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Biochemical characterisation of the tumour suppressor PH
domain-containing leucine-rich repeat protein phosphatase 2
(PHLPP2)

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Abstract

In the exploration of PH domain Leucine-rich Repeat Protein Phosphatase 2 (PHLPP2), initial studies by Gao et al. identified this phosphatase as a regulator of Protein Kinase B (PKB, also known as Akt) signalling. PHLPP2 was shown to have a domain structure comprising a Ras-associated (RA) domain, pleckstrin homology (PH) domain, a leucine-rich repeat (LRR) domain, a Protein Phosphatase 2C (PP2C) domain, and a PDZ-binding motif. It was reported to specifically dephosphorylate the regulatory hydrophobic motif of Akt, suggesting a role in cell survival and growth pathways. However, comprehensive purification and biochemical profiling suggest that the observed phosphatase activity in earlier studies came from co-purified Protein Phosphatase 2A (PP2A), a known Akt phosphatase, rather than PHLPP2 itself. The use of okadaic acid (OA), an inhibitor of PP2A, as well as removal of PP2A with Phosphatase-Inhibitor Beads (PIBs), revealed no intrinsic PHLPP2 phosphatase activity post-purification. Furthermore, we experimentally determined that the catalytic domain of PHLPP2 anchors a single Zn^{2+} metal ion and we present a model for the pseudo-catalytic site of PHLPP2 using molecular dynamics (MD) and quantum mechanical (QM) methods. In conjunction with the lack of PHLPP2 activity in phosphatase assays, we raise the hypothesis that PHLPP2 may be a pseudophosphatase, lacking intrinsic enzymatic activity under the tested conditions. This insight necessitates a re-evaluation of PHLPP2's role, inviting more rigorous experimental designs to clarify its biological function.

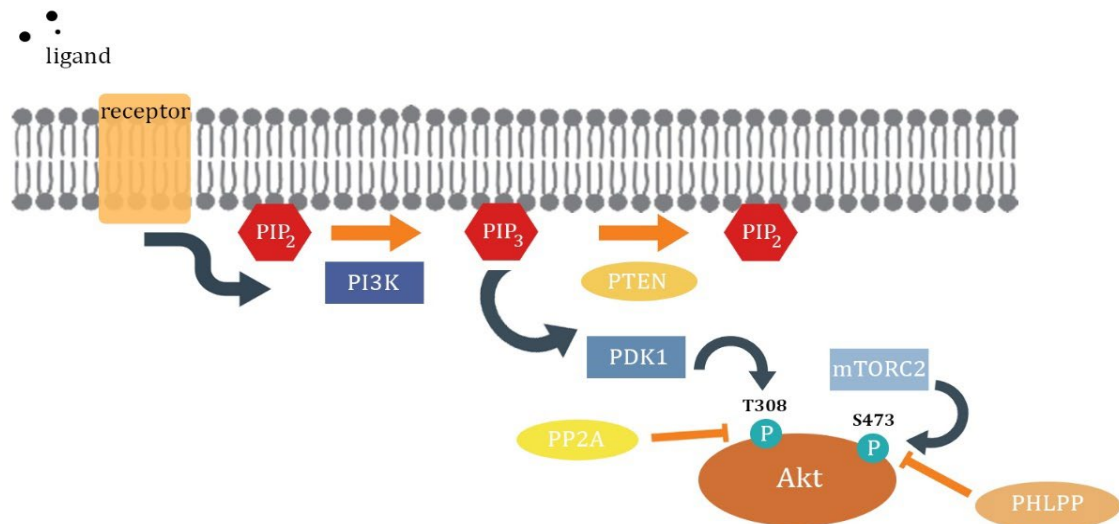
1. Introduction

The Phosphoinositide-3-Kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway is essential for controlling important biological processes such as growth, differentiation, metabolism, and cell survival (Manning & Toker, 2017). The hormone insulin, which is essential for the metabolism of fats and carbohydrates, initiates this pathway by binding to its receptor (Chen et al., 2022). This activation of the PI3K/Akt signalling cascade results in numerous physiological reactions such as increased cell uptake of glucose, prevention of programmed cell death (apoptosis), promotion of lipogenesis, and suppression of gluconeogenesis in the liver (Manning & Toker, 2017).

Phosphoinositides, more specifically phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P₃] are crucial for the activation of Akt (Manning & Toker, 2017). These phospholipids are located in the cell membrane and Phosphoinositide 3-kinase (PI3K) initiates this process (Burke, 2018). Phospholipidinositol-4,5-bisphosphate [PI(4,5)P₂] is converted into PIP₃ by PI3K in response to external signals, such as growth factors, including insulin. Akt is subsequently recruited to the cell membrane by binding to PIP₃ through its PH domain, setting the stage for its subsequent activation by other kinases (Figure 1 a) (Rong et al., 2001).

Full activation of Akt requires phosphorylation at two key residues: serine 473 (S473) in the hydrophobic motif (HM) and threonine 308 (T308) in the activation loop (AL) (Figure 1 b). Phosphoinositide-dependent kinase-1 (PDK1) and mTOR Complex 2 (mTORC2) are responsible for this phosphorylation at T308 and S473 respectively (Dangelmaier et al., 2014; Zhao et al., 2014). This is essential for the activation of Akt which then influences a variety of substrates involved in transcription, cell migration, glucose metabolism, apoptosis, and proliferation.

a)



b)

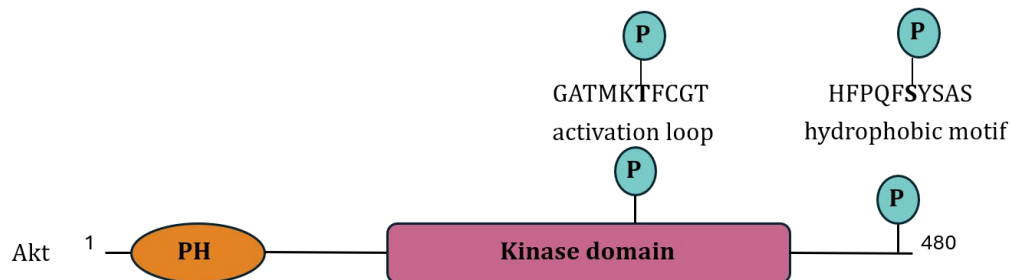


Figure 1. Akt signaling pathway:

a) Akt/PI3K signaling pathway; Ligand binding to a receptor activates PI3K, converting PIP₂ to PIP₃, facilitating Akt's PH domain-mediated membrane anchoring. Akt is phosphorylated at T308 by PDK1 and at S473 by mTORC2, achieving full activation. PTEN and PP2A negatively regulate the pathway by dephosphorylating PIP₃ and Akt, respectively, while PHLPP specifically dephosphorylates Akt at S473,

b) Domain organization of Akt highlighting its Phosphorylation Sites AL and HM.

Furthermore, disruption of the Akt pathway has been linked to a number of diseases, including cancer and metabolic diseases. Akt hyperactivation is often seen in cancer as a result of mutations in upstream regulators such as PI3K. Likewise, loss of the tumor suppressor gene Phosphatase and tensin homolog (PTEN), which functions as a brake on the PI3K/Akt pathway, leads to its dysregulation (Nowak et al., 2019).

To combat this dysregulation, the abundance of active Akt within the cell must be controlled. This is partially regulated by phosphatases, which dephosphorylate Akt on its AL and HM, rendering it inactive (Shi, 2009). Two phosphatases have been previously reported to dephosphorylate Akt; PH domain Leucine-rich Repeat Protein Phosphatase (PHLPP2) and Protein Phosphatase 2A (PP2A) and they therefore perform a vital part in ensuring the precise modulation of the Akt signalling pathway (Gao et al., 2005). PP2A has been reported to dephosphorylate T308 located in the AL, while PHLPP2 has been reported to specifically dephosphorylate S473 site within the HM. This dynamic equilibrium between the activities of kinases and phosphatases, is essential for the preservation of cellular homeostasis and functionality (Kuo et al., 2008).

This results in varied effects on the amplitude and duration of Akt pathway activity. PHLPP2 is a 147 kDa protein comprised of 4 domains: a RA domain, PH domain, LRR domain, and a PP2C phosphatase domain. (Figure 2 a). Although a crystal structure for PHLPP2 has not yet been determined, its structure can be predicted using AlphaFold (Jumper et al., Nature 2021) (Figure 2 b). The RA domain may influence interactions with GTPases, affecting cell signaling. The PH domain's known ability to bind to membranes provides clues into PHLPP2's subcellular localization. The LRR domain could mediate protein interactions, affecting PHLPP2's activity. The PP2C domain is responsible for its phosphatase activity, regulating the Akt pathway, and the PDZ-binding motif suggests potential interactions with PDZ domain-containing proteins, influencing further PHLPP2's localization and function.

a)



b)

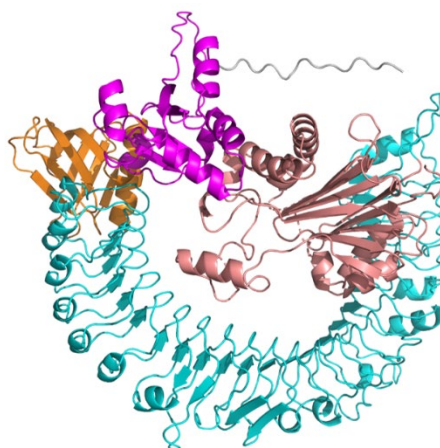


Figure 2. Domain organization of human PHLPP2

a) *Domain organization of human full length PHLPP2: RA domain, PH domain, 23 LRR, PP2C catalytic domain and PDZ binding motif*

b) *Alpha fold prediction of human full length PHLPP2: RA domain (pink), PH domain (orange), 23 LRR(blue), PP2C domain (teal)*

PHLPP2 is reported to dephosphorylate the HM of Akt at the S473 location, which enzymatically deactivates Akt. A previous study showed that while the isolated PP2C domain of PHLPP2 can act on both T308 and S473, full-length PHLPP2 shows a clear preference for S473 (Gao et al., 2005.) This attenuation of kinase activity has major consequences for cellular processes such as cell cycle regulation and death.

