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Structural analyses of inositol phosphate second messengers bound to signaling effector proteins.

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Abstract

The higher-order inositol phosphate second messengers inositol tetrakisphosphate (IP₄), inositol pentakisphosphate (IP₅) and inositol hexakisphosphate (IP₆) are important signaling molecules that regulate DNA-damage repair, cohesin dynamics, RNA-editing, retroviral assembly, nuclear transport, phosphorylation, acetylation, crotonylation, and ubiquitination. This functional diversity has made understanding how inositol polyphosphates regulate cellular processes challenging to dissect. However, some inositol phosphates have been unexpectedly found in X-ray crystal structures, occasionally revealing structural and mechanistic details of effector protein regulation before functional consequences have been described. This review highlights a sampling of crystal structures describing the interaction between inositol phosphates and protein effectors. This list includes the RNA editing enzyme “adenosine deaminase that acts on RNA 2” (ADAR2), the Pds5B regulator of cohesin dynamics, the class 1 histone deacetylases (HDACs) HDAC1 and HDAC3, and the PH domain of Bruton’s tyrosine kinase (Btk). One of the most important enzymes responsible for higher-order inositol phosphate synthesis is inositol polyphosphate multikinase (IPMK), which plays dual roles in both inositol and phosphoinositide signaling. Structures of phosphoinositide lipid binding proteins have also revealed new aspects of protein effector regulation, as mediated by the nuclear receptors Steroidogenic Factor-1 (SF-1, NR5A2) and Liver Receptor Homolog-1 (LRH-1, NR5A2). Together, these studies underscore the structural diversity in binding interactions between effector proteins and inositol phosphate small signaling molecules, and further support that detailed structural studies can lead to new biological discovery.

Introduction:

Higher-order inositol phosphates are important second messenger signaling molecules that regulate a wide array of cellular processes, including but not limited to DNA-damage repair

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(Hanakahi et al., 2000; Hanakahi and West, 2002), cohesin dynamics (Ouyang et al., 2016), RNA-editing (Macbeth et al., 2005), virus assembly (Dick et al., 2018), protein phosphorylation (Lee et al., 2013; Wang et al., 2015), bacterial protein acetylation (Zhang et al., 2016), eukaryotic histone acetylation (Watson et al., 2012), histone crotonylation (Kelly et al., 2018), nuclear transport (Alcázar-Román et al., 2006; Miller et al., 2004) and ubiquitination (Scherer et al., 2016), among others. These molecules have been shown to be required factors in maintaining the structural fold of effector proteins (Macbeth et al., 2005) and the enzymatic activity of important cellular enzymes (Watson et al., 2012). Thus it is important to understand how these small signaling molecules regulate such a diverse array of cellular processes.

The enzymes that generate higher order inositol phosphate molecules are a series of well-known kinases and phosphatases that have a high degree of conservation from yeast to humans. One of the most important of these enzymes is inositol polyphosphate multikinase (IPMK, ipk2, Arg82) a central enzyme in all higher-order inositol phosphate synthesis. IPMK was originally identified as a transcriptional co-regulator in yeast (Bercy et al., 1987; Dubois et al., 1987) and only later shown to be a kinase (Odom et al., 2000; Saiardi et al., 2001). IPMK has both kinase-dependent (Blind et al., 2012; Fu et al., 2018; Hatch et al., 2017; Maag et al., 2011; Odom et al., 2000; Resnick et al., 2005; Sang et al., 2017; Seeds et al., 2015; Steger et al., 2003; York et al., 1999) and kinase-independent (Ahmed et al., 2015; Bang et al., 2012; Kim et al., 2013, 2011; Kim and Snyder, 2011; Xu et al., 2013b, 2013a) roles in regulating transcription (Malabanan and Blind, 2016). Although IPMK has no specific protein kinase activity (Blind et al., 2012; Seacrist and Blind, 2018; Shears and Wang, 2019), *in vitro* IPMK can phosphorylate the phosphoinositide lipid PI(4,5)P2 (PIP2) to generate PI(3,4,5)P3 (PIP3) in membrane vesicles and micelle systems with reasonable enzyme kinetic parameters (Blind et al., 2012; Resnick et al., 2005). IPMK can also phosphorylate PIP2 while the lipid is bound in 1:1 stoichiometry to a particular non-membrane protein transcription factor, with excellent enzyme kinetics (Blind et al., 2012). IPMK is also critical for full production of PIP3, and full regulation of PIP3 downstream signaling, in several cell lines and whole animal mouse models.

