-inositol symporters: SMIT1 and SMIT2, encoded by *SLC5A3* and *SLC5A11* genes, respectively (Schneider, 2015). Only one proton-inositol symporter has been identified, which is HMIT (H^+-*myo*-inositol symporter) encoded by the *SLC2A13* gene (Uldry et al., 2001) (Fig. 4).

Both types of inositol transporters belong to the Solute Carrier (SLCs) family of transporters, which represents the largest group of transporters with >400 of members subdivided into several dozens of different families and are primarily but not exclusively dependent on ion (sodium) or electrochemical (proton) gradient to transport specific metabolites. The solute carrier family 5 includes 12 different members and actively transport metabolites using a sodium gradient. Within the SLC5 transporter family, two transport inositol (SLC5A3-11), five transport glucose (SLC5A1-2-4-9-10), and the rest transport diverse non-sugar metabolites such as biotin, acetylcoline, or small acidic molecules like lactate and pyruvate (Cannizzaro et al., 2019). The solute carrier family 2 includes 13 different members (Holman, 2020; Uldry and Thorens, 2004). Twelve of them carry out bi-directional transport of glucose or fructose by facilitated diffusion down a concentration gradient and represent the glucose transporter family GLUT1-12 (SLC2A1-12) (Byers et al., 2017). The remaining member is HMIT (SLC2A13) that couples proton to inositol transport and is able to transport inositol against its concentration gradient (Uldry et al., 2001). However, the subcellular localization of HMIT is controversial. The HMIT protein contains an endoplasmic reticulum retention signal and an internalization motif. Only after mutagenizing these amino acid sequences, it was possible to assess the inositol transport capability of HMIT using Xenopus oocyte (Uldry et al., 2001). Intact HMIT is localised on intracellular vesicles (Uldry et al., 2004) or on membranous structures around the nucleus (Di Daniel et al., 2009). Hence, it was speculated that HMIT is either present at an undetectable but active amount on the plasma membrane (Fu et al., 2012), or that it

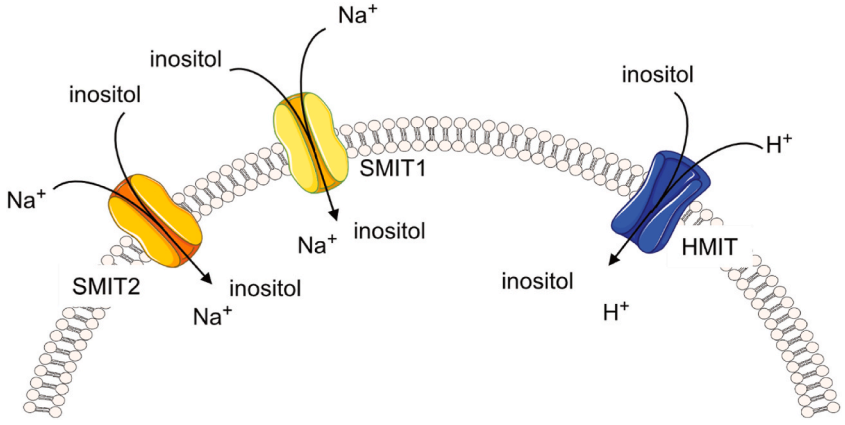

Fig. 4. Mammalian inositol transporters
Three active membrane transporters have been identified. The sodium-inositol symporter SMIT1 (Sodium/Myo-Inositol Cotransporter 1), also known as SLC5A3 (Solute Carrier Family 5 Member 3) was drawn in yellow, and SMIT2 (Sodium/Myo-Inositol Cotransporter 2), also known as SLC5A3 (Solute Carrier Family 5 Member 11) was represented in orange. Proton-inositol symporter HMIT (Proton Myo-Inositol Symporter), also known as SLC2A13 (Solute Carrier Family 2 Member 13) was drawn in blue.

is translocated to the plasma membrane in a signal-dependent manner (Uldry et al., 2004).

Interestingly, although inositol transporters cannot transport glucose, they exhibit some flexibility in terms of the inositol isomer transported. SMIT1 can transport both *myo*- and *scyllo*-inositol and SMIT2 has similar affinity for D-*chiro*- and *myo*-inositol (Hager et al., 1995; Lin et al., 2009). Likewise, HMIT can transport *myo*-inositol, *scyllo*-, *muco*- or *chiro*-inositol (Uldry et al., 2001).