This specificity highlights the therapeutic promise of targeting PHLPP2 in situations where Akt signaling is inappropriately regulated, such as in certain cancers, and is crucial for the precise control of Akt activation. Due to a loss of PHLPP2 levels in human cancers, PHLPP2 has been classified as a tumour suppressor gene and PHLPP2 has been explored in non-cancer contexts, such as promoting immune cell activation and enhancing cell survival in neurodegenerative diseases (Sierecki et al., 2010).

With its complicated structure and function, a second phosphatase, PP2A, belonging to a different catalytic fold than PHLPP2, and which dephosphorylates the AL of Akt at the T308, further demonstrates the complex nature of cellular regulation. PP2A is a holoenzyme complex composed of a regulatory subunit (B, B', B'', or B'''), a catalytic subunit C, and a structural scaffold component A. This complex can perform specific dephosphorylation actions on a variety of substrates and cellular processes (Dzulko et al., 2020) Particularly, the regulatory B subunits provide substrate specificity and control the location and activity of the PP2A holoenzymes, which affects a wide range of biological processes (Seshacharyulu et al., 2013). The variety and adaptability of PP2A's holoenzyme structures (Figure 3), highlight the essential function of this protein in cellular signalling pathways which includes Akt.

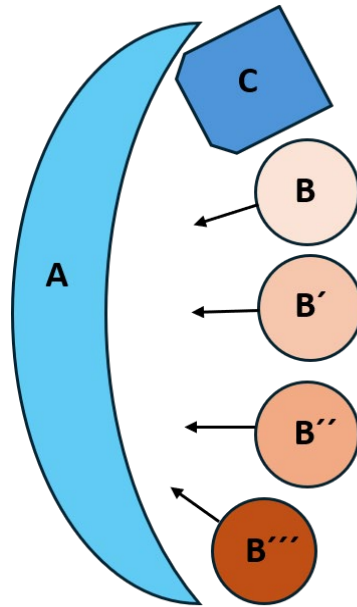


Figure 3. PP2A holoenzyme, diverse regulatory sub units

Understanding the complex interactions between kinase and phosphatase activities in cellular signalling is essential because of the ubiquitous and dynamic regulation of PP2A within the cell, which can result in a variety of pathological conditions, including cancer (Seshacharyulu et al., 2013). Comprehension of this tricky equilibrium between these opposing players can create opportunities for therapeutic approaches in conditions where dysregulated Akt signalling is present (Yadav et al., 2017; Gangula & Maddika, 2013).

To understand PHLPP2, it is crucial to explore the diversity and mechanisms of various phosphatases in detail. By examining their structural variations, regulatory mechanisms, and functional diversity, we can achieve better interpretation of PHLPP2's role within cellular signalling networks.

The human protein phosphatome, which is composed of 264 phosphatases identified by the study of 189 genes, is a large and complex network of enzymes that coordinate dephosphorylation processes in different physiological settings (Chen et al., 2017). Phosphatases also differ structurally and functionally from one another. In comparison with kinases there are 538 kinases encoded by the human genome. Phosphatases can be classified into 10 unique structural folds, which range from cysteine-based classes (CC1, CC2, CC3) to metal-dependent protein phosphatases (PPM) and beyond (Figure 4). Each fold represents a unique evolutionary solution to the challenge of substrate

dephosphorylation, with specific folds like the PPM being pivotal for enzymes requiring metal ions for their catalytic activity.

This classification reveals the evolutionary creativity and adaptability of these proteins (Chen et al., 2017).

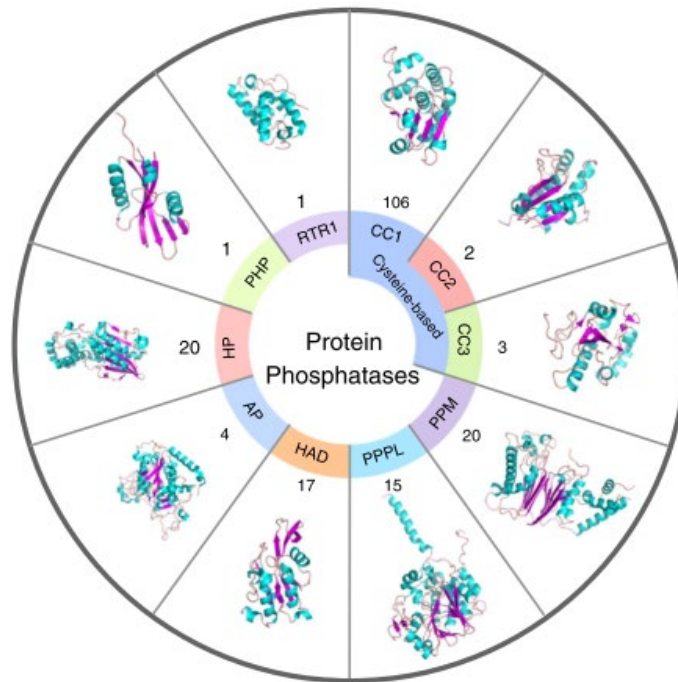


Figure 4. Human protein phosphatases are from 10 structural folds (Chen et al., 2017)

PHLPP2 is a member of the PPM (Metal-dependent Protein Phosphatase) family, reported to be manganese-dependent, meaning it requires metal ions in its catalytic site for its phosphatase activity (Grzechnik et al., 2016). The PPM family members' need for metal ions highlights the various tactics phosphatases use to achieve substrate specificity and catalytic efficiency (Kamada et al., 2020).

Members of PPM phosphatases have two or three divalent metal ions bound at their catalytic site. Metal ions M1 and M2 form a closely-spaced cluster in the active site of PPM phosphatases, which is critical for binding and coordinating phosphate ions, peptides with phosphoserine (pSer) and phosphothreonine (pThr). These ions facilitate the catalytic activity by jointly coordinating a bridging water molecule or hydroxide ion, proposed to act as the nucleophile in an S_N2 phosphate monoester hydrolase reaction. Additionally, the M1-M2 cluster helps stabilize the reaction's transition state by coordinating water molecules. The role of the M3 metal ion is suggested that it might be involved in positioning an activated, metal-bound water molecule to facilitate protonation of the serine/threonine leaving group. This is essential for breaking the phosphate monoester bond during the enzyme's action (Debnath et al., 2018).

The human phosphatome's diversity is further complicated by the presence of pseudophosphatases. Reports of experimental phosphatase activity led to the classification of PHLPP2 and its isoform PHLPP1 as functional phosphatases (Gao et al., 2005). However, later evolutionary analyses revealed that both proteins have substitution mutations in key residues required for metal ion coordination, suggesting that they function as pseudophosphatases (Chen et al., 2017). Despite the presence of those mutations, experimental data continue to support their role in phosphatase activity.

It is necessary to look into PPM1A (Protein Phosphatase, Mg²⁺/Mn²⁺ Dependent 1A), PHLPP2's closest structurally defined relative, in order to obtain a better understanding of PHLPP2 and its function within the phosphatome.

PPM1A's crystal structure provides insight into the coordination of metal ions within its catalytic site (Figure 5), showing how conserved residues enable the exact positioning of key metal ions, such as Mn²⁺, which enables the enzyme's catalytic activity and stability (Debnath et al., 2018).

Beyond just sharing the same evolutionary lineage within the PPM family, PPM1A and PHLPP2 are comparable. Although the precise coordination and function of these ions may vary, both phosphatases show evidence of being dependent on metal ions for their catalytic activity. The first two metal ions (M1 and M2) in PPM1A are essential to the enzyme's activity; residues D60, D239, and D282 coordinate M1, while D38, D60, and G61 coordinate M2 (Pan et al., 2015;). These ions play a crucial role in the activation of water molecules, which makes the nucleophilic assault on the phosphate group of the substrate easier and guarantees effective catalysis (Das et al., 1996). Furthermore, PPM1A requires a third metal ion (Su et al., 2011), which is coordinated by D146, D239 and D243 (Debnath et al., 2018). This emphasizes the complexity of metal ion dependency and suggests that PHLPP2's mechanism, although with its own specific structural and functional details, may be similar. The necessity of this third metal ion for modulating substrate specificity highlights the complex regulation of phosphatase activity, which may be influenced by the cellular concentration of metal ions within cells. This component of regulation could suggest a more general theme in the phosphatome, where precise control over dephosphorylation processes is ensured by enzyme activity that is precisely adjusted in response to cellular circumstances (Priya et al., 2021).

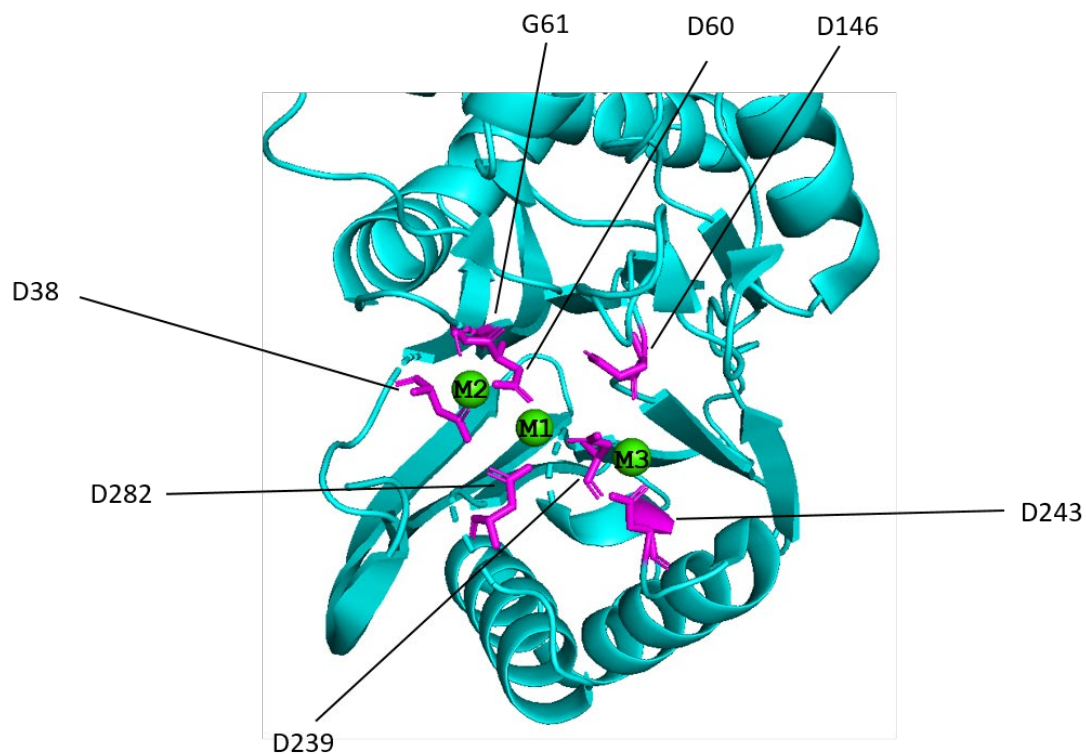


Figure 5. Crystal structure of PPM1A catalytic site; metal ions M1, M2, and M3 with Coordinating Amino Acids

Therefore, the investigation of PPM1A not only adds to our understanding of the diversity of the human phosphatome but also highlights the delicate balance that phosphatases maintain in cellular signalling networks.