The first kinase substrates of IPMK to be identified were phosphorylated inositols, and IPMK is required for full production of the full set of higher-order inositol phosphates. More specifically, IPMK is required for Ins(1,4,5,6)P4 (IP4) and Ins(1,3,4,5,6)P5 (IP5) synthesis in mammalian cells (Fujii and York, 2005; Leyman et al., 2007; Maag et al., 2011; Resnick et al., 2005; Seeds et al., 2005; York et al., 1999). IPMK does not directly generate IP6, however IP6 levels are greatly diminished or absent upon loss of IPMK depending on the system, as IP5 is used by the enzyme IPK1 to generate IP6. Further, the kinase IP3K mediates production of Ins(1,3,4,5)P4 (note difference with Ins(1,4,5,6)P4) by acting on the Ins(1,4,5)P3 generated by phospholipase C activity on the phosphoinositide lipid PI(4,5)P2 (PIP2), through an alternative pathway (Irvine et al., 1986). The intricacies of inositol phosphate production have been reviewed in detail elsewhere (Hatch and York, 2010; Kim et al., 2017; Shears et al., 2017; York, 2006), this review focuses on the structural biology of higher-order inositol phosphates bound to effector proteins elucidated by X-ray crystallographic structural analyses.

X-ray crystallographic diffraction analysis is a high resolution analytical technique that uses the short wavelength of X-rays diffracted by all atoms within a crystalized sample to determine the electron density within the regular repeating crystal lattice or asymmetric unit. Crystallization of small molecules from complex solutions can also be used as a preparatory technique to remove chemical impurities, while X-ray diffraction is an analytical method. Macromolecular X-ray crystallographic diffraction uses the same concepts from small molecule crystallography but applies it to complex proteins. In order to produce a three dimensional structure of a protein, the protein sequence must be fit into the electron density, a process called refinement. The electron density reflects all ordered atoms in a crystal, and so refinement can also define the position of any well-ordered small molecule ligands bound to the protein, intentionally or otherwise. In practice, these ligands are often bound and co-purified before crystallization of the entire protein-ligand complex is attempted. In other cases, it is often possible to “soak” pre-formed crystals of the apo-protein with the ligand of interest. The information gleaned from crystal structures of protein-ligand complexes can provide critical information elucidating mechanistic details of how ligands regulate protein function, and are very important for biomedical applications such as structure-based drug design.

Since X-rays are diffracted by all molecules within the crystal lattice, including molecules or ligands not knowingly incorporated by the investigator, it has occasionally happened that “unexpected” electron density has been observed in structures during refinement. This has occurred on several occasions for the higher-order inositol phosphates generated by IPMK and IPK1 in particular. The focus of this review is on the structural biology of effector proteins where X-ray crystallographic analyses have revealed atomic-resolution details of higher-order inositol phosphate binding sites, in a few cases even before functional consequences of the inositol phosphate binding were known. Also discussed are the implications and influence structural biology has on genetic and biochemical studies of the enzymes that generate higher-order inositol phosphates, including IPMK and IPK1.

1.1 A single, stoichiometric IP6 molecule found unexpectedly in the Pds5B crystal structure.

Pds5B is a subunit of the cohesin complex which can form ring-like structures around chromosomes, resulting in sister chromatid cohesion, transcriptional regulation and chromosomal compaction. Pds5B stimulates release of cohesin from chromosomes, and is thus considered an overall negative regulator of cohesin DNA-binding function. In X-ray crystallographic structural analyses of Pds5B, a well-ordered IP6 molecule from the insect cell expression system was unexpectedly found in the “jaw” region of the Pds5B protein (Fig 1), a region required for interaction with cohesin (Ouyang et al., 2016). These authors show IP6 is required for Pds5B interaction with cohesin *in vitro*, and mutants of Pds5B at the IP6 binding site fail to bind cohesin in living cells (Ouyang et al., 2016). This crystal structure and functional data indicate IP6 is required for Pds5B interaction with cohesin, a prerequisite for direct Pds5B regulation of cohesin. However, regulation of Pds5B by IP6 has never been tested in inositol depleted cells, so it will be interesting to determine if Pds5b functions are altered in mammalian cells depleted of IP6.