As mentioned earlier, SMIT1 plays a key role in osmoregulation. Indeed, the expression of *SMIT1* is upregulated under hypertonic stress and is under the control of TonEBP (Tonicity-responsive Enhancer Binding Protein) transcription factor (Rim et al., 1998; Shin et al., 2012). *SMIT1*$^{-/-}$ knockout mice die from respiratory failure a few hours after birth. Like the *IMPA1*$^{-/-}$ knockout mice, feeding heterozygous mothers with additional inositol rescued the lethality phenotype of the homozygous offspring (Buccafusca et al., 2008). Surviving adult *SMIT1*$^{-/-}$ knockout mice exhibited reduced level of inositol in the brain, neurological disorders, and alterations in bone formation/structure (Dai et al., 2011). Mice model for *SMIT2* remains to be developed. HMIT was believed to be the primary inositol transporter of neurons. However, cortical neurons from *HMIT*$^{-/-}$ mice, which do not express *SMIT1-2* either, were still able to uptake inositol (Di Daniel et al., 2009). This surprising discovery suggests that additional, yet unknown inositol transporter(s) must exist.

Since inositol can be both synthesized endogenously and imported from the environment, yet a degradation pathway is missing in most cell types, it is reasonable to speculate that a highly regulated exit pathway is present to facilitate inositol cellular homeostasis. However, surprisingly little is known about such a pathway. Electrophysiological studies in the early 1990s proposed the existence of a volume-sensitive organic osmolyte and anion channel (VSOAC), also known as volume-regulated anion channel (VRAC), which allows the cellular exit of anions but also of uncharged metabolites such as inositol (Goldstein and Davis, 1994; Jackson and Strange, 1993; Strange et al., 1993). VRAC channels are heteromers formed from various combinations of LRRC8A (Leucine Rich Repeat Containing 8 Family Member A) and other LRRC8 proteins (B, –C, -D, or -E) (Ghouli et al., 2022). Recently, a VRAC containing LRRC8D subunit has been reported to transport non-polar metabolites including inositol in response of cell swelling (Lutter et al., 2017). However, cell swelling is a rare event that only occurs as an acute response to hypoxia, or to direct damages on the cell membranes by lipid peroxidation or pore-forming toxins (Miller and Zachary, 2017). Hence, VRAC-mediated inositol export is unlikely to be relevant to normal cells, and so future studies are required to uncover the alternative export pathway(s) of inositol.

The strikingly balanced influx and efflux of radiolabelled inositol was recently characterized in primary cortical neurons (Saiardi and Mudge, 2018). This study used steady-state as well as pulsed ^{3}H-inositol labelling to study inositol fluxes and revealed that newly imported inositol can be rapidly used for the synthesis of phosphoinositides. More importantly, the rate of inositol efflux was increased upon carbachol- or potassium chloride-induced depolarization to such a degree suggesting that most inositol coming from the hydrolysis of phosphoinositides equilibrates rapidly with extracellular inositol. Therefore, inositol influx and efflux are not just balanced in unstimulated neurons but are also rapidly coupled to the recycling of inositol trough the phosphoinositides cycle (Saiardi and Mudge, 2018). Since neuronal activities induce synaptic vesicle release and membrane recycling through the endocytic pathway, the authors hypothesized that inositol could enter and exit cells via a vesicular intermediate (Saiardi and Mudge, 2018). This theory awaits experimental validation.

5. Perspective

The above paragraphs, which do not intend to provide an exhaustive review of the literature, discussed key aspects of inositol synthesis, degradation, import and export. Unlike some species of bacteria, the vast majority of higher eukaryotes and mammalian cell types do not degrade inositol to use the carbon backbone for energy production. This unique metabolic stability makes inositol an ideal precursor for generating a myriad of phosphorylated signalling molecules (Livermore et al., 2016). Like other precursors of signalling molecules, the cellular concentration of inositol must be tightly regulated to cope with the changing demands of the cell. In the absence of a degradation mechanism, inositol cellular homeostasis is achieved by the balancing acts between influx through the plasma membrane, endogenous synthesis, and cellular export. Unfortunately, most of the studies up to date focussed on the three pathways separately and future works are required to gain a comprehensive view on how they cooperate with each other to achieve inositol homeostasis.

