IP6K

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Inositol and its phosphate metabolites play a pivotal role in several biochemical pathways and gene expression regulation: inositol pyrophosphates (PP-IPs) have been increasingly appreciated as key signaling modulators. Fluctuations in their intracellular levels hugely impact the transfer of phosphates and the phosphorylation status of several target proteins. Pharmacological modulation of the proteins associated with PP-IP activities has proved to be beneficial in various pathological settings. IP7 has been extensively studied and found to play a key role in pathways associated with PP-IP activities. Three inositol hexakisphosphate kinase (IP6K) isoforms regulate IP7 synthesis in mammals. Genomic deletion or enzymic inhibition of IP6K1 has been shown to reduce cell invasiveness and migration capacity, protecting against chemical-induced carcinogenesis. IP6K1 could therefore be a useful target in anticancer treatment.

Keywords: cancer progression ; ip6k1 ; inositol-hexakisphosphate

1. Introduction

Inositol is a ubiquitous polyol involved in a number of essential processes in living organisms. *Myo*-inositol is physiologically the most important of nine isomers and is the precursor of a bewildering number of complex inositol-containing molecules, including inositol phosphates [1][2]. Inositol compounds are essential for many biological functions in living cells: membrane biogenesis [3], trafficking [4], signal transduction, and regulation of gene expression [5]. Inositol phosphates are prominent mediators of these processes. Inositol-1,4,5-trisphosphate (IP_3) has been widely investigated as an intracellular second messenger [6][7][8]. It is metabolized to a large number of additional inositol polyphosphates that also function as cell signals [9]. Among these, inositol hexakisphosphate (IP_6), also known as phytic acid, is the most abundant inositol polyphosphate found in eukaryotes, identified as the principal phosphate-storage molecule in plant seeds [10][11]. It is involved in regulation of trafficking [12] as well as in several nuclear events [13][14]. Inositol hexakisphosphate is the building block to which successive phosphate groups are added to yield inositol pyrophosphates (PP-IPs) [15][16], where as many as one or two energetic di(β)phosphates bonds are crammed around the six-carbon inositol ring [17]. This class of molecule recently gained appreciation as critical modulators of a huge number of “signaling” pathways [18][19]. As proof of concept, PP-IPs show high turnover as their intracellular levels fluctuate significantly in various pathological disorders, including cancer [20].

2. IP_6Ks: Balance, Activity, and Regulation in Physiological Homeostasis and Cancer

IP_6Ks have been identified in several organisms [21][22][23]. In mammals, the three isoforms identified [24][25] have distinct sequences that are selectively involved in protein–protein interactions and post-translational modifications [25]. These regions of IP6Ks protein sequence regulate the activity, stability, subcellular distribution, and target proteins of IP_6Ks [26][27]. The isoforms also differ in tissue expression. In humans, IP_6K1 is widely expressed, while IP_6K2 is higher in the breast, thymus, colon, adipose tissue, testis, prostate, and smooth muscle. In heart and skeletal muscle, IP_6K3 is the most expressed form [28]. The IP_6Ks belong to the same family of inositol phosphate kinases as IP_3K (IP_3-kinase) and IPMK (inositol phosphate multikinase), all characterized by a common PxxxDxKxG motif in the inositol binding region [29]. On the contrary, $PPIP_5K1$ and $PPIP_5K2$—homologs of the yeast enzyme Vip1—do not belong to the inositol phosphate kinase family, as they have a histidine acid phosphatase-like domain in the C-terminal portion of the protein in addition to the kinase domain [30].

IP_6Ks can phosphorylate IP_6 to 5-IP_7 and IP_5 to PP-IP_4 [31]. It is arguable that the relative affinities of a given IP_6K for IP_6 over IP_5 vary in different organisms, from yeast to mammals. For instance, in humans, IP_6K2 displays a 20-fold higher affinity for IP_6 than for IP_5, while IP_6K1 shows a 5-fold higher K_M (concentration of substrates when the reaction reaches half of Vmax) for IP_6 than for IP_5 [21].