2. Specific Aims

Despite its reported role in dephosphorylating Akt, the precise mechanisms by which PHLPP2 selectively targets this kinase for dephosphorylation and the subsequent regulatory actions are not understood.

Historically in studies such as Sierecki et al., 2014 the biochemical characterization of PHLPP2 has predominantly relied on single-step purification processes, as affinity GST-tagging. It may not be sufficient when purifying enzymes, due to the potential for possible contaminations. Consequently, several critical aspects of PHLPP2's activity remain experimentally unverified. Notably, while it is established that okadaic acid, a common inhibitor of different phosphatases such as PP1 and PP2A, does not inhibit PPM family phosphatases including PHLPP2, direct experimental evidence confirming its lack of effect on PHLPP2 is lacking.

Additionally, despite the known requirement of metal ions for PHLPP2 activity, there remains a significant gap regarding the precise identity of the metal ions integral to its catalytic site. Previous studies have suggested possible roles for Mn_{2+} or Mg_{2+} , consistent with other members of the PPM family, yet definitive experimental evidence identifying the specific metal ion associated with PHLPP2 remains elusive. This absence of concrete data limits our understanding of how metal ion coordination influences the phosphatase's structure and enzymatic function.

Aim 1: Determine the specific metal ion coordinated by PHLPP2 in its catalytic site

While PHLPP2 has been reported to be manganese-dependent, this has not been experimentally confirmed. This thesis aims to definitively identify the metal ion coordinated by PHLPP2 within its catalytic site and to determine its stoichiometry. Additionally, analysis of specific mutants that are likely involved in metal ion coordination.

Aim 2: Elucidate the Substrate Specificity of PHLPP2

To understand how PHLPP2 differentiates and targets specific motifs over others, this study aims to:

- Characterize the recognition and binding of PHLPP2 to the AL and HM of Akt using peptide substrates. This includes assessing the phosphatase activity towards phospho peptides that mimic these regions.

Aim 3: Determine the Mechanism of Action of PHLPP2

- Validate the phosphatase activity of PHLPP2 using a variety of in vitro assays, including malachite green and para-nitrophenyl phosphate (pNPP) assays, to measure phosphatase activity under different conditions.
- Conduct comparative analyses with known phosphatases and utilisation of okadaic acid as an inhibitor of other phosphatases, to determine if any observed activities are intrinsic to PHLPP2 or due to contaminants.

Additionally, the unresolved structure of PHLPP2 prompts further speculations, is there a possibility of existence of distinct conformations, active and inactive that could play a role in regulatory functions. Utilizing mass photometry, this thesis aims to determine if different oligomeric states of PHLPP2 are present, which could provide clues about its functional diversity and mechanism of action.

3. Preliminary Data

Preliminary data collected by Katharina Maria Siess, a former PhD student, have provided us important findings into PHLPP2's activity.

Siess examined the enzymatic phosphatase activity of purified, recombinant PHLPP2 against its synthetic substrates, phosphopeptides encompassing the HM and the AL of Akt. Contrary to existing literature, her findings indicated an unexpected preference of PHLPP2 for the AL over the HM (Figure 6), suggesting a potential revaluation of the enzyme's substrate specificity and catalytic behaviour could be necessary.

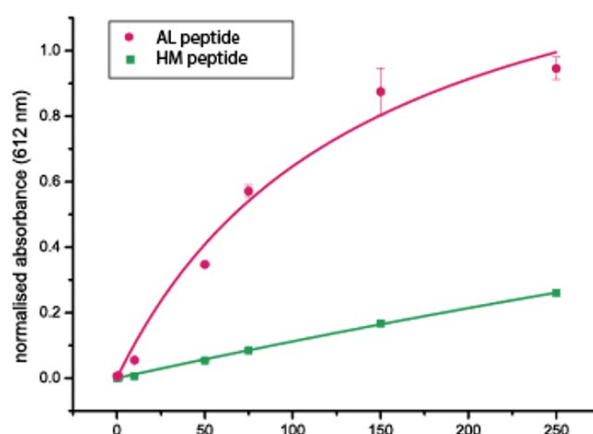


Figure 6. PHLPP2 phosphatase Activity on Akt; Hydrophobic Motif and Activation Loop peptides

Siess next tested the enzymatic activity of both wild-type (WT) and a mutant (D1024N) of PHLPP2 using a malachite green assay (Figure 7). This D1024 residue is highly conserved among PPM-family phosphatases and mutation to asparagine is reported to cause loss of phosphatase function (Pan et al., 2013). Contrary to expectations, the Malachite green assay showed that the D1024N mutant not only maintains activity but also shows more phosphatase activity than the PHLPP2 wild-type. This unexpected finding suggests that the activity observed in the assay may have come from a contaminant rather than PHLPP2 itself, and motivated us to consider the possibility of other mechanisms or interactions being involved.

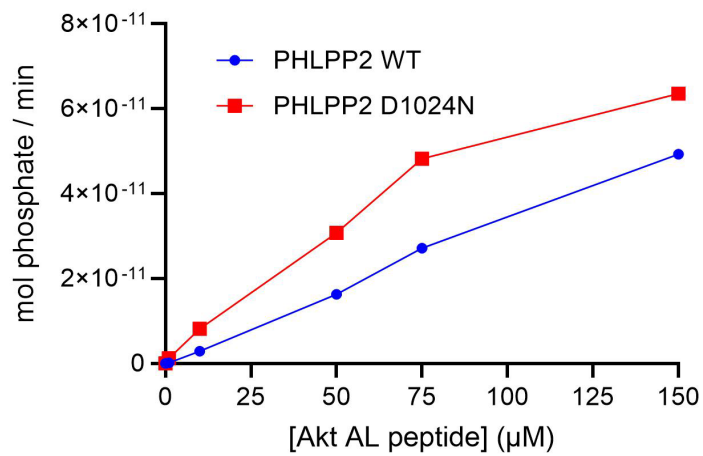


Figure 7. Phosphatase Activity of WT PHLPP2 and D1024N Mutant Against Akt Activation Loop Peptide

Given the lack of experimental data and together with the observation which a former PhD student reported it is crucial to re-examine all previously published data on PHLPP2, as our understanding of this phosphatase's role in cellular signalling may require fundamental revisions.

4. Materials and Methods

4.1. Protein expression in Sf9 insect cells

Six-well plate wells were created with 3 mL of medium containing 1×10^6 cells per well in order to transfect Sf9 insect cells. To generate a transfection mixture, 2.5 μ g of bacmid DNA was mixed with 100 μ L of antibiotic-free ESF media and 7.5 μ L of FuGENE transfection reagent (Promega) in 50 μ L of the same medium. The mixture was incubated for 15 to 30 minutes at room temperature, before 100 μ L of this mixture was introduced to each well for transfection. The transfected cells were examined for YFP fluorescence after 4–5 days. Following collection and filtering, the V0 virus-containing supernatant was utilized to infect a small-scale culture (25 mL at 1×10^6 Sf9 cells/mL). The culture was collected to extract the V1 virus after cell viability fell below 90% (500 x g centrifugation at room temperature for 10 minutes). Next, a larger scale culture of 1 L of Sf9 cells was infected with 1 mL of the V1 virus at a density of 1×10^6 cells/mL. When viability fell below 90%, the cells in this bigger culture were collected by centrifugation at 4000 rpm for 15 minutes at room temperature. The collected cell pellets were processed straight into lysis buffer or frozen in liquid nitrogen and kept at -80°C for later use.

4.2. Protein purification

Protein purification was carried out in a three-step process, starting with Glutathione-S-transferase (GST) affinity purification, followed by ion exchange chromatography, and concluding with size exclusion chromatography (SEC).

4.2.1. GST affinity purification

Pellet (1L Sf9 culture) was lysed in lysis buffer containing 50 mM Tris pH 7.5, 150 mM KCl, 1 mM TCEP, 2 mM benzamidine, 1 mL protease inhibitor stock, 2 mM MgCl_2 , 2 μ L DNase, and 0.25% Chap for approximately 1h at 4°C in the cold room and then spun down (30 min, 18k rpm, 4°C). Clarified supernatant was incubated with 2 ml glutathione Sepharose 4B beads for 2h at 4°C . The beads were washed 3x with 50 ml purification buffer (50 mM Tris pH 7.5, 150 mM KCl, 1 mM TCEP + 0.25% CHAPS), followed by TEV cleavage at 4°C overnight.

4.2.2. Ion exchange chromatography

PHLPP2 was recovered from the beads by gravity filtration and diluted to 50 mM KCl with QA buffer (50 mM Tris pH 7.5, 1 mM TCEP) before loading onto a 1 ml Q column equilibrated in buffer QA. PHLPP2 was eluted with a linear KCl gradient. Fractions containing PHLPP2 were diluted to 150 mM KCl and validated by SDS-PAGE. Subsequently, the fractions were concentrated to approximately 400 μ L in a 50 kDa MWCO Centricon concentrator.