We particularly would like to emphasize that although inositol cellular exit is potentially a key contributor to inositol homeostasis, the underlying molecular pathways are virtually uncharacterised. This area of research is worthy of future explorations, because it is both important to our understanding of inositol homeostasis and to our knowledge of how inositol is absorbed as a nutrient by the intestine. A recent study using chicken small intestine (Rohm et al., 2022) highlighted the unclear and unforeseen complexity of inositol intestinal transport. The key missing information is the identities of the transporters deployed to export inositol across the basolateral membrane of the intestine to enter the bloodstream. While SMIT1/2 and HMIT may be involved in the absorption of inositol from the luminal side of the intestine, they cannot function against the sodium or proton gradients which is characteristic of transport across the basolateral membrane. Therefore, future works are required to identify the transporters that release inositol from the intestine into the bloodstream.

Many of the lacunas in our understanding of inositol cellular homeostasis stemmed from the methodological challenges to study this important sugar. While it is relatively easy to study inositol cellular entry by following the cellular accumulation of a radioactive tracer (^{3}H-inositol or ^{14}C-inositol), studying inositol exit is technically difficult, since cells must be pre-labelled with radioactive inositol. The recent development of stable isomers of inositol such as $^{13}C_6$-Inositol would circumvent the tedious use of radioactive tracers and allow the use of mass spectrometry (MS) (Qiu et al., 2020) or ^{13}C-NMR (Harmel et al., 2019) techniques to trace $^{13}C_6$-Inositol cellular entry. Moreover, while the use of MS to follow a stable isotopic tracer is relatively easy, it has limited use in the study of endogenously synthesized inositol. Glucose and inositol have identical mass, and so a simple MS protocol would not be able to

distinguish between the two sugars. Protocols that take advantage of the different chemistry of glucose (a reducing sugar) and inositol have been developed but are complicated by the extra derivation steps (Ratiu et al., 2019). The recent development of selective HILIC-amide columns capable of resolving polar compound as saccharide (Nguyen et al., 2020) could pave the way to developing simple LC-MS approaches that allow simultaneous studies of endogenous inositol synthesis and inositol plasma membrane fluxes, thereby improving our understanding of their relative contributions to inositol homeostasis.

The compartmentalization of phosphoinositide signalling pathways is an established concept (Di Paolo and De Camilli, 2006; Hammond and Balla, 2015), as membranes of different organelles carry distinct combinations of phosphoinositides. This compartmentalization is maintained by differential localizations of inositol-lipid kinases and phosphatases and is easy to conceptualise since phosphoinositides are membrane-bound and so cannot freely diffuse into the nearby soluble space. Although inositol and inositol phosphates are water-soluble, several works suggest that these molecules may also be compartmentalized. The influx and efflux of inositol observed in stimulated neurons are rapidly coupled to the recycling of phospholipase C (PLC)-generated IP_3 to inositol (Saiardi and Mudge, 2018), suggesting that a compartmentalized phosphoinositide synthesis mainly uses inositol acquired from the extracellular milieu. Biochemical compartmentalization of the lipid component of the phosphoinositide cycle (Michell, 1975) has been recently detected, based on the finding that PLC-generated diacylglycerol (DAG) is the main source of the lipid backbone for the re-synthesis of phosphoinositol after cell stimulation (Barneda et al., 2022; Kim et al., 2022). Therefore, a PLC-important phosphatidylinositol pool must be coupled with dedicated inositol plasma membrane fluxes.