Furthermore, measurement of IP_6Ks has advantages with respect to direct quantitation of PP-IPs. Estimation of inositol PP-IPs suffers from a number of problems, including intrinsically higher chemical reactivity and a higher degradation rate, which can be ascribed to the intrinsic acidic phosphatase domain of $PPIP_5K$ and to the hydrolytic activity exerted by DIPP (diphosphoinositol-phosphate phosphohydrolase) proteins [32]. Indeed, previous studies have been unable to detect a change in PP-IPs in response to biochemical/metabolic stimuli [17], although further investigations have provided compelling evidence in support of this hypothesis [33]. On the other hand, noncatalytic functions of IP_6K could make tricky the association with PP-IPs signaling. It has also been demonstrated that PP-IPs turn over rapidly (recruiting up to 50% of the IP_6 pool), depending on chemical (ATP and fluoride) stimulus [16] or during specific cell phase transitions, such as those of the cell cycle [34].

The activity of IP_6K is closely coupled to activation of G protein signaling. G protein-coupled receptor (GPCR) activation through overexpression of $G_{\alpha q}$ fosters phospholipase-C-dependent release of IP_3 by phosphatidyl-inositol-bisphosphate (PIP_2) cleavage [35]. In turn, the increased availability of IP_3 provides the substrate for inositol kinases to produce a plethora of inositol phosphates (chiefly, IP_6 and IP_5) and inositol pyrophosphates (PP-IPs). Overexpression of IP_6K only results in a minimal increase in PP-IPs, even in the presence of high levels of IP_5 and IP_6, while when IP_6K is overexpressed together with GPCR activation, a significantly increased release of PP-IPs has been recorded [35]. These findings suggest a cooperative network linking GPCR and IP_6Ks, which can tune inositol metabolism by acting as an “IPK-dependent IP code” [35]. This hypothesis has contributed to a revision of the role traditionally attributed to IP_6. It is widely agreed that inositol hexakisphosphate displays a bewildering number of physiological and pharmacological activities [10]. However, the IPK-dependent IP code hypothesis may substantiate the suggestion made 20 years ago by Shears [12] who proposed that the critical importance of IP_6 may depend on being a tipping point between IP_3 and the successive generation of IPs. Indeed, increasing evidence in recent years has provided sound confirmation that it is the further phosphorylation of IP_6 to IPs that yields physiologically active metabolites [36]. Any factor that potentiates IP_3 release through phospholipase-C activation is likely to reduce PIP_2 levels while promoting inositol phosphokinase (IP_6K) activity. Accordingly, phospholipase-C and IP_6K both seem to play a potentially critical role in several biological pathways.

2.1. IP_6K1

IP_6K1 has been implicated in biological processes, such as energy metabolism, insulin signaling, trafficking, chromatin remodeling, cell migration, cancer metastasis, and neutrophil functions.

Recent studies suggest that in IP_6K1-KO mice models, IP_6K1 suppression increases energy expenditure by stimulating the protein kinase AMPK [37][38]. AMPK and Akt are significantly modulated under insulin stimulation [39]. IP_6K1 could modulate AMPK and Akt activities by interfering with insulin release. The link between IP_6K1 and Akt merits detailed discussion. Akt resides in the cytosol in an inactive conformation and translocases to the plasma membrane after cell stimulation. The Akt pleckestrin homology domain has a high affinity for PIP_3, which promotes Akt translocation to the membrane [40]. The Akt/PI3K interaction causes conformational changes and subsequent PDK1-dependent phosphorylation at the Thr^{308} kinase domain. However, full activation requires a further phosphorylation at S473, catalyzed by several enzymes, including PDK2 and ILK. IP_7 competitively binds to the PH domain, thus preventing its phosphorylation and activation by PDK1. Notably, IP_7 strongly inhibits Akt activation, with an IC50 of 20 nM, close to the Kd (35 nM) displayed by PIP_3 in respect to the PH domain of Akt [41]. IP_6K1 knockout leads to increased PDK1-dependent Akt activation, determining a plethora of biochemical consequences for metabolic regulation, not yet well investigated. Indeed, after glucose stimulation and subsequent increase in the ATP/ADP ratio, a significant increase in IP_7 was observed. In detail, IP_7 production by IP_6K1 inhibits the stimulatory effect of IP_6 on AMPK. The response of IP_7 to the increase in ATP/ADP ratio occurs a few minutes (10–30) after the stimulus. In turn, IP_7 associates with the Akt PH domain, preventing interaction with PIP_3 and therefore reducing Akt membrane translocation and consequent insulin-stimulated glucose uptake. This mechanism involves feedback, whereby increased availability of ATP drives the system to inhibit glucose uptake by modulating insulin transduction by blocking Akt membrane recruitment [42][43][44]. This regulation may also be indirectly affected by IP_7-promoted nuclear localization of LKB1. Nuclear transfer of LKB reduces LKB cytosolic activity, thus hindering AMPK phosphorylation and activation [45]. It is worth noting that RNAi silencing of IP_6K1 blocks IP_7 and insulin release after glucose stimulation. In IP_6K1-KO models, changes in the intracellular IP_6/IP_7 ratio increase AMPK activation [46]. Conversely, Akt signaling is significantly increased, leading to a decrease in GSK3b phosphorylation, and augmented protein translation. Reduction in GSK3b phosphorylation increases its catalytic activity and is likely be followed by a surge in adipogenesis and diminished glycogen levels [47]. Indeed, after insulin stimulation, IP_7 decreases (from 33% to 60%) in IP_6K1 knockout hepatocytes, whereas Akt and GSK3β increase, improving glucose tolerance, presumably due to a decrease in hepatic glucose production [48]. Conversely, overexpression of IP_6K1 finally impairs insulin-signaling transduction, whereas IP_6K1 silencing may lead to insulin hypersensitivity, as observed in IP_6K1 KO mice. As proof of concept, a number of animal models of insulin hypersensitivity share the common biochemical signature