4.2.3. Size exclusion chromatography

The sample was loaded on a Superdex 200 column equilibrated in SEC buffer (20 mM Tris pH 7.5, 150 mM KCl, 1 mM TCEP). Following SEC, peak fractions were identified and collected based on SDS-PAGE analysis. The sample was concentrated to 1 mg/mL in a 50 kDa Molecular Weight Cut-Off (MWCO) Centricon, flash frozen in liquid N₂ and stored at -80°C.

4.3. ICP-MS

Samples were stored at -80 °C prior to measurement. All vials and materials which came into contact with the sample, blank and standard solutions were pre-cleaned via soaking for 24 h in 10% and 24 h in 1% nitric acid followed by flushing with distilled water and drying under a class 100 laminar flow clean bench. Measurements were performed under clean room conditions (class 10000). Between the sample runs 1% formic acid solution was injected to eliminate carry over, followed by one buffer blank for assessment of possible contamination.

A NexSAR Speciation Analysis Ready HPLC system from Perkin Elmer (Massachusetts, USA) controlled by Clarity 8.8 software was used for the size exclusion separation. The mobile phase consisted of 50 mmol/L ammonium acetate at pH 7 and the flow rate was set to 0.4 mL min⁻¹. The injection volume for injection of samples, blank solutions and standards onto the ACQUITY UPLC Protein BEH 450Å SEC column (Waters™) was 15 µL. For elemental detection, the system was combined with an NexION 2000 quadrupole ICP-MS from Perkin Elmer (Massachusetts, USA). The reaction cell was filled with oxygen to induce the formation of ³²S¹⁶O⁺ and circumvent the ¹⁶O²⁺ interference on the most abundant sulfur isotope ³²S. Zinc was detected as ⁶⁶Zn. Bovine Cu/Zn-SOD was employed to determine the intensity ratio between the ⁶⁶Zn and the ³²S¹⁶O signals. The stoichiometric zinc ratio was calculated from the number of sulfur atoms per protein. ICP-MS parameters and data evaluation were optimized according to (Hann et al. 2004). pH measurements were conducted with an Education Line EL20 pH-Meter with a micro electrode (inLab Micro, Mettler, Toledo, USA).

4.4. Mass photometry

Mass photometry experiments were carried out on a Refeyn TwoMP instrument, data was acquired using AcquireMP (Refeyn) and processed using DiscoverMP (Refeyn). Microscope coverslips (24 x 50 mm) were cleaned with both 100% isopropanol and Milli-Q water, in a sonicating water bath for 5 mins, and dried with a nitrogen stream. Chambers were made by sticking silicone gaskets (3 x 1 mm) on top of the slides. Mass calibration was performed using NativeMark Unstained Protein Standard (Invitrogen), by fitting a contrast-to-mass calibration curve to 66, 146, 480 and 1048 kDa standards. Purified full length PHLPP2 was diluted to 100 nM in buffer (20 mM Tris pH 7.5, 150 mM KCl, 1 mM

TCEP), filtered through 0.22 μm PES filters, and loaded into the coverslip chambers. Data was collected for 60 s at a 1 kHz frame rate.

4.5. Differential Scanning Fluorimetry

Protein of 0.5 mg/mL concentration was prepared and mixed in a 1:100 ratio with SYPRO orange dye (ThermoFisher Scientific). This mixture was tested both with and without 1 mM EDTA. The experiment was conducted in a SEC buffer at pH 7.5. A thermocycler (BioRad) was employed to incrementally increase the temperature from 0 to 90 $^{\circ}\text{C}$, in steps of 0.5 $^{\circ}\text{C}$.

4.6. Malachite Green Assay

Malachite green assays were performed according to the Sigma-Aldrich protocol. PHLPP2 was serially diluted two-fold from 500 nM to 0 nM, while PP2A was serially diluted ten-fold from 10000 pM to 0 pM. Each was incubated with Akt AL and HM peptides at a concentration of 0.4 mM in a gel filtration buffer consisting of 20mM Tris (pH 7.5), 150 mM KCl, and 1 mM TCEP. Assays were conducted with and without the addition of 25 nM okadaic acid. Following a 1-hour incubation period, the working reagent was added to terminate the phosphatase reactions. The absorbance of samples was measured in a 96-well plate format at 620 nm absorbance using a TECAN 500 infinite plate reader.

4.7. Peptide Fingerprinting Mass Spectrometry

Peptides were separated on an Ultimate 3000 RSLC nano-flow chromatography system (Thermo-Fisher), using a pre-column for sample loading (Acclaim PepMap C18, 2 cm $\times$ 0.1 mm, 5 μm , Thermo-Fisher), and a C18 analytical column (Acclaim PepMap C18, 50 cm $\times$ 0.75 mm, 2 μm , Thermo-Fisher), applying a segmented linear gradient from 2% to 35% and finally 80% solvent B (80 % acetonitrile, 0.1 % formic acid; solvent A 0.1 % formic acid) at a flow rate of 230 nL/min over 60min. Eluting peptides were analyzed on an Exploris 480 Orbitrap mass spectrometer (Thermo Fisher), which was coupled to the column with a FAIMS pro ion-source (Thermo-Fisher) using coated emitter tips (PepSep, MSWil).

For analysis of peptides obtained from PIB eluates, the mass spectrometer was operated in DIA mode with the FAIMS CV set to -45, the survey scans were obtained in a mass range of 350-1200 m/z, at a resolution of 60k at 200 m/z and a normalized AGC target at 300%. 31 MSMS spectra with variable isolation width between 13 and 24 m/z covering 349.5-1200.5 m/z range including 1 m/z windows overlap, were acquired in the HCD cell at 30% collision energy at a normalized AGC target of 1000% and a resolution of 30k. The max. injection time was set to auto.

For analysis of peptides obtained from purified, recombinant PHLPP2, the mass spectrometer was operated in DDA mode with two FAIMS compensation voltages (CV) set to -45 or -60 and 1 s cycle time

per CV. The survey scans were obtained in a mass range of 350-1500 m/z, at a resolution of 60k at 200 m/z, and a normalized AGC target at 100%. The most intense ions were selected with an isolation width of 1.2 m/z, fragmented in the HCD cell at 28% collision energy, and the spectra recorded for max. 100 ms at a normalized AGC target of 100% and a resolution of 15k. Peptides with a charge of +2 to +6 were included for fragmentation, the peptide match feature was set to preferred, the exclude isotope feature was enabled, and selected precursors were dynamically excluded from repeated sampling for 20 seconds.

Raw data were processed using Spectronaut software (version 18.3, <https://biognosys.com/software/spectronaut/>) with the DirectDIA+ workflow or FragPipe (version 19.1) (Kong et al. 2017). The Uniprot *Spodoptera frugiperda* proteome (version 2023.03), PHLP2_HUMAN protein sequence, as well as a database of most common contaminants were used. The searches were performed with full trypsin specificity and a maximum of 2 missed cleavages at a protein and peptide spectrum match false discovery rate of 1%. Carbamidomethylation of cysteine residues were set as fixed, oxidation of methionine and N-terminal acetylation as variable modifications. The cross-run normalization was turned off and all other settings were left as default.

Computational analysis was performed using Python and the in-house developed Python library MsReport (version 0.0.19) (Hollenstein et al. 2023). Protein intensity less than 1000 was set to missing to remove the low-quality quantification values. LFQ protein intensities reported by SpectroNaut were log2-transformed and normalized across samples using the ModeNormalizer from MsReport. The missing normalized LFQ intensity values were imputed by drawing random values from a normal distribution after filtering out contaminants, proteins with less than 2 peptides and less than 1 quantified values in at least one group. Using MsReport, iBAQ intensities were calculated from the raw intensities reported by FragPipe.

The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifiers PXD045891 and PXD045978.

4.8. Phosphatase inhibitor beads

PIBs were prepared according to (Brauer et al. 2021).

4.9. Para-nitrophenyl phosphate (pNPP) Assay

PHLPP2 and Lambda Phosphatase were serially diluted two-fold from 1000 nM to 0 nM and incubated with 20 mM pNPP substrate in a gel filtration buffer (20 mM Tris, pH 7.5, 150 mM KCl, 1 mM TCEP). The assays, performed with and without 25 nM okadaic acid, were terminated using 5 M NaOH. Absorbance was measured at 405 nm in a 96-well plate using a TECAN 500 infinite plate reader.

4.10. Sample preparation for peptide fingerprinting

Pellet of 1L Sf9 culture was lysed in 20 mL lysis buffer (2 mM benzamidine, 200 μ L Protease inhibitor stock, 2mM MgCl₂, 1 mM TCEP, 0.4 μ L benzonase, 0.25% Chaps) for approximately 1 h at 4°C in the cold room and then spun down (30 min, 18k rpm, 4°C). The clarified supernatant was assessed for protein concentration using the Bradford assay, and two equal aliquots (1 mg/mL) were prepared. As a control, 0.5 μ M okadaic acid was added to one of the samples. The two lysate samples (1 mL each) were then incubated with 20 μ L of phosphatase inhibitor beads (PIBs) at 4°C for 1 hour. After incubation, the supernatant was carefully removed, and the PIBs were washed three times with 0.5 mL of Lysis buffer. Subsequently, the washed beads were incubated with 120 μ L of 2% SDS for 1 hour at 65°C and the eluates collected using Pierce Micro-Spin columns.