The cytosolic pathway leading to the formation of IP_6 is dependent on the inositol monophosphates generated by ISYNA1 (Desfougeres et al., 2019). Thus, it is reasonable to propose that the lipid and cytosolic metabolic pathways of inositol metabolism may be compartmentalized, or at least partially physically separated. Of note, inositol kinase activities have not been described in mammalian cells, and our own work suggested that such activities are undetectable in mammalian cell extracts (Saiardi lab unpublished). Therefore, inositol imported from the extracellular environment must first become a phosphoinositide, and only after PLC-mediated cleavage, IP_3 could be metabolized into other inositol phosphates. Overall, the above arguments are supportive of the idea that the inositol acquired from the environment and that synthesized endogenously might influence different pools of phosphoinositides and inositol phosphates. Furthermore, it remains unknown which route is the primary source of free inositol that performs osmoregulation. Ostensibly, imported inositol is likely to fulfil this role because inositol transporter SMIT1 is involved in cellular response to osmotic stress (Kwon et al., 1992; Shin et al., 2012), however, ISYNA1 expression is also controlled by osmotic changes (Chauvin and Griswold, 2004) and so endogenously synthesized inositol may also contribute. Crucially, the inositol pool used for osmoregulation is likely to be separated from the pool/s controlling the synthesis of the phosphorylated signalling molecules, because the latter requires far lower amount of inositol. The larger pool of inositol regulating osmolality might minimally influence inositol phosphates cycle/s that do not lead to the full dephosphorylation to inositol. This idea is supported by the observation that IP_6 and IP_2 are metabolically linked in multiple inositol polyphosphate phosphatase 1 (MINPP1) knockouts (Ucuncu et al., 2020). The very recent and thoughtful $^{13}C_6$-NMR analysis of MINPP1 activity revealed a distinctive IP_6 catabolism to IP_2 (Trung et al., 2022). Hence, an inositol phosphate cycle IP_2 to IP_6 and back to IP_2 would be only minimally influenced by fluctuations within the pool of inositol serving as an osmolyte. While more experimental work is required to test the hypothesis, these considerations should stimulate research efforts aimed at studying different pools of inositol and its derived molecules inside the cell.

Although biochemical approaches have provided hints for the existence of different pools of inositol, ultimately, we will need imaging tools to 'visualise' these pools by microscopy. Bio-sensors, like those used to detect specific phosphoinositides (Wills et al., 2018), or more accurate techniques that allows direct imagining of inositol by chemical-physical properties such as Raman spectromicroscopy (Kolozsvari et al., 2015) or multi-isotope imaging mass spectrometry (MINS) (Saiardi et al., 2014), will revolutionise our knowledge on inositol homeostasis and soluble inositol-phosphates signalling.

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Author declaration

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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CRediT authorship contribution statement

Xue Bessie Su: Conceptualization, Writing – original draft, Writing – review & editing. **An-Li Andrea Ko:** Visualization, Writing – review & editing. **Adolfo Saiardi:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interests. The funding bodies do not have any role in the study design.

Data availability

No data was used for the research described in the article.

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Abbreviations

CDIPT	CDP-diacylglycerol-inositol 3- phosphatidyltransferase
CSF	cerebrospinal fluid
DAG	diacylglycerol
DIP	di-*myo*-inositol-phosphate
GLUT	glucose transporter family
GPI	glycosylphosphatidylinositol
HMIT	proton/*myo*-inositol co-transporter
IMPA	*myo*-inositol-monophosphatase
INO1	inositol requiring (budding yeast inositol-3-phosphate synthase)
IP	*myo*-inositol phosphate
IP_5	inositol pentakisphosphate
IP_6	inositol hexakisphosphate, phytic acid
IPS	inositol phosphate synthase
ISYNA1	inositol-3-phosphate synthase 1 (mammals)
ITPK1	inositol tetrakisphosphate 1-kinase
LC-MS	liquid chromatography-mass spectrometry
LRRC8	leucine riche repeat containing 8 family member
MDCK	canine kidney cells
MINPP1	multiple inositol polyphosphate phosphatase 1
MINS	multi-isotope imaging mass spectrometry
MIOX	inositol oxygenase
MIPS (IPS)	L-*myo*-inositol-3-phosphate synthase
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
PI	phosphatidylinositol
PLC	phospholipase C
PIP	phosphoinositide
$PI(4,5)P_2$	phosphatidylinositol (4,5) bisphosphate
PIS	phosphatidylinositol synthase
PP-IP	*myo*-inositol pyrophosphate
SLC	solute carrier family of transporters
SMIT1/2	sodium/*myo*-inositol co-transporter −1 or −2
VRAC	volume-regulated anion channel
VSOAC	volume-sensitive organic osmolyte and anion channel

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