of an increased tier of Akt activation and translocation [49]. Furthermore, in mouse embryo fibroblasts (MEFs), IP_6K1-induced energy expenditure inhibition leads to reduction of glycolysis via IP_7-mediated destabilization of the interaction between the transcriptional activators of glycolytic genes (GCR1 and GCR2) [50].

Although IP_6K2 proves sensitive to ATP/ADP fluctuations and may induce IP_7 synthesis, it is unlikely that it could act as a sensor of energy requirements, as does IP_6K. This apparent conundrum can be explained if we consider the cell compartmentalization of IP_6K. In fact, while IP_6K1 is usually found in the cytosol and nucleus, IP_6K2 is almost all in the nucleus [51].

2.2. IP_6K2

A number of studies suggest an essential role for IP_6K2 in cell death, migration, cancer metastasis, and progression. IP_6K2 activity sensitizes a number of cancer cells, including OVCAR3, HeLa, HEK293, PC12, and HL60, to apoptosis [52][53][54][55]. Deletion of IP_6K2 prevents apoptotic consequences of γ-irradiation or β-interferon addition to ovarian cancer cells, while overexpression of IP_6K2 significantly raises cell death rate under the same conditions [53]. Overexpression of IP_6K2 augments the cytotoxic effects of many cell stressors, whereas transfection with a dominant negative IP_6K2 decreases cell death. It is noteworthy that the apoptosis surge is associated with increased synthesis of IP_7 and transfer of IP_6K2 from nuclei to mitochondria, while no changes are recorded in the intracellular localization of the other IP_6K isoforms [52]. In detail, IP_6K2 directly mediates IFNβ-induced apoptosis [52] by enzymically regulating p53 activity and by increasing expression of the Apo2L/TRAIL ligand that initiates apoptosis through death-receptor signaling. Namely, HSP90 physiologically binds IP_6K2 and inhibits its catalytic activity. By interfering with HSP-IP_6K2 binding, HSP90 fosters IP_6K2 activation that ultimately leads to increased cell apoptosis [56]. Nuclear localization of IP_6K2, promoted by interaction with HSP90, is a mandatory step for establishing proper IP_6K2-p53 binding. [57]. Indeed, IP_6K2 has been demonstrated to directly modulate p53-dependent apoptosis. Gene disruption of IP_6K2 in colorectal cancer cells selectively impairs p53-mediated cell death and favors cell cycle arrest [57]. This interaction suppresses phosphorylation of the cell cycle arrest regulator (p21) and its transcription, while enhancing p53-mediated apoptosis [58]. This implies that IP_6K2 acts as a switching factor, driving p53 activity towards apoptosis rather than cell cycle arrest. It should be noted that although IP_6K2 regulates p53 by direct binding, its catalytic activity generating IP_7 is essential for its influence on p53 signaling. It has also been observed that IP_6K2 can promote apoptosis independently of its enzyme activity. By interacting with TRAF2, IP_6K2 interferes with apoptosis and nuclear factor kappa β (NF-kβ) signaling, thus affecting the release of tumor necrosis factor α (TNFα) [27]. The proapoptotic activity of IP_6K2 is successfully antagonized by heat-shock proteins (HSPs). Overall, these findings suggest that IP_6K2 actively participates in the regulation of the Apo2L/TRAIL cell death pathway. Moreover, PP-IPs modulate cell death and telomere length in yeast by antagonizing the homolog of ataxia telangiectasia mutated (ATM) kinase, a regulator of the DNA damage response and apoptosis in mammals [59].