The samples were mixed with 5 μ L 100mM Tris HCl pH 8.5 and heated at 95°C for 5min. Then disulfide bonds were reduced with 4 μ L of 250 mM dithiothreitol (DTT) for 30 min at room temperature before adding 4 μ L of 500 mM iodoacetamide and incubating for 30 min at room temperature in the dark. The remaining iodoacetamide was quenched with 2 μ L of 250 mM DTT for 10 min. Sera-MagSpeed Beads mix (GE Life Sciences, cat. nos. 45152105050350 and 65152105050350) was added to the sample at beads:protein ratio of 10:1 and mixed with ethanol to reach 50% final ethanol concentration. The tubes were kept in a thermomixer for 10min at 1000rpm for 24min to let protein bind to the beads. Then the supernatant was removed using a magnetic rack and the beads were washed with 180 μ L 80% ethanol in water off the rack. And the wash procedure was repeated 3 more times. Afterwards, 30 μ L 50mM ammonium bicarbonate with 120ng trypsin (Trypsin Gold, Promega) was added to the beads and incubated at 37°C 1000rpm overnight. The beads were settled on a magnetic rack and the supernatant was transferred to a new tube. The beads were rinsed with 30 μ L ammonium bicarbonate and sonicated for 30s and the supernatant was pooled to the supernatant from the previous step. Then the samples were centrifugated at 20000g for 1min and the supernatant was transferred to a new tube to remove any remaining beads. Trifluoroacetic acid was added to reach a final concentration of 0.5 %.

For the analysis of recombinant PHLPP2, ~5 μ g protein was mixed with 15 μ L 8M urea 50mM ammonium bicarbonate. Disulfide bonds were reduced with 0.8 μ L of 250 mM dithiothreitol (DTT) for 30 min at room temperature before adding 0.8 μ L of 500 mM iodoacetamide and incubating for 30 min at room temperature in the dark. The remaining iodoacetamide was quenched with 0.4 μ L of 250 mM DTT for 10 min. 100 μ L 50mM ammonium bicarbonate was added to dilute the urea to 1M. The protein was digested with 160ng trypsin (Trypsin Gold, Promega) at 37°C overnight. The digest was stopped by adding trifluoroacetic acid to reach a final concentration of 0.5 % and the peptides were desalted using C18 Stagetips (Rappsilber, Mann, and Ishihama 2007)

4.11. Molecular Dynamics simulation

Molecular dynamics (MD) simulations of full-length PHLPP2 were carried out using the GROMACS version 2023, employing the all-atom Amber99SB-ILDN force field and the TIP4P-D water model. The initial configuration of the full-length PHLPP2 protein was obtained from an AlphaFold prediction for PHLPP2. Based on the active site architecture of structural homologs and evolutionary conservation of metal-coordinating residues, we manually placed the Zn^{2+} ion in the M2 position of the putative active site. To prepare the system we performed an energy minimization, constant volume equilibration (NVT) and constant pressure equilibration (NPT). Minimization and equilibration established the stability of the Zn^{2+} ion in the active site. Stability was confirmed by a production simulation of a single copy system. The system was run for 10 ns in a cubic water box of 8 nm x 8 nm x 8 nm with 0.15 M NaCl, NaCl concentration was selected to mimic the experimentally confirmed conditions for protein stability. Due to inherent limitations molecular mechanics cannot approximate adequately the quantum behavior of the active sites containing metal ions. To overcome this limitations, we imposed distance restraints between coordinating atoms and the zinc ion, as well as imposing distance restraints between the coordinating atoms. The restraints were necessary to preserve the tetrahedral geometry of coordinating atoms around the zinc ion. Simulations with restrictions were then run for a total length of 100 ns. A leap-frog algorithm was used for integration under periodic boundary conditions. In both energy minimization and production runs, neighbor-lists were updated every 20 steps, following a Verlet-scheme based grid-search approach. The bonds involving H atoms were constrained using LINCS. Temperature control was achieved via a V-rescale thermostat, with a relaxation time of 0.1 ps, while pressure was controlled using a Parrinello-Rahman approach. Compressibility for the barostat was set to 4.5×10^{-5} 1/Pa, and the relaxation time was 2 ps. Coupling was done separately for water and protein in all cases. A twin-range spherical cut-off (1.0 nm/1.2 nm) was used for van der Waals interactions, while electrostatics were treated using the Particle-Mesh Ewald method with a real space cut-off of 1.2 nm, 0.16 nm grid and cubic interpolation. During the production simulation global RMSD (Root mean square deviation) has remained stable at 0.2-0.25 nm after 10 ns, RMSD was calculated compared to the final structure of NPT equilibration. To determine the stability of the active site we calculated the RMSD of the coordinating atoms relative to the atom positions of the structural homologue. For further, quantum, molecular modeling we had taken a randomized structure from the pool of the smallest active site RMSD structures. The chosen structure had persisted in the minimal active site RMSD range for at least 1 ns as a cutoff, in order to avoid stochastic and unrealistic structures. The structures and trajectories were visualized using PyMol.

4.12. ONIOM method

Calculations were performed with the Gaussian 16 (Revision C.01) quantum chemistry software package (Dapprich et al., 1999). To reduce the computational time given the size of the system, we employed the ONIOM method (Rappaport et al., 2007) as implemented in the Gaussian 16 program. A two-layer QM/QM method that combines different QM methods is applied: a high-level of accuracy for the atoms three bonds away from the zinc ion and a low level for the rest of the systems – cut from the corresponding initial structures (crystal structure of a 1:1:1 FGF23-FGFR1c- α -Klotho Ternary Complex, PDB 5W21, and MD generated model) at approximately 12 Å in radius around the metal cation where the amino acid chains logically end with methyl groups. In all calculations for the α -Klotho model system, a DFT function (M062X, B3LYP, or ω B97XD) was adopted for the inner layer, while a semi-empirical method (PM3) was used for the treatment of the real system. Different basis sets (6-31G(d,p), 6-31+G(d,p) and 6-31+G(3d,p)) in conjunction with the selected DFT functions were assessed for an accurate prediction of structural parameters (Zn-S and Zn-O bond lengths). All the methods tested predicted Zn-S bond lengths in the range 2.32-2.40 Å, and the average lengths of the three Zn-O bonds were 1.96-2.02 Å. It was concluded that both oniom(ω B97XD/6-31G(d,p):PM3) and oniom(B3LYP/6-31+G(3d,p):PM3) methods are suitable for proper description of the geometry of the α -Klotho Zn center.

5. Results

5.1. Protein Purification

To elucidate the biochemical properties, metal ion specificity and enzymatic activity of PHLPP2, it is crucial to obtain a pure and homogeneous sample of PHLPP2. The expression of the full-length PHLPP2 protein was done in baculovirus-infected Sf9 insect cells. The purification process for PHLPP2 (Figure 8), was carried out through a three-step purification protocol, starting with GST-affinity chromatography. Following this, the GST tag was enzymatically cleaved from PHLPP2 using TEV protease. Subsequently, the cleaved PHLPP2 was subjected to ion exchange chromatography (IEX) to separate PHLPP2 based on its net charge (Figure 9 b). Peak fractions from all purification steps were analysed via SDS-PAGE (Figure 9 a). The final step involves size exclusion chromatography (SEC) to further purify PHLPP2 by molecular weight (Figure 9 c). SEC also acts as a quality control check to ensure PHLPP2 is not aggregated. This multi-step purification protocol is designed to yield PHLPP2 with a high degree of purity suitable for enzymatic assays. Mass spectrometry data (Figure 9 d), shows that what we have purified is indeed PHLPP2, corresponding to the expected molecular weight of PHLPP2 (147 kDa).

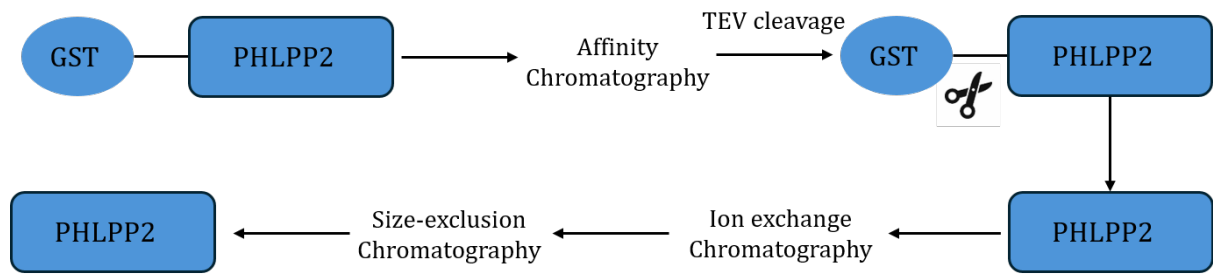


Figure 8. Schematic illustration of Three-Step PHLPP2 purification Process Involving GST, Anion Exchange and Size Exclusion Chromatography