Pds5b is also known to regulate transcription by regulating formation of “DNA loops” by cohesin (Wutz et al., 2017). DNA-loops are three dimensional chromatin “loop” structures which form at sites where the transcription factor CTCF binds DNA (Busslinger et al., 2017; Wendt et al., 2008). The DNA loops provide a three-dimensional architecture to chromatin, allowing the formation of topological domains with chromatin and bringing sequences close together in 3D space. Data gathered by several groups has led to the intriguing “DNA Loop Extrusion Hypothesis” to explain the genetic evidence suggesting CTCF, cohesin and Pds5 collaborate to regulate DNA loops (Wutz et al., 2017). In this model, cohesin uses its ATPase activity to processively extrude along DNA until a CTCF site is reached. At CTCF sites, cohesin processivity is stalled in a Pds5-dependent manner, establishing the boundary of the DNA loop. In cells depleted of Pds5 proteins, cohesin fails to stop at CTCF sites, processively “walking through” CTCF which results in much longer DNA-loops, mis-localization of cohesin away from CTCF sites in chromatin and altered gene expression patterns.

However, it remains unknown how cohesin detects proximity to CTCF, and why Pds5 is required for this process. One of the enzymes responsible for IP6 production in mammalian cells has a long history linked to chromatin regulation, inositol polyphosphate multikinase (IPMK) (Ahmed et al., 2015; Bang et al., 2012; Bercy et al., 1987; Blind et al., 2012; Dubois et al., 1987; Fu et al., 2018; Kim et al., 2013, 2011; Kim and Snyder, 2011; Maag et al., 2011; Odom et al., 2000; Resnick et al., 2005; Sang et al., 2017; Seeds et al., 2015; Shen et al., 2003; Steger et al., 2003; Willhoft et al., 2016; Xu et al., 2013a, 2013b; York et al., 1999). IPMK is required to produce the precursor molecules needed for IP6 synthesis in mammalian cells, and loss of IPMK alters gene expression in yeast (Steger et al., 2003) and mammalian cells (Resnick et al., 2005). It would be interesting to determine how IPMK might regulate Pds5B, or if the IPK1 kinase most directly responsible for production of IP6 in mammalian cells might regulate DNA loop extrusion by altering Pds5B activity. One might predict that depletion of cellular IP6 might prevent Pds5B from functioning to inhibit cohesin processivity at CTCF sites, resulting in dysregulated DNA loop size, altered histone marks and ultimately in dysregulated gene expression (Busslinger et al., 2017; Davidson et al., 2016; Wendt et al., 2008; Wutz et al., 2017).

1.2 A single, stoichiometric IP6 molecule found unexpectedly in the core of the ADAR2 crystal structure.

The RNA-editing enzyme “adenosine deaminase that acts on RNA 2” (ADAR2) converts adenosine to inosine by hydrolytic deamination of RNA. X-ray crystallographic studies of the catalytic domain of ADAR2 expressed in *Saccharomyces cerevisiae* yeast revealed a well ordered IP6 molecule locked within the highly basic core of the ADAR2 protein (Macbeth et al., 2005), along with 29 well-ordered water molecules (Fig 2). The resolution of this structure was 1.7Å, so the electron density representing the IP6 molecule could be identified and properly assigned to IP6 during the refinement process, despite the IP6 being unexpectedly bound to ADAR2. The highly integrated nature of the IP6 molecule into the structure of ADAR2 suggested if the ADAR2 protein were expressed in yeast lacking IP6, the catalytic activity of ADAR2 would be diminished. Indeed, when ADAR2 enzyme was purified from *ipk1* yeast, the *in vitro* enzymatic activity decreased to background levels

(Macbeth et al., 2005). Thus, ADAR2 was discovered by crystallographic structural analyses to bind and be regulated by IP6. It will be interesting to determine if cellular loss of IPMK or IPK1 alters RNA-editing capacity of ADAR2 globally, as the entire family of ADAT1 deaminases requires IP6 for tRNA editing activity (Macbeth et al., 2005).