As strong as IP_6K2-mediated apoptosis may be, IP_6K2 participation in the regulation of such functions through its nuclear [60], mitochondrial [53], and cytosolic [54][61] localization requires further investigation.

As observed in IP_6K1-KO models, IP_6K2-KO, too, reduces cell–cell adhesion, growth, spreading, metastasis, and FAK phosphorylation in cancer cells. The molecular mechanisms so far proposed include LKB1 sequestering in the nucleus and inhibition of cytosolic phosphatase activation, and consequently, FAK dephosphorylation [59]. Remarkably, IP_6K1 and IP_6K2 both favor sequestering of LKB into the nucleus in an inactive form [45][61].

The tumor suppressor LKB1 is credited with inhibiting FAK activation [62] and enhancing E-cadherin expression [63], thus inhibiting motility and invasiveness. These findings strongly suggest that LKB1 plays a critical role in controlling the balance between cell–cell and cell–matrix adhesion. In addition, by modulating AMPK activity, LKB1 interferes with a number of critical metabolic processes [64]. Interaction with two subunits of the heterotrimeric holoenzyme (STRAD and Mo25) in the cytosol leads to phosphorylation of LKB1 at serine-428 and then activation by PKCδ [65]. This finding is worth mentioning as it suggests that IP_6K2/IP_7 can fine tune the activity of “constitutive” kinases, like PKCδ and CK2 [37], as previously indicated.

Indeed, a number of results have clearly established that deletion of IP_6K1 or IP_6K2 reduces cell migration, while IP_6K2-KO, quite paradoxically, reduces tumor volume [66]. IP_6K2-KO cells display almost total loss of IP_8 levels, whereas only a small decrease in IP_8 levels was recorded in IP_6K1-KO [58][67]. It is tempting to speculate that persistent IP_7 synthesis, even at a lower rate, is mandatory for apoptosis, as previously suggested. However, somewhat paradoxically, complete suppression of IP_6K2 enhances development of carcinoma of the gastrointestinal tract in mice [68], probably because IP_6K2-dependent pyrophosphate synthesis may in turn activate p53 and protein kinase CK2, thus promoting apoptosis [2]. In IP_6K2 knockout mice, a substantial increase in tumorigenesis in response to 4-nitroquinoline-1-oxide, a UV-mimetic carcinogen, has been observed [69]. These findings provide indirect confirmation of the link between IP_6K2 and p53, as

p53-mediated apoptosis is required for apoptosis induced by UV-mimetic factors. However, unlike p53 knockouts models, the IP_6K2 mutants do not develop spontaneous tumors. This apparently odd behavior suggests that IP_6K2 may only influence p53 proapoptotic activity when the system is exposed to a carcinogen stressor but does not directly entail "spontaneous" carcinogenesis.

In ovarian carcinoma cells, IP_6K2 deletion confers protection against interferon alpha (IFNα)-induced cell death, whereas overexpression of IP_6K2 enhances the apoptosis rate promoted by IFNα and/or γ-irradiation [70]. Yet some controversial results have also been reported, since under estradiol stimulation, β-catenin-induced oncogenesis significantly increases IP_6K2 gene expression downstream of the Wnt/β-catenin signaling pathway [71]. Overexpression of IP_6K2 presumably leads to increased pyrophosphate synthesis, reducing cell levels of IP_6, which may in turn contribute to the transformed phenotype. On the other hand, suppression of IP_6K1 confers protection against tumors experimentally induced with carcinogens [72].

Although these findings are still preliminary, they suggest that IP_6K1 and IP_6K2 can exert opposite effects in carcinogenesis. It is also likely that the effects of IP_6K2 on cancer cells are disjointed, i.e., IP_6K2 probably enhances apoptosis while increasing the acquisition of an invading/migrating phenotype. IP_6K2 may, therefore, act as a tumor suppressor in the initiation stage but contribute to metastatic spread by enacting EMT at later stages. It is worth underlining that similar dual roles have been observed for TGF-β1 [73].