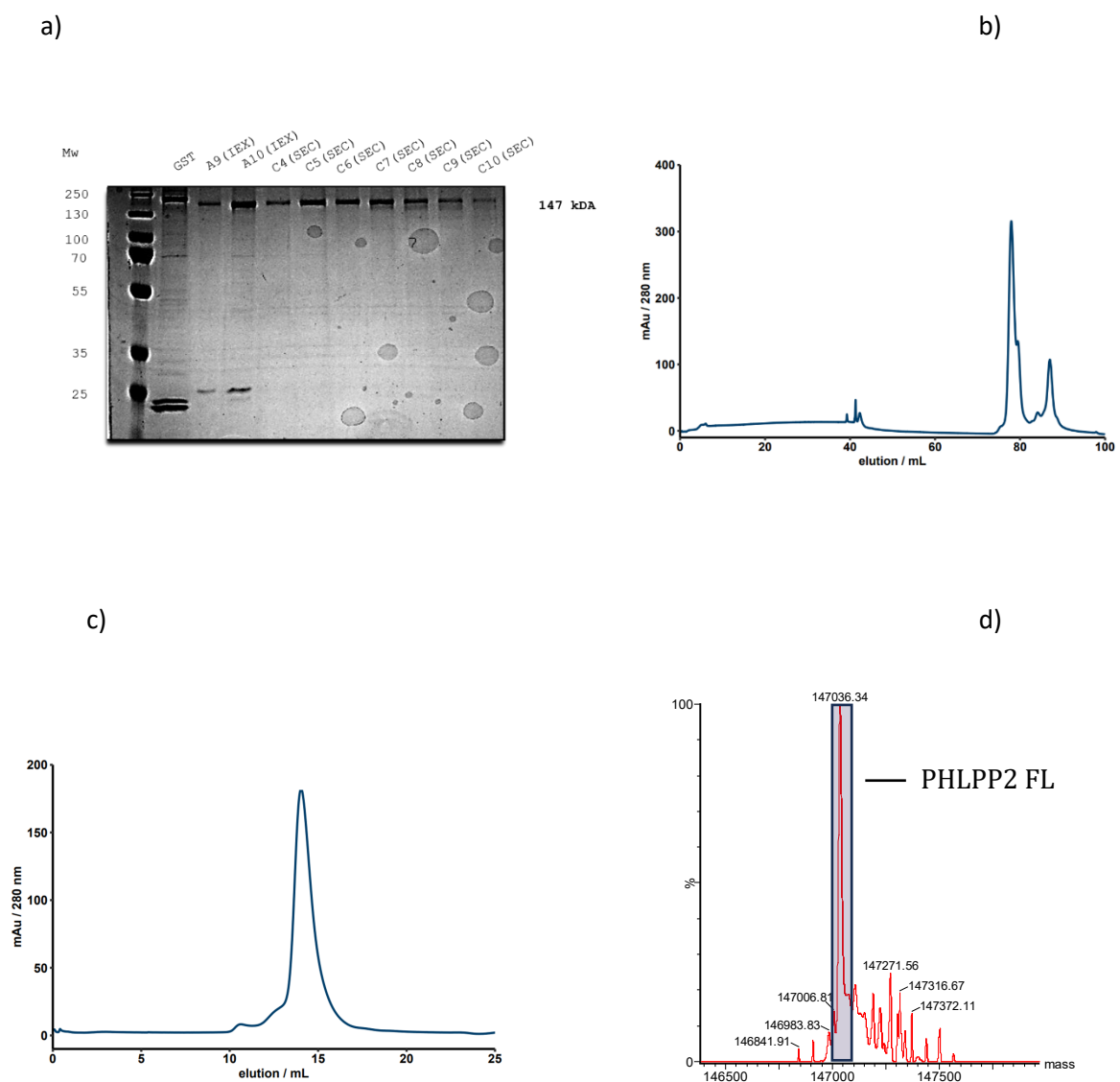


Figure 9. Human PHLPP2 Full Length purification protocol

a) SDS gel of PHLPP2: Lane 1 shows the ladder control, lane 2 Shows GST-PHLPP2 at ca. 150 kDa, lanes 3-4 shows PHLPP2 Ion Exchange fractions, lanes 5-11 shows different fractions of Size exclusion chromatography.

b) Chromatogram illustrating the ion exchange chromatography profile of PHLPP2i

c) Chromatogram illustrating the size exclusion chromatography profile of PHLPP2

d) Mass spectrometry of full length PHLPP2, indicating correct size of 147 kDa

Mass photometry was utilised to further assess the oligomeric state of PHLPP2 and its distribution (Figure 10). The dominant peak at approximately 136 ± 18.7 kDa closely aligns with the anticipated molecular weight of one PHLPP2 molecule, indicating that PHLPP2 is predominantly monomeric. A secondary peak at approximately 265 ± 24 kDa is indicative of a dimeric population of PHLPP2 particles, with the mass approximating twice that of the monomeric form. The presence of this peak suggests the possibility of PHLPP2 to form dimers in the examined conditions.

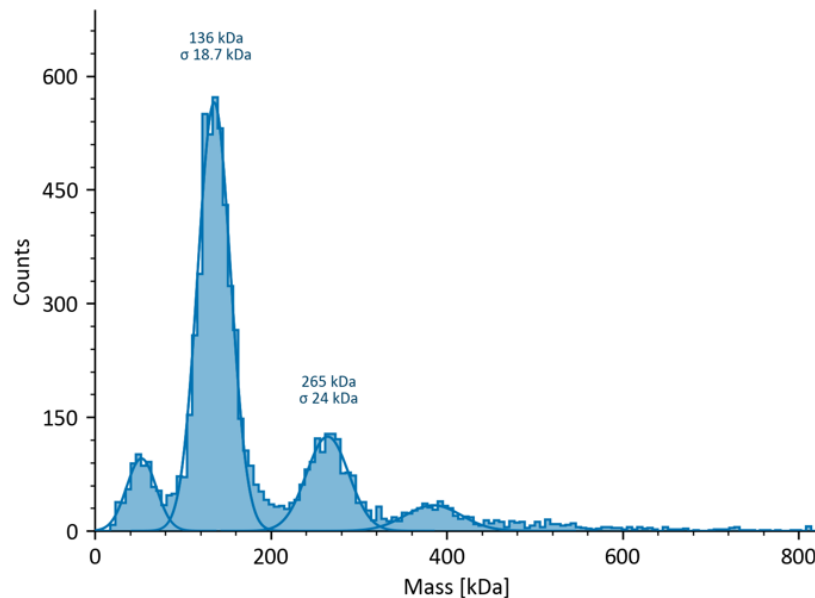


Figure 10. Mass Photometry Analysis of PHLPP2 indicating Monomer Purity and Potential Dimer Formation

Further experiments are needed to determine whether the observed dimerization of PHLPP2 is physiologically relevant. It should also be noted that the mass photometry measurements were carried out at a PHLPP2 concentration of 12.5 nM, which is slightly higher than the endogenous PHLPP2 concentration in the cell (Cho et al., 2022). Additional investigations, such as cross-linking studies, co-immunoprecipitation, and cryo-electron microscopy, could provide more direct evidence of PHLPP2 dimer formation and its stability under physiological conditions. Moreover, the functional implications

of such dimerization remain unclear. Understanding whether the dimeric state of PHLPP2 influences its activity, interaction with substrates, or its role in signalling pathways requires further study.

5.2. Implication of metal ion in PHLPP2

Metal ions play a pivotal role in the biological function of phosphatases, acting as essential cofactors that are crucial for the catalytic activity of these enzymes by influencing both substrate binding and the phosphoryl transfer reaction (Das et al., 1996). Phosphatase activity is dependent on the precise coordination and binding of these metal ions within the enzyme's active site, which can include ions such as zinc, manganese, magnesium and others (Bellomo et al., 2018). Understanding the specific metal ions bound to phosphatases like PHLPP2 is not only fundamental to grasping their biochemical mechanisms but also critical for therapeutic targeting and inhibitor design. Despite the established importance of metal ions in phosphatases, there has been a gap in experimental data confirming the identity of the ions bound to PHLPP2. Gao et al., 2005; and Sierrecki & Newton, 2014 have suggested that PHLPP2 coordinates Mn^{2+} ions, yet there is no experimental confirmation of this.

5.2.1. Detection of metal ion

In collaboration with Stephan Hann at Boku University, we wanted to confront the hypothesis that PHLPP2 has a manganese metal ion bound to its catalytic site. To find out experimentally which metal ion is bound we used Size Exclusion Chromatography coupled with Inductively Coupled Plasma Mass Spectrometry (SEC-ICP-MS), (Hann et al., 2004). The method involves using the enzyme superoxide dismutase (SOD) as a calibrant, extracted from bovine red blood cells. SOD has a well-characterized active site containing one copper and one zinc ion. A standard curve generated from the known metal ion concentrations in SOD, as measured by ICP-MS, enable us to precisely determine the metal ion within PHLPP2.

Figure 11. show peak areas for sulfur (^{32}S), copper (^{65}Cu), and zinc (^{66}Zn) in ICP-MS counts. Subsequent calculations of the sulfur to copper (S/Cu) and sulfur to zinc (S/Zn) ratios were compared with the known stoichiometry of SOD1 (4:1:1 for S:Cu:Zn). Analysis of three biological replicates of PHLPP2, each measured in triplicate to maximize precision, revealed consistent counts for sulfur and zinc. The persistent S/Zn ratio across all replicates, coupled with the no detectable signals for iron, copper, manganese or calcium, shows the association of PHLPP2 with Zn^{2+} ion. The stoichiometry for zinc in PHLPP2 was a 1:1 ratio, indicating a one-to-one correspondence between PHLPP2 molecules and Zn^{2+} ion.

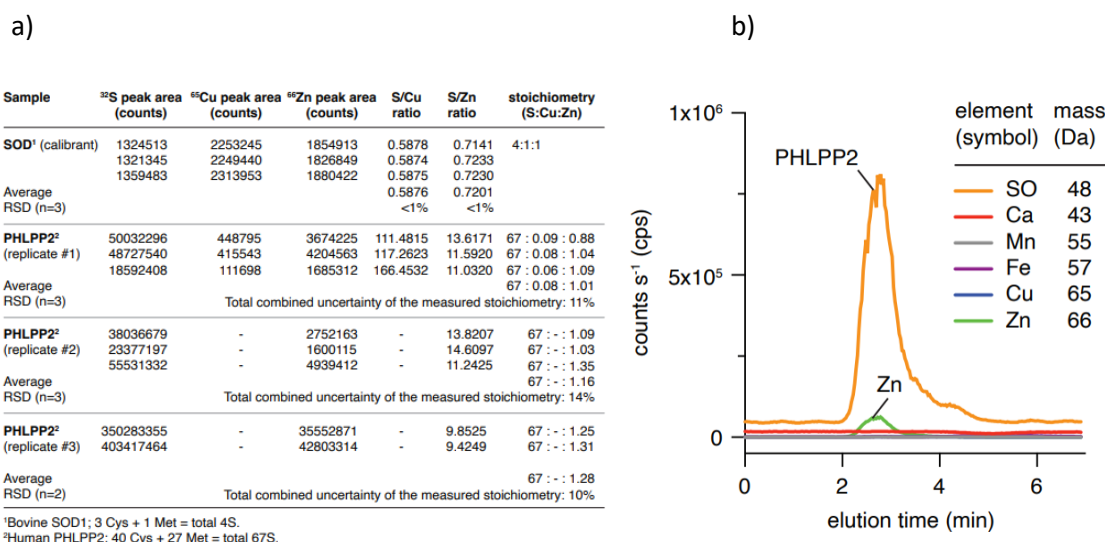


Figure 11. Characterization of Metal Ion binding to PHLPP2 by SEC ICP-MS Analysis