1.3 Higher-order inositol phosphates found unexpectedly in crystal structure of class 1 HDACs.

The Class 1 HDACs (*HDAC1*, *HDAC2* and *HDAC3*) are histone “eraser” enzymes that deacetylate lysine residues on histone tails, resulting generally in downregulated transcription (Kelly and Cowley, 2013). HDACs are brought to chromatin by corepressor adapter proteins. The adapter corepressors function to mediate the interaction between the HDAC enzyme and a DNA-binding transcription factor (Wang and Tsai, 2008). There are several known corepressors for HDACs, including MTA1 and “Arginine-Glutamate dipeptide repeats” (RERE) (Chen et al., 1996; Humphrey et al., 2001; Wagner et al., 1998; Wood et al., 2000). Structural analyses of HDAC3 expressed this target protein using a transient transfection system in HEK293 cells. This protein was crystalized, and an unexpected IP4 molecule was able to be unambiguously identified as Inositol 1,4,5,6 tetraphosphate. The IP4 resides close to the HDAC active site, buried between the HDAC enzyme and the corepressor NCOR2 (SMRT) in the 2.1Å crystal structure of this complex (Fig 3A–B). In the HDAC crystal structures, the inositol molecule contacts both the corepressor and the HDAC enzyme (Watson et al., 2012). Although initial interaction analyses were consistent with IP4 mediating interaction between HDAC3 and NCOR2, later evidence suggested the inositol molecule acts primarily as an allosteric activator of the HDAC active site (Millard et al., 2013; Watson et al., 2016), as the large protein-protein interaction interface between the HDAC and corepressor proteins is very stable (Millard et al., 2013).

Following the initial discovery of IP4 in the HDAC3:NCOR2 complex, a series of papers from Prof. John Schwabe’s group (Millard et al., 2013; Watson et al., 2016, 2012) showed both HDAC1 and HDAC3 (Fig 3C,D) are activated *in vitro* by any inositol containing three adjacent phosphates (Watson et al., 2016), including several synthetic unnatural inositol analogs, as well as IP4 and IP5 made by IPMK, and IP6 which requires IPMK for synthesis in cells, but is actually synthesized by the IPK1 enzyme. The IPMK product IP4 (Inositol 1,4,5,6 P4) co-purifies with HDAC3 from the HEK cell expression system (Watson et al., 2012) and activates both HDAC1 and HDAC3 (Watson et al., 2016). Inositol 1,3,4,5,6 pentakisphosphate (IP5) and IP6 were also shown to activate HDAC1 and HDAC3 with slightly better potency than IP4 (Watson et al., 2016). All the crystallographic evidence suggests these higher-order phosphorylated inositols bind to the same site, close to the active site (Fig 3B and Fig 3D), and all function to increase HDAC enzyme activity *in vitro*. There is no evidence HDACs other than Class 1 are regulated by inositols, and while HDAC8 is considered a Class 1 HDAC no inositol was found in the crystal structure of HDAC8 (Vannini et al., 2007). Mutations at the inositol binding site decrease HDAC activity *in vitro* (Arrar et al., 2013; Millard et al., 2013; Watson et al., 2016) and in cells (Jamaladdin et al., 2014), but it remains unclear if wild-type HDACs have altered activity in cells depleted of inositols (Fu et al., 2018). It will be important to determine if cellular depletion of inositol

phosphates can alter HDAC activity in mammalian cells, as has been shown in yeast (Worley et al., 2013).

1.4 Bruton's tyrosine kinase (Btk) is activated by IP6, crystallographic analysis reveals two distinct IP6 binding sites in one PH domain.

Btk is an important tyrosine kinase known to be activated by PIP3-mediated recruitment to the plasma membrane via the Btk PH domain. While studying the auto-inhibited structure of Btk, work from John Kuryian's lab showed IP6 can activate Btk kinase activity, however IP4 could not. The 2.3Å crystal structure of the Btk PH-TH domains bound to IP6 revealed 4 molecules of IP6 bound to each Btk dimer (Fig 4A). Two IP6 molecules were bound to the canonical PIP3-binding site, as might be expected due to similarity with the PIP3 headgroup, however 2 molecules of IP6 were also seen at a "peripheral" binding site, which had not been seen in other PH domains (Fig 4B). Although the peripheral site IP6 molecule interacts with two Btk PH domains, mutation of residues critical for IP6 binding at this site not only decreased Btk activity in the context of the full length Btk, but also decoupled Btk from IP6 stimulation of kinase activity. These data suggest the peripheral IP6-binding site functionally regulates Btk activity. Recently, work from Mike Sheetz's lab used the Btk-PH domain as a biosensor to detect increases in nuclear PIP3 induced by DNA damage, altering AKT recruitment to these sites dependent on the kinase IPMK. It will be interesting to disentangle the roles of IP6 vs. PIP3 signaling in this system, given that IPMK is required for full production of both the IP6 and PIP3 signaling molecules in mammalian cell lines (Resnick et al., 2005) and in mouse embryonic fibroblasts (Maag et al., 2011) and has transcriptional regulator roles (Hamann and Blind, 2018).