2.3. IP_6K3

IP_6K3 is highly expressed in mouse and human myotubes and muscle [74]. Its physiological role is relatively unexplored. High levels of expression have been detected in the brain. Purkinje cells regulate motor learning and coordination, and IP_6K3 deletion alters these functions. Abnormalities in cell size and spine density are detected, perturbed by dysfunctional IP_6K3 binding of adducin and spectrin, two cytoskeletal proteins involved in the morphogenesis of dendritic trees [74]. Regarding other IP_6Ks, IP_6K3 seems to participate somehow in glucose metabolism. Indeed, IP_6K3-null mice exhibit lower blood glucose and reduced insulin levels, associated with increased plasma lactate levels. These findings suggest that downregulation or suppression of IP_6K3 can enhance glycolysis. However, IP_6K3 suppression is followed by a significant reduction in pyruvate dehydrogenase kinase-4 (PDK4) [75]. Since PDK4 depresses glucose oxidation by inhibiting conversion of pyruvate to acetyl coenzyme A (acetyl-CoA), it is paradoxical that IP_6K3 suppression does not lead to an increase in glucose oxidation.

3. Future Perspectives

Growing interest focused on IPs has shed light on their biological functions and corresponding deregulation issues. Among IPs, IP_7 plays a significant role in cell metabolic balance, ATP production, and phosphate homeostasis. From these studies, IP_6Ks emerge as key regulators of IP_7 intracellular levels in physiological and pathological processes (Figure 2).

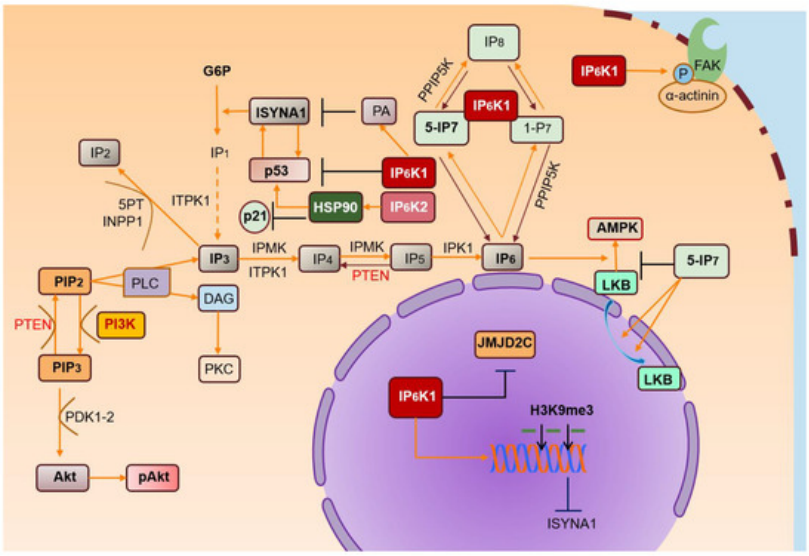

Figure 2. IP_6Ks and their pathways. IP_3 is metabolized in many inositol polyphosphates, of which IP_6 is the most abundant. IP_6Ks produce IPs (IP_7) starting from IP_6. IP_6K/IP_7 levels are crucial for regulating various biological processes. IP_6K1 binds α-actinin localized at focal adhesions, promoting its phosphorylation by FAK and regulating cell migration. IP_6-stimulated AMPK activation is inhibited by high levels of IP_7, reducing cytosolic localization of LKB. High PA levels

promote nuclear IP_6K1 translocation, inhibiting ISYNA1, and consequently, de novo biosynthesis of myo-inositol. Nuclear IP_6K1 interacts with JMJD2C and induces its dissociation from chromatin, increasing H3K9me3 levels and inhibiting transcription of target genes. Likewise, IP_6K2 may localize in the nucleus, downstream of its interaction with HSP90. In turn, nuclear IP_6K2 localization promotes binding to p53, suppressing p21 activation and transcription.

Interest in the development of molecular factors that can (selectively) interrogate and manipulate the cell actions of inositol pyrophosphates, especially by modulating IP_6Ks and $PPIP_5Ks$, is gaining momentum [70]. Targeting these pathways could be helpful in certain diseases but also potentially dangerous. For example, knockout experiments on IP_6Ks highlighted a worse situation in mice, sensitizing the animals to chemical tumorigenesis [69], lung inflammation [76], and loss of motor learning, coordination, and fitness [74][77]. It is therefore crucial to determine whether pharmacological inhibition of IP_6Ks is safe enough to pursue clinical investigations.

Studies based on gene deletion assays are unlikely to provide useful data, since more than 900 genes are altered by deletion of IP_6K homolog (Kcs1) in *S. cerevisiae* [78]. The range of this genetic penetration probably highlights the functional polyvalence of IP_6Ks, which presumably have both catalytic and scaffolding functions, as already demonstrated for inositol pentakisphosphate kinase [79] and inositol polyphosphate multikinase [80]. A more promising approach may focus on specific cell-permeant inhibitors of PP-IPs or on “physiological” modulators of IP_6Ks, an approach that at least in principle would not be flawed by secondary genetic changes or interference with IP_6K scaffolding functions.

The compound N2-(m-trifluorobenzyl)N6-(p-nitrobenzyl)purine (TNP) has been shown to bind specifically to IP_6Ks by competing with ATP for the same binding site. As a result, TNP reduces IP_7 levels by inhibiting the kinase and phosphatase activities of IP_6Ks. Within 2 h of treating various cell types with 10–30 μM TNP, levels of IP_7 fell by 60–90% [81][82], and IP_8 synthesis was also significantly reduced [83]. As expected, IP_6 levels increased proportionally by as much as 40%.

TNP does not efficiently cross the blood–brain and blood–testis barriers. In fact, chronic TNP administration (15 weeks, 10 mg/kg/day) in mice does not lead to neuronal or reproductive abnormalities [84]. However, TNP could interfere with the metabolism of other drugs by inducing modifications in drug signaling or increasing Ca^{2+} and Zn^{2+} levels [85].

TNP inhibitory activity discriminates between IP_6Ks and other inositol phosphate kinases (IPMKs and IP_3Ks). The catalytic site of the IP_6K family is structurally related to that of IPMKs and IP_3Ks, though IP_6Ks have around 100-fold lower affinity for ATP than do the latter [82]. Higher TNP values are therefore required to efficiently neutralize IP_3K (IC50 0.47 μM for IP_6Ks versus 18 μM for IP_3K). However, TNP displays some off-target effects, including ERK phosphorylation, which in principle is not mediated by IP_6Ks. The use of TNP to investigate the intracellular functions of IP_6Ks is therefore debatable. To minimize undesirable effects, it could be useful to develop safe and selective inhibitors of IP_6K isoforms for investigating the specific role sustained by the different IP_6K isoforms.

Regarding carcinogenesis, IP_6K1 and IP_6K2 activities presumably drive cells and tissues towards opposite outcomes. As previously reported, IP_6K1 joins in Akt signaling, and its knockout decreases IP_7 synthesis, resulting in enhanced PDK-dependent phosphorylation of Akt activation. Hyperactivation of Akt (~10- to 50-fold) [86][87] is known to enable tumorigenesis [88]. However, IP_6K1-KO is only associated with a minimal increase in Akt activation in mice [37], insufficient to enact neoplastic development [37]. Indeed, it has been reported that deletion of IP_6K1 protects against chemical tumorigenesis and metastasis [67], although the mechanisms underlying the effect are still unknown. Instead, IP_6K2-KO sensitizes to chemical tumorigenesis and probably increases the occurrence of spontaneous cancer [72].

Acronyms: 1-IP_7 (1-diphospho-2,3,4,5,6-pentakisphosphate); 5-IP_7 (5-diphospho-1,2,3,4,6-pentakisphosphate); Akt (protein kinase B); AMPK (5’ AMP-activated protein kinase); DAG (diacylglycerol); FAK (focal adhesion kinase); H3K9me3 (histone 3 lysine 9 trimethylation); IP_2 (inositol-2-phosphate); IP_3 (inositol-3-phosphate); IP_4 (inositol-4-phosphate); IP_5 (inositol-5-phosphate); IP_6 (inositol-hexakisphosphate or phytic acid); IP_6K1 and IP_6K2 (inositol hexakisphosphate kinase 1/2); IPK1 (inositol-pentakisphosphate 2-kinase); IPMK (inositol polyphosphate multikinase); ISYNA1 (d-3-myoinositol-phosphate synthase); JMJD2C (Jumonji domain-containing protein 2C); LKB (liver kinase B1); P (phosphate group); PA (phosphatidic acid); PI3K (phosphatidylinositol 3-kinase); PIP_2 (phosphatidyl-inositol-4,5-biphosphate); PIP_3 (phosphatidylinositol-3-phosphate); PKC (protein kinase C); PLC (phospholipase C); $PPIP_5K$ (inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase); PTEN (phosphatase and tensin homolog); P_8 (1,5-bis-diphosphoinositol 2,3,4,6-tetrakisphosphate); G6P (glucose-6-phosphate); IP_1 (inositol-1-phosphate, *myo*-Inositol); ITPK1 (inositol-tetrakisphosphate 1 kinase).

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