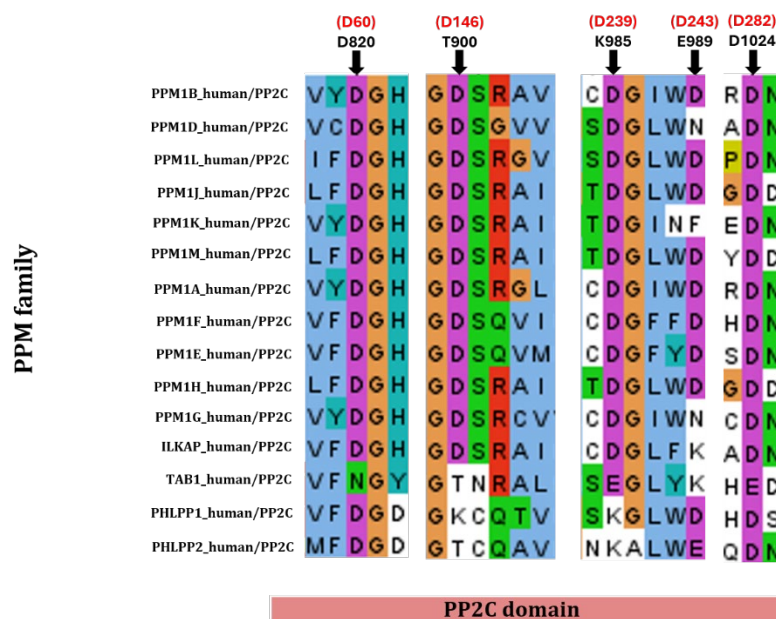
- a) Quantitative Analysis of PHLPP2 Stoichiometry by SEC ICP-MS: The data represent three biological replicates, indicating a consistent 1:1 stoichiometry of zinc to protein ratio across measurements
- b) SEC-ICP MS Chromatogram of PHLPP2, chromatogram highlights presence and specific binding of Zn Metal Ion to PHLPP2 in comparison to other potential metal ions

5.2.2. Metal Ion Coordination; Insights

PHLPP2, a member of the PPM (protein phosphatase Mg^{2+}/Mn^{2+} - dependent) family, displays unique metal-binding properties that diverge from the canonical members such as PPM1A. Notably, PHLPP2 lacks conservation of key residues that typically coordinate the binding of metal ions essential for phosphatase activity. Based on ICP-MS data, which revealed a 1:1 stoichiometry of Zn^{2+} ion bound to PHLPP2, we set out to identify the potential amino acid residues which would be responsible for coordinating the Zn^{2+} ion within its catalytic site, thus shedding light on its unique enzymatic mechanism. The comparative analysis, using the AlphaFold2-predicted structure of PHLPP2's phosphatase domain (Figure 13), demonstrated a close alignment with PPM1A.

The conserved metal ion binding sites within PP2C domain, M1, M2 M3, which are integral to the catalytic function in PPM1A and other PPM phosphatases, are not all conserved in PHLPP2 (Figure 12 a). As exemplified by PPM1A (Debnath et al., 2018), for which we have detailed crystallographic data (Figure 4), the residues coordinating the metal ions in PPM1A (D60, D146, D239, D243, and D282) are conserved among the majority of the family members. This conservation underlines the evolutionary importance of these residues in maintaining the structural integrity and catalytic function of the phosphatases. Notably, PHLPP2 and TAB1, which has been reported to be a pseudophosphatase (Chen et al., 2017; Conner et al., 2006), deviate from this conservation pattern, suggesting a possible divergence in their metal ion coordination or a distinct functional adaptation.

a)



b)

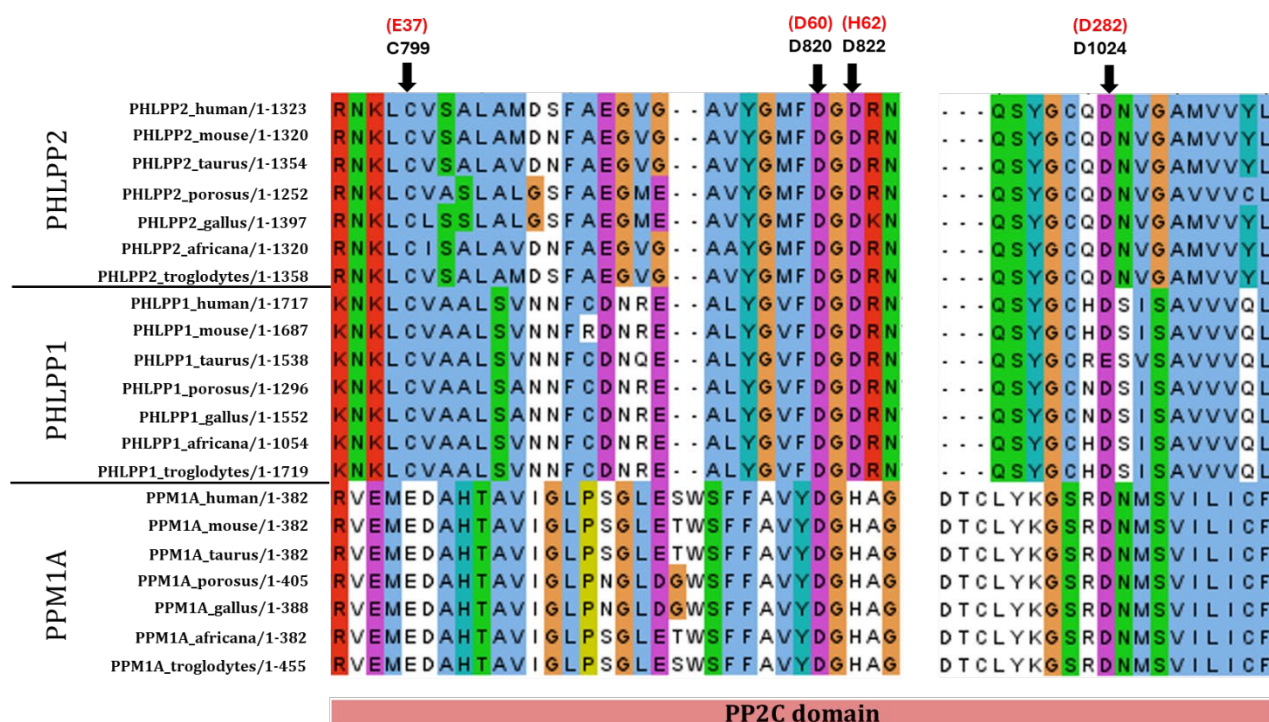


Figure 12. Conservation of PP2C Catalytic Residues among PPM members

- a) Conservation of Metal Ion Coordination Residues within the PPM Phosphatase Family: Emphasis on PPM1A and Deviations in PHLPP2 as Highlighted in Red
- b) Conservation of suspected catalytic site among PHLPP2, PHLPP1 and close Relative PPM1A

Through structural modelling, we identified a quartet of residues—C799, D820, D822, and D1024—as the most probable coordinators of a Zn^{2+} ion within the PHLPP2 structure, aligning with the M2 metal ion binding site of PPM1A (Figure 13). The exclusion of alternative sites M1 and M3 as potential binding zones is supported by the presence of K985, which introduces electrostatic repulsions that makes metal ion binding at these sites energetically unfavorable. This reinforces the unique suitability of the M2 site, where D820 and D1024 are conserved and optimally positioned to participate in metal coordination. The only other residues in proximity to the M2 site that possess side chains capable of coordinating zinc are C799 and D822. C799 contains a sulfur atom, while D822 includes an oxygen atom, both of which have lone pairs of electrons needed for forming coordinate bonds with Zn^{2+} ion. Zinc typically adopts a tetrahedral coordination geometry, due to 4 sp^3 orbitals created by hybridization of the empty 4s^2 orbital (in Zn^{2+} ion, the 2 electrons are lost from the 4s orbital) with the 3 empty 4p orbitals. This hybridization arrangement allows for the optimal spatial distribution of electron pairs around the Zn^{2+} ion, consistent with the principles of VSEPR (Valence Shell Electron Pair Repulsion) theory, which predicts tetrahedral geometry based on the minimization of electron pair repulsion. The selection of these four residues for Zn^{2+} ion coordination is due to their chemical compatibility with Zn^{2+} ion and their spatial arrangement conducive to forming a stable tetrahedral complex.

In an intriguing parallel, PHLPP2's D820 and D1024 correspond to PPM1A's D60 and D282, while C799 and D822 align with E37 and H62, respectively (Figure 12 b).

A closer examination of the sequence alignment reveals that while residues like D820 and D1024 are conserved across the phosphatase family, C799 and D822 are distinctive to PHLPP (Figure 12 b). This conservation pattern led us to think that these residues could be involved in Zn^{2+} ion coordination within PHLPP2. Additional potential zinc-binding residues such as cysteine, histidine, aspartate, or glutamate were not found in proximity to the M2 site, further supporting our hypothesis.

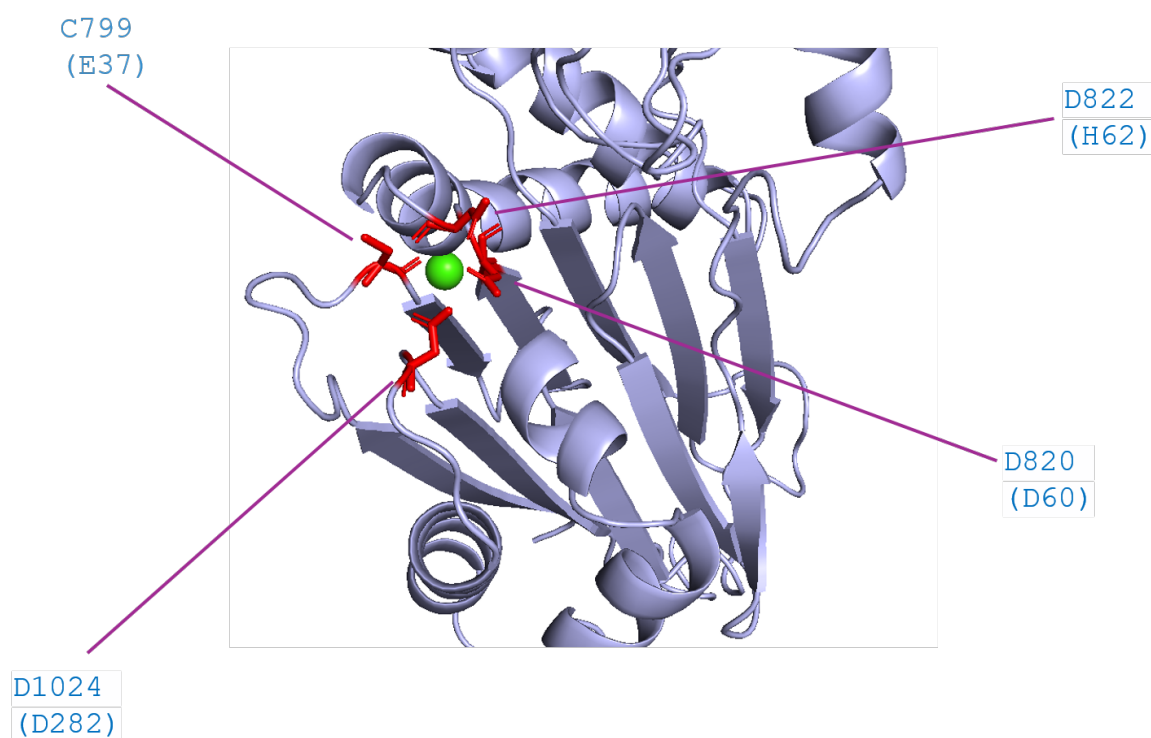


Figure 13. PHLPP2 catalytic domain, predicted residues involved with coordination of Zn^{2+} . PHLPP2 residues: C799, D820, D822, D1024 are aligned with PPM1A residues: E37, D60, H62, D282

5.2.3. PHLPP2 stability

Upon discovering a Zn^{2+} ion is bound within the catalytic site of PHLPP2, coupled with its conservation patterns in comparison to other members of the PPM family, we engineered two specific mutants D820A and D1024N to explore this anomaly further. These mutants were generated using the same purification strategy as for the WT PHLPP2. We then assessed their thermal stability through Differential Scanning Fluorimetry (DSF) experiments. DSF can be used to detect increases in the melting temperature of the protein of interest as an indicator of protein stability. We also included ethylenediaminetetraacetic acid (EDTA), as a chelating agent to try to extract metal ions Zn^{2+} from PHLPP2, to assess whether Zn^{2+} has an effect on PHLPP2 stability.

The data (Figure 14 a) indicates similar SEC elution profiles for all constructs suggesting that the mutations do not significantly alter the solubility or purification characteristics of the mutants as

compared to the wild type. Furthermore, they have similar thermal stability in the presence and absence of 1 mM EDTA as depicted in Figure 14 b).

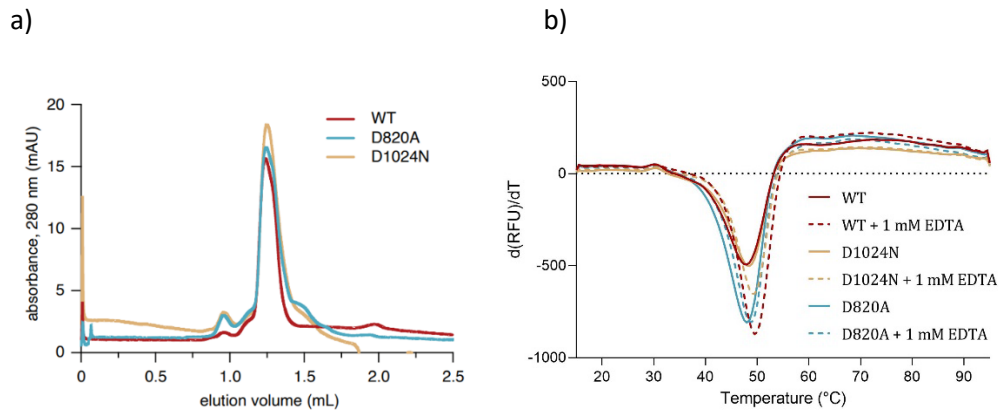


Figure 14. Stability Analysis of PHLPP2 Wild-Type, D820A and D1024N mutant

- a) *Micro-SEC Chromatogram: Displaying elution profiles for wild-type PHLPP2 and mutants D820A and D1024N. No significant shift in peak elution indicates relative stability of WT and its mutants.*
- b) *Differential Scanning Fluorimetry (DSF) analysis of PHLPP2 and its Mutants upon addition of chelating agent EDTA. The results suggest that the wild-type and mutant proteins, D820A and D1024N, maintain their thermal stability upon incubation with 1 mM EDTA*

The DSF experiment yields insights that are open to interpretation. The thermal stability observed in the mutants D820A and D1024N, compared to the WT PHLPP2, suggests three possibilities: first, the mutations themselves do not compromise the structural integrity of the zinc-binding site. Second EDTA, despite its chelating properties, is insufficient to effectively extract zinc from the site. The last possibility is that the bound Zn^{2+} ion is not critical for the overall thermal stability of PHLPP2. These mutants could only be purified in much lower yields, suggesting that they may be less stable in a cellular environment.

5.3. Computational modelling

Without crystallographic or cryo-electron microscopy evidence to clarify PHLPP2's active site structure, we turned to computer modelling to hypothesize how Zn^{2+} ion could be coordinated. The experimentally determined crystal structure, which includes a C1D3 zinc finger (G. Chen et al., 2018), establishes a precedent for such configurations.

We investigated computationally the zinc-binding site of PHLPP2, hypothesizing that the residues C799, D820, D822, D1024 coordinate the Zn^{2+} ion are crucial for the enzyme's catalytic site. Initial molecular dynamics (MD) simulations provided a structural base, but with limited precision in

modelling the electronic environment due to the homogeneous and uniformly charged sphere representation of atoms. Deviations from the optimal bond lengths were observed, specifically the 3 O-Zn distances are shorter than ideal (1.8-1.9Å) and in contrast S-Zn distance is longer than ideal (3.0Å). Moreover, D820 was observed to bind Zinc bi-dentally (Figure 15). However what MD simulations shows us is that the metal ion maintained relative stability and compatibility with the protein matrix.

Due to limitations with the molecular dynamics method and difficulty to achieve a more accurate electronic characterization, we employed the “Our own N-layered Integrated molecular Orbital and Molecular mechanics” (ONIOM) approach.

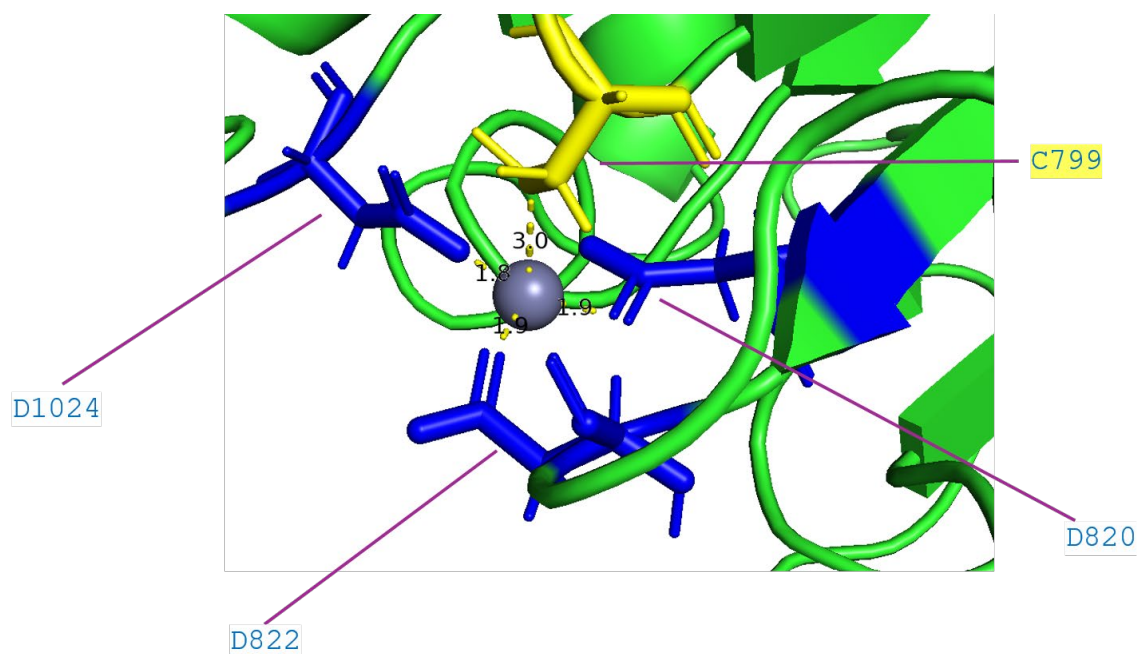


Figure 15. MD simulation analysis of the catalytic domain of PHLPP2. Residues D1024, D822, D820 are highlighted in blue, they form bond lengths of 1.8-1.9 Ångström with Zn^{2+} . Residue C799 is depicted in yellow, it forms bond length of 3.0 Ångströms with Zn^{2+} .

The ONIOM framework is a hybrid quantum mechanical method that treats atoms within a certain proximity to the Zn^{2+} ion with a higher level of theory, while distal atoms are modelled using a less computationally intensive semi-empirical method. In particular, the sulfur atom of C799 was modelled in its deprotonated state, reflecting its experimentally observed interaction with Zn^{2+} ions. The resulting model, evaluated (Figure 16) against protein structures predicted by AlphaFold2, revealed a near-tetrahedral geometry of the zinc coordination site, with O-Zn and S