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Linking Phosphoinositides to Proteins: A Novel Signaling Pipeline

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Abstract

Phosphoinositide (PIP_n) signaling plays pivotal roles in myriad biological processes and is altered in many diseases including cancer. Canonical PIP_n signaling involves membrane-associated PIP_n lipid second messengers that modulate protein recruitment and activity at membrane focal points. In the nucleus, PIP_n signaling operates separately from membranous compartments defining the paradigm of non-canonical PIP_n signaling. However, the mechanisms by which this non-membranous nuclear PIP_n pool is established and mediates stress signaling is poorly understood. The recent discovery of a p53-signalosome by *Chen et al. (Nature Cell Biology 2022)* represents a new PIP_n signaling axis that operates independently from membrane structures where PIP_ns are dynamically linked to nuclear p53 and modified by PIP_n kinases and phosphatases, allowing the activation of a nuclear PI 3-kinase/Akt pathway that is entirely distinct from the canonical membrane-localized pathway. Here, we will discuss emerging insights about the non-canonical PIP_n pathway, which links PIP_ns to a growing number of cellular targets and highlight the similarities/differences with its canonical counterpart. We will also discuss potential therapeutic targets in this non-canonical PIP_n pathway, which is likely to be deregulated in many diseases.

Keywords: Phosphoinositide, Signalosome, PIPn linked proteins, Cancer, PI3K, Nucleus

In Brief

The canonical phosphoinositide (PIP_n) signaling pathway on membranes has well established roles in cancer and has been successfully targeted. The recent discovery of a membrane-independent PIP_n signaling pathway anchored on nuclear proteins that operates independently of the canonical pathway has ushered in a new PIP_n signaling paradigm with novel therapeutic targets for diverse diseases.

The Canonical PIP_n Pathway: Membranes Lead the Way

Phosphatidylinositol (PI) is the lipid precursor to all seven distinct phosphoinositide (PIP_n) phosphoisomers. PI phosphate (PIP) kinases phosphorylate the *myo*-inositol headgroup on the 3-, 4-, and 5-hydroxyl positions to generate the specific PIP_n isomers [1]. The monophosphates (PI3P, PI4P, and PI5P), diphosphates (PI3,4P_2, PI4,5P_2, and PI3,5P_2) and triphosphate (PI3,4,5P_3) can then bind to and regulate proteins and enzymes involved in most cellular pathways [2]. The PI substrate platform combined with diverse enzymatic

interactions and intracellular compartmentalization conveys robust signaling that involves generation, interconversion, and depletion of specific PIP isomers in a highly regulated fashion. In turn, effectors bind the PIP_ns and are activated with spatial and temporal specificity which is directly or indirectly involved in the regulation of a broad array of biological processes and impacts many diseases.

De novo PI synthesis occurs at the endoplasmic reticulum (ER) and begins with glycerol-3-phosphate (G-3-P) uptake [3]. G-3-P is then converted into lysophosphatidic acid (LPA) by G-3-P acyltransferases, and LPA acyltransferases subsequently convert LPA into phosphatidic acid (PA) [3,4]. A series of metabolic reactions then leads to the formation of cytidine diphosphate diacylglycerol (CDP-DG) from PA which is used in combination with inositol to form PI in a reaction catalyzed by PI synthase [3,5]. Following synthesis, PI localizes to multiple membrane compartments via membrane trafficking and PI transfer protein (PITP) courier mechanisms allowing for compartmentalized signaling throughout the cell [6]. PIP_ns are generated by the actions of multiple kinases and phosphatases on the 3-, 4-, and 5-hydroxyl residues of the inositol head. The type II PI3Ks phosphorylate PI at the 3-hydroxyl position to generate PI3P, while the type II and III PI4Ks phosphorylate the 4-hydroxyl to generate PI4P [7]. There are at least two mechanisms for generating PI5P: phosphorylation of PI by PIKFYVE or dephosphorylation of $PI4,5P_2$ by type I and II $PI4,5P_2$ 4-phosphatases [8]. $PI4,5P_2$ is generated via phosphorylation of PI4P and PI5P by the type I and II PIP kinases, respectively, and dephosphorylation of $PI3,4,5P_3$ by PTEN [9,10]. The final PIP_n, $PI3,4,5P_3$, can be synthesized by two enzyme families: the p110α/β catalytic subunit of the class I PI3K and IPMK [11,12].