1.5 Structural biology of phosphoinositide lipid binding proteins revealed by X-ray crystallography.

Nuclear receptors are ligand-regulated transcription factors with important roles in transcriptional regulatory networks (Blind et al., 2012; Crowder et al., 2017; Haliyur et al., 2018; Hamann and Blind, 2018). The nuclear receptor and transcription factor Steroidogenic Factor-1 (SF-1, *NR5A1*) binds many phospholipids, and X-ray crystal structures of SF-1 bound to PIP2 and PIP3 showed the phosphorylated inositol headgroup forms a new interaction surface on SF-1 (Fig 5), which functionally changes interaction of the nuclear receptor with a transcriptional coactivator *in vitro* (Blind et al., 2014). These X-ray crystal structures confirmed earlier docked models of SF-1 bound to PIP2 or PIP3 (Blind et al., 2012), which suggested the inositol headgroups would be highly solvent exposed, and might be accessible to lipid signaling enzymes (Blind et al., 2012). Indeed, IPMK is able to directly phosphorylate the PIP2 phospholipid bound to SF-1, and PTEN is able to dephosphorylate PIP3 bound to SF-1 (Blind et al., 2012). Importantly, no inositol phosphate has ever been shown to compete with phospholipids for binding to SF-1, although free inositol phosphates can compete for IPMK kinase activity with PIP2 bound to SF-1 (Blind et al., 2012). It will be interesting to use current synthetic biology approaches to determine if SF-1 is bound to inositol phosphates or phosphoinositides in bacteria expression systems (Clarke et al., 2019; Saiardi et al., 2018). Thus both PIP2 and PIP3 regulate SF-1 activity by a new mechanism, elucidated largely by X-ray crystallographic analyses of this nuclear receptor.

The closest homolog to SF-1 is Liver Receptor Homolog-1 (LRH-1, *NR5A2*), which also had been shown to bind to phosphoinositide lipids. The crystal structure of the complex of LRH-1 bound to PIP3 (Fig 6A) revealed many more changes to these static X-ray crystal structure snapshots than had been observed with SF-1 (Sablin et al., 2015). Although the PIP3 headgroup is shifted when comparing the SF-1 vs. LRH-1 bound PIP3 structures (Bryant and Blind, 2019), it is difficult to make any comparisons as PIP3 in the LRH-1 structure happens to lie in a crystal lattice interface. The shift in position of PIP3 headgroup bound to LRH-1 compared to SF-1 (Fig 6B) is thus most likely due to steric clashing imposed by the crystal lattice. However, since SF-1/PIP3 complex is bound to the transcriptional coactivator peptide PGC1a, while the LRH-1/PIP3 complex is bound to the corepressor peptide of DAX-1, it remains formally possible allosteric effects of different coregulator peptides might induce the conformational changes seen in these structures. What is clear from the LRH-1 structure bound to PIP3 is that PIP3 binds in the same conserved hydrophobic ligand binding pocket in LRH-1 and SF-1. Further structural and more importantly functional analyses will be required to clarify the role of phosphoinositides in regulating LRH-1 function in living cells.

Conclusions:

X-ray crystallographic analyses of higher-order phosphorylated inositol and phosphoinositide lipid binding protein effectors have provided valuable insight into the role these small signaling molecules play in mammalian cellular physiology. When coupled with eukaryotic expression systems, structural studies have occasionally revealed mechanism even before function has been established, truly highlighting the power of structural biology. Thus, structural biology can play an important, yet underappreciated, role in biological discovery.

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Figure 1: Pds5B crystal structure (PDB:5HDT) reveals IP6 binding site.

A. The overall 2.7Å crystal structure of human Pds5B (residues 21–1120), showing the IP6 acquired natively from the insect cell expression system. **B.** Close-up of IP6 binding site, showing interactions between IP6 phosphates and Pds5B basic residues surrounding the IP6-binding pocket. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).

Figure 2: ADAR2 crystal structure (PDB: 1ZY7) reveal IP6 binding site.

A. The overall 1.7 Å crystal structure of the catalytic domain of human ADAR2 (residues 299–701), showing the IP6 acquired natively from the yeast (*Saccharomyces cerevisiae*) expression system. **B.** Close-up of IP6 binding sites, showing interactions between IP6 phosphates basic residues of ADAR2 core surrounding the IP6-binding pocket. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).

Figure 3: Class 1 HDAC crystal structures (PDB:5ICN HDAC1 and PDB:4A69 HDAC3) reveal IP4 and IP6 binding site.

A. The overall 2.1 Å crystal structure of full-length human HDAC3 in complex with the deacetylase activation domain of NCOR2 (SMRT) corepressor depicted in light pink, showing the inositol tetrakisphosphate (1,4,5,6)P4 (IP4) acquired natively from the HEK293 cell expression system. IP4 is an enzyme product of the inositol polyphosphate multikinase (IPMK). **B.** Close-up of the inositol tetrakisphosphate (1,4,5,6)P4 (IP4) binding site, showing interactions between IP4 phosphates and basic residues of the HDAC3 and NCOR2 proteins which form the IP4-binding pocket. **C.** The overall 3.0 Å crystal structure of full-length human HDAC1 in complex with the ELM2-SANT domain from the MTA1 corepressor depicted in light pink, showing the IP6 acquired natively from the HEK293 cell expression system. **D.** Close-up of the IP6 binding site, showing interactions between IP6 phosphates and basic residues of the HDAC1 and MTA proteins which form the IP6-binding pocket. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).

Figure 4: Bruton's tyrosine kinase (Btk) crystal structure (PDB:4Y94) reveal a novel IP6 binding site.

A. The overall 2.3Å crystal structure of bovine Btk PH-TH domain (residues 1–172) showing the IP6, which had been ectopically added prior to crystallization. Note Btk is a dimer, and the IP6 resides between in the dimers in a peripheral binding site. Monomer Btk(1) shown in pink, monomer Btk(2) shown in gray. The other two IP6 molecules are bound to the canonical PIP3 / IP4 binding site. **B.** Close-up of the IP6 in the peripheral binding site between monomers Btk(1) and Btk(2), showing interactions between IP6 phosphates and basic residues from the Btk(1) and Btk(2) peripheral IP6 binding site. **C.** Same as in B, rotated 180°. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).

Figure 5: Steroidogenic Factor-1 (SF-1) ligand binding domain (LBD) crystal structures bound to phosphoinositide lipids reveal PIP2 (PDB:4QK4) and PIP3 (PDB:4QJR) binding sites.

A. Superposition of the overall crystal structures of human SF-1 (residues 218–461) complexed with a peptide representing the transcriptional coactivator PGC1α (cyan) and either the lipid PI(4,5)P2 (PIP2) at 2.8Å or the lipid PI(3,4,5)P3 (PIP3) at 2.4Å, showing binding sites for the phosphoinositide lipids. **B.** Close-up of the PIP2 binding site, showing position of the axial 2-position going into the plane of the page. **C.** Close-up of the PIP3 binding site, showing position of the axial 2-position going out of the plane of the page. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).

Figure 6: Liver Receptor Homolog-1 (LRH-1) ligand binding domain (LBD) crystal structure bound to phosphoinositide lipid reveals PIP3 (PDB:4RWV) binding site.

A. The overall 1.9Å crystal structure of human LRH-1 (residues 297–539) complexed with a peptide representing the transcriptional coactivator DAX-1 (cyan) and the phosphoinositide lipid di-palmitoyl (C16:0) PI(3,4,5)P3 (PIP3) ectopically added prior to crystallization, showing binding sites for the PIP3 lipid. **B.** Close-up of the PIP3 binding site, showing position of the axial 2-position going out of the plane of the page. Water molecules depicted as red spheres, all images generated in Pymol (Schrödinger).