PIP_n degradation is mediated by either phosphatases or phospholipase C isoforms [13]. The most studied phosphatase, phosphatase and tensin homolog (PTEN), dephosphorylates $PI3,4,5P_3$ into $PI4,5P_2$. PTEN is present in the cytoplasm, nucleus, and nucleoli, and its nuclear re-localization is triggered by genotoxic stress [14]. Notably, PTEN loss/mutation is a major driver of tumorigenesis and correlates with therapeutic resistance in patients [15]. Src homology 2 domain-containing inositol polyphosphate 5'phosphatases (SHIP1 and SHIP2) dephosphorylate $PI3,4,5P_3$ to generate $PI3,4P_2$ at the plasma membrane, in the nucleus and nucleoli [14,16]. Another 5-phosphatase, oculocerebrorenel syndrome of Lowe (OCRL-1) catalyzes the degradation of $PI3,4,5P_3$ into $PI3,4P_2$ and of $PI4,5P_2$ into PI4P at the plasma membrane, in Golgi, endosomes and lysosomes [10]. Similarly, INPP4 phosphatases (INPP4A, INPP4B) dephosphorylate PI3, $4P_2$ into PI3P [16]. Type I and type II PI4, $5P_2$ phosphatases dephosphorylate PI4, $5P_2$ into PI5P [14,16]. The myotubularin (MTM)/myotubularin-related (MTMR) group is a large family of phosphoinositide 3-phosphatases that dephosphorylate PI3P and PI3, $5P_2$ to PI and PI5P, respectively, in autophagosomes and endosomes [16]. Through this extensive metabolic regulation, the different PIP_n isomers function as distinct cellular signals that promote or inhibit protein recruitment, interaction and activities which drive various biological processes. While PI metabolism and signaling in the cytosol is decidedly complex, a non-canonical PIP_n signaling pathway in non-membranous nuclear regions was recently discovered that employs both shared and distinct machinery for nuclear PIP_n signaling **(Figure 1)**.

The canonical PIP_n signaling paradigm is exemplified by the PI 3-kinase (PI3K)/Akt pathway which imparts cancer cells with cell stress resistance, metastatic potential, sustained proliferation, and therapeutic resistance [17]. In this pathway, membrane-associated PI is sequentially phosphorylated by PIP kinases to generate PI4, $5P_2$ [18]. PI3Ks then recognize and phosphorylate PI4, $5P_2$ into PI3,4, $5P_3$ marking the initiating and key signaling event in the pathway [19]. Until recently, this cascade was thought to occur only at the plasma membrane [20-22]. However, several recent reports have shown a critical, perhaps predominant role for endosomal membranes where the PIP kinases are scaffolded on IQ motif containing GTPase activating protein 1 (IQGAP1) to enhance signaling efficiency [20,22]. After $PI3,4,5P_3$ generation by PI3Ks regardless of cellular location, protein domains such as the pleckstrin homology (PH) domain on Akt localize towards and bind to $PI3,4,5P_3$ where they can be activated by other PH domain-containing kinases including PDK1 and mTORC2 [21,23,24]. More comprehensively reviewed elsewhere [17,24], these fundamental concepts span many PIP_n signaling pathways [25]. The elucidation of canonical PI3K/Akt signaling and function has resulted in the development of PI3K inhibitors and their successful clinical use [24]. Unfortunately, these discoveries also established dogmas for PIP_n signaling that allowed some PIP_n pathways to go unconsidered [20-22], especially in the nucleus [14,26-29].

The Non-Canonical PIP_n Pathway: Linking PIP_ns to Proteins

PIP_n signaling has since been shown to extend beyond the canonical membrane signaling paradigm exemplified by the PI3K/Akt pathway [14,29-32]. The main distinctions between the canonical and non-canonical pathways are the cellular location and role of membrane structures in PIP_n signaling [27,28,33-35]. While the existence of nuclear PIP_ns has been established [32], their regulation and biological relevance is an emerging area of research. Non-canonical PIP_n signaling in the nucleus was originally met with skepticism due to the lack of membrane structures [30,31]. Of the seven PIP_n lipid phosphoisomers, six have been detected in the nucleus [14]. Akt is also activated in nuclei, implicating a key role for $PI3,4,5P_3$ generation in the non-membranous nucleoplasm [27,36]. There is now evidence for a complex signaling axis in the nucleus involving multiple PIP_n-regulated protein/enzyme targets [29,37,38]. Founding members include Speckle targeted PIPKIα regulated-poly(A) polymerase (Star-PAP) and

Steroidogenic Factor-1 (SF-1), but the list of nuclear PIP_n-associated targets continues to expand [33,39,40]. Recently, nuclear PIP_ns were shown to be linked to the tumor suppressor p53, both wild-type and mutant, in response to cellular stress [28]. Interestingly, these nuclear protein-PIP_n linkages are distinct from the PH, C2, FYVE (Fab 1, YOTB, Vac 1 and EEA1) and polybasic domain protein-PIP_n interactions [41]. It is now clear that p53 has two distinguishable interactions with PIP_ns: first, binding of $PI4,5P_2$ to the C-terminal polybasic domain of p53, and second, a PIP_n linkage that is resistant to denaturation and lipid extraction [26-28]. Specifically, linked PIP_n isomers persist after denaturation and SDS-PAGE, co-migrating with p53, are detected by multiple PIP_n isomers monoclonal antibodies, and are metabolically labelled with [^{3}H]*myo*-inositol [26,27]. Protein-PIP_n interactions on membranes utilize the negatively charged membrane as an anchor to tether signaling components. This dynamic often depends on interactions between the protein and the negatively charged bilayer, and the protein and the PIP_n [10,16,42-44]. In the absence of a membrane, p53 seems to mimic the structural contributions of a membrane by binding PIPKIα which converts p53-PI4P complexes into p53-$PI4,5P_2$ [28]. This modification then recruits the small heat shock proteins (sHSPs) αB-crystallin and HSP27 which stabilize p53 providing an alternative model for PIP_n signaling **(Figure 1)** [28].

Building on this foundation, Chen *et al.* demonstrated that these nuclear p53-PIP_n complexes do not simply convey stability, but that the PIP_ns linked to p53 participate in signal propagation, designated the p53-signalosome [27]. This discovery is the first link between p53, the most mutated protein in all cancers, and the PI3K/Akt pathway, the second most mutated pathway in all cancers [45]. In parallel to the canonical PI3K/Akt pathway, p53-PIP_n complexes are sequentially modified to generate p53-PI4P, then p53-$PI4,5P_2$, before inositol polyphosphate mutikinase (IPMK) acts as a nuclear PI3K to generate p53-$PI3,4,5P_3$ complexes in the nucleus. p53-$PI3,4,5P_3$ complexes recruit PH domain proteins including Akt and its activating enzymes (PDK1 and mTORC2) which co-immunoprecipitated with p53 and increased association after IMPK activation. Once in proximity, this resulted in membrane-independent increases

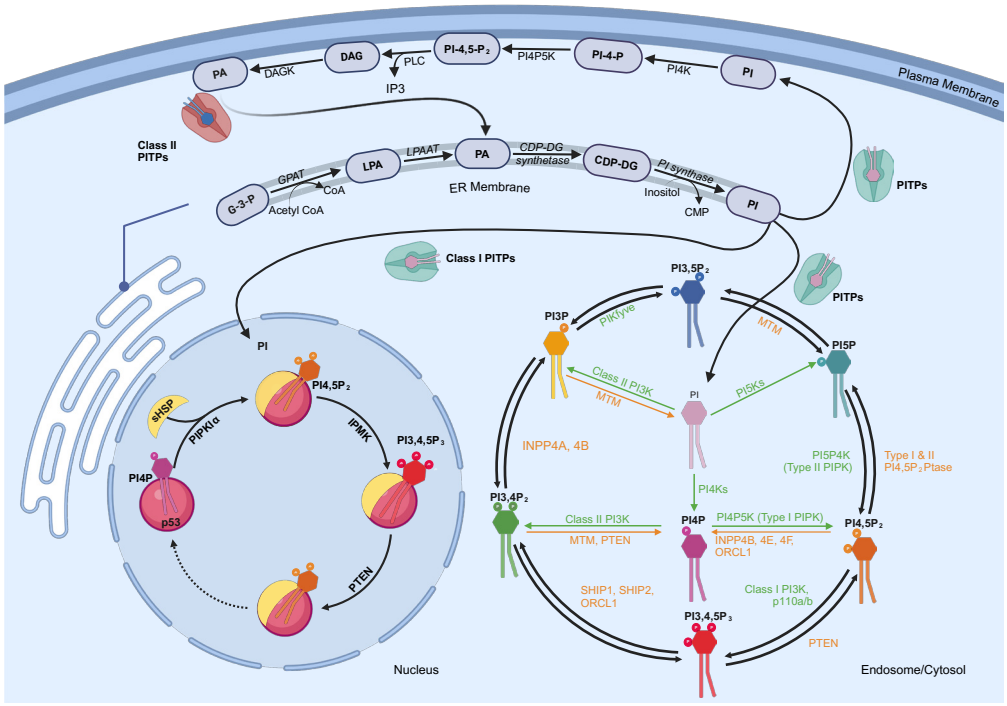

Figure 1. Canonical and non-canonical PIP_n signaling. PI is synthesized in the ER where it is exported to various cellular compartments conveying spatially distinct signaling using specific kinases. PI: Phosphatidylinositol; PI4K: Phosphatidylinositol-4-Kinase; PI3K: Phosphatidylinositol-3-Kinase; PI5K: Phosphatidylinositol-5-Kinase; PI3P: Phosphatidylinositol-3-Phosphate; PI4P: Phosphatidylinositol-4-Phosphate; PI5P: Phosphatidylinositol-5-Phosphate; PI4P5K: Phosphatidylinositol-4-Phosphate 5-Kinase; PI3,5P: Phosphatidylinositol-3,5-Biphosphate; $PI4,5P_2$: Phosphatidylinositol-4,5-Biphosphate; PI3,4P: Phosphatidylinositol-3,4-Biphosphate; PI3,4,5P: Phosphatidylinositol-3,4,5-Triphosphate) PLC: Phospholipase C; IP3: Inositol Triphosphate; DAG: Diacylglycerol; DAGK: Diacylglycerol Kinase; PA: Phosphatidic Acid; G-3-P: Glycerol-3-Phosphate; GPAT: G-3-P Acyltransferase; LPA: Lysophosphatidic Acid; LPAAT: LPA Acyltransferase; CDP-DG: Cytidine Diphosphate DAG; CMP: Cytidine Monophosphate; PITP: Phosphatidylinositol Transfer Proteins; PIKfyve: Phosphoinositide Kinase, FYVE-type Zinc Finger Containing; MTM: Myotubularin; INPP4: Inositol Polyphosphate-4-Phosphatase; PTEN: Phosphatase and Tensin Homolog; OCRL1: Oculocerebrorenal Syndrome of Lowe; SHIP: Inositol Polyphosphate-5-Phosphatase; sHSP: Small Heat-Shock Proteins; PIPKIα: Phosphatidylinositol 4-Phosphate 5-Kinase Type-1 alpha; IPMK: Inositol Polyphosphate Multikinase.

in pAkt (S473 and T308) Western Blot and immunofluorescent staining in the nucleus **(Figure 2)**. Similarly, PTEN acts in an analogous fashion as it does in the membrane pathway to dephosphorylate nuclear p53-PI3,4,5P_3 into p53-PI4,5P_2, turning off Akt activation in the nucleus [27] as it does in the canonical pathway. The interconversion of these protein-PIP$_n$ complexes was established using orthogonally validating SDS-PAGE co-migration and proximity ligation assays for the specific PIP$_n$ isomers after modulation of the upstream and downstream kinases [27]. The p53-signalosome is generated and maintained by ordered interactions between p53 and the PIP$_n$ regulatory enzymes [27]. This demonstrates that protein-PIP$_n$, as well as protein-protein interactions are required for activating Akt. Given the interactions between p53 and the requisite PIP kinases, protein targets like p53 may scaffold the kinase activity for the coupled PIP$_n$s like the role of IQGAP1 for endosomal signaling [20,22]. In this way, IQGAP1 and p53 have the capacity to act as signaling pipelines starting with PI and ending with PI3,4,5P_3. Despite the overlapping components and concepts, it is important to note that this nuclear pathway is resistant to clinical inhibitors targeting cytosolic PI3Ks that are structurally dissimilar to IPMK, highlighting the translational relevance of the non-canonical PIP$_n$ pathway [27].

While the discovery of the non-canonical PIP$_n$ signaling pathway has led to several new molecular insights, perhaps the most profound is the concept of protein-PIP$_n$ complexes. Both the foundational project defining p53-PI4,5P_2 [28] and subsequent discovery of p53-PI3,4,5P_3-mediated nuclear Akt activation [27], underscore the idea that structure and specificity can be conveyed to PIP$_n$ signaling events by proteins, not just membranes. In the non-membranous nucleoplasm, it is plausible that protein-membrane interactions are substituted with protein-protein interactions to confer similar structural orientations and preserve the protein-PIP$_n$ binding required for activation **(Figure 3).** While several groups have

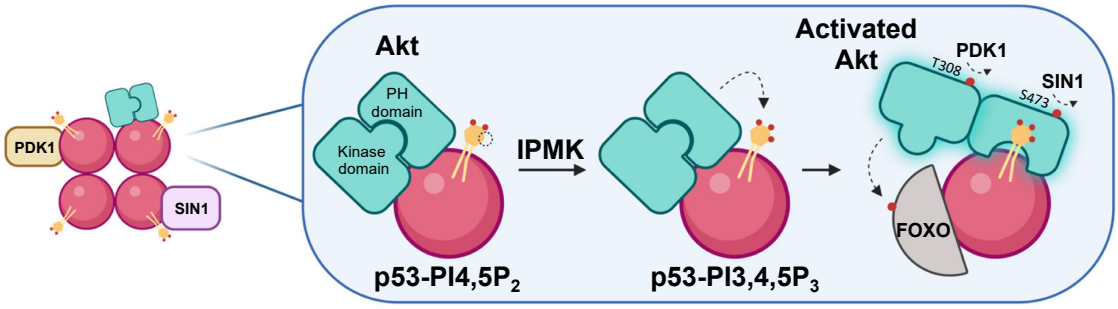

Figure 2. The p53 signalosome. Inactive Akt interacts with p53-PI4,5P_2. IMPK acts as a nuclear PI3K generating p53-PI3,4,5P_3 complexes which induces pleckstrin homology (PH) domain binding and activation of Akt by PDK1 and Sin1 resulting in FOXO phosphorylation. All components of this nuclear pathway interact with p53 allowing specific regulation of p53-PIP$_n$ signaling and activity.

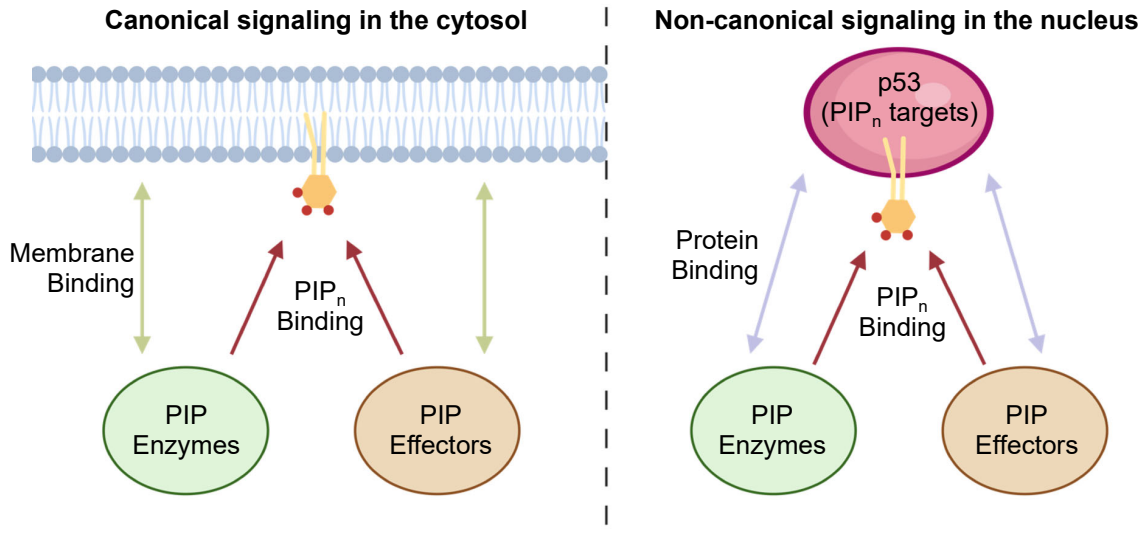

Figure 3. Canonical and Non-canonical PIP$_n$ signaling. PIPn enzymes and PIP$_n$ effectors bind PIPn lipids regardless of location. The membrane structure and binding in canonical PIP signaling is replaced with protein structure and binding in non-canonical PIP$_n$ signaling. This gives p53 and other target proteins regulatory impact for interactions with enzymes and effectors.

observed biological connections between nuclear PIP_ns and the cell cycle [46], oxidative stress [14,35], and the DNA damage response [34], the concept of nuclear PIP_n signaling has lacked mechanistic insights until recently. The p53-signalosome offers a plausible mechanistic framework for the observed nuclear signaling events and the biological prerequisite for structure and stability needed by PIP_ns to be recognizable and modifiable via PIP_n-linked proteins.

Additionally, p53-signalosome-dependant Akt activation, independently or in combination with other oncogenic nuclear proteins regulated by linked PIP_ns, has clear translational implications. Upwards of 40% of all cancers harbor PI3K specific mutations which drive tumorigenesis [47-49]. Cancer cells often overcome therapeutic interventions such as PI3K inhibition by circumventing the targeted pathways [50]. It is plausible that in the context of PI3K inhibition, cancer cells may upregulate the non-canonical nuclear pathway to maintain Akt activation and avoid cell death, leading to therapeutic resistance and tumor recurrence. Dysregulated PI3K/PTEN signaling not only drives tumor resistance/growth but also influence brain function. Specifically, continuous activation of PI3K caused by PTEN mutations lead to increased oxidative stress, decreased cytochrome c oxidase activity and elevated levels of mitochondrial DNA deletions, resulting in energy stress in the cerebellum and hippocampus [51]. Additionally, PTEN null mice exhibit impaired socializing function, such as a lack of preference for social interaction and learning, shortened nest-forming activity and abnormally high levels of anxiety [52]. These finding have since led to PI3K inhibitors being studied in a variety of brain diseases, such as Huntington's disease, depression, Parkinson's disease, and Alzheimer's disease [53]. Since increased Akt activity significantly contributes to autistic behavior, PI3K inhibitors are also being investigated to suppress the PI3K/AKT/mTOR pathway and rescue autistic phenotypes [53]. In the context of neurodegenerative disease, non-canonical PIP_n signaling via protein-PIP_n complexes may also contribute to their pathogenesis as has been demonstrated in cancer models.

While the therapeutic potential of nuclear PIP_ns remains an active area of ongoing investigation, the identification of PIP_n targets clearly highlights potential avenues for combating cancer [27,28,34,54]. p53-PIP_n complexes contribute to fundamental oncogenic phenotypes. p53-PI4,5P_2 complexes generated by PIPKIα recruit sHSPs which can stabilize mutant p53 and contribute to mutant p53 oncogenicity [28,55], Downstream, p53-PI3,4,5P_3 complexes are generated by IPMK and suffice to promote Akt activation and further tumorigenic phenotypes [27]. In support of these observations, siRNA targeting of IPMK reduced Akt activity, chemoresistance and metastatic potential in cancer cells [27]. While this strategy limits nuclear Akt activation, other mutant p53 oncogenic mechanisms putatively remain stabilized via p53-PI4,5P_2-sHSP stability. Indeed, this concept was more recently demonstrated after identifying an upstream regulator of p53-PIP_n complexes where targeting this mechanism largely reduced both p53 protein levels, as well as nuclear Akt activation; however, the corresponding chemosensitivity and decreased metastatic potential cannot be solely attributed to the p53-signalosome [26]. Despite the many hurdles that remain for investigating non-canonical PIP_n signaling in the future, there are numerous regulatory mechanisms that have yet to be defined. The diverse PI kinases involved in canonical signaling implicate many kinases beyond those included here to be involved in non-canonical PIP_n signaling, some of which may have greater therapeutic applications for diseases like cancer. In parallel, a greater structural understanding of these target-lipid-kinase interactions will undoubtedly promote a shift in the fundamental comprehension of PIP_n signaling in cells.

The technical methodology used to define protein-PIP_n complexes provides additional insights. Specifically, these publications include evidence of p53-PIP_n linkages by SDS-PAGE, western blotting and metabolic labelling [26-28]. Conventional protein interactions with PIP_ns do not withstand the denaturing conditions of SDS-PAGE and free PIP_n species migrate well below the loading dye of a protein lysate. However, the PIP_ns linked to p53 co-migrate with p53 on the protein gels, indicating a tight association consistent with a posttranslational modification (PTM). This putative protein modification is currently being chemically defined, and the implications of a PIP_n PTM has broad biological and therapeutic relevance. The long history of research on membrane-bound PIP_ns has revealed an impressively diverse array of biological processes linked to these lipids [56]. Defining the nature of the p53-PIP_n linkage may help identify the enzymatic requirement for forming these unique protein-PIP_n complexes. The discovery that PIP_ns are linked to proteins as a putative PTM to regulate their stability and function points to a fundamentally new way that PIP_ns transduce stress signals that is just now being explored.

Despite these recent advances and the exciting potential, they hold, many critical questions remain for non-canonical PIP_n signaling. PI is synthesized in the ER but clearly has roles in the nucleus, implicating a transport mechanism that was very recently identified [26] but has yet to be fully characterized. Additionally, the chemical definition of the p53-PIP_n linkage and the requisite enzymatic machinery remain an active area of research. While p53 constitutes the founding member of these unique protein-PIP_n linkages, other critically important proteins that are modified or PIPylated by PIP_ns have since been identified [54,57] warranting further study to define the PIPylome, how these protein targets are regulated, and what their biological/pathobiological functions may be. Indeed, it is of great interest to study non-canonical PIP_n signaling in the context of PI3K inhibition and develop therapeutic interventions that might synergize with PI3K inhibitors or potentially target both the canonical and non-canonical

nuclear pathways. In conclusion, the work by Chen *et al.* and others has identified an unanticipated dimension for PIP_n signaling operating in the nucleus and anchored on proteins which regulates fundamental biological processes and likely contribute to many diseases including cancer **(Figure 3)**.

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Competing Interests

The authors declare no conflict of interest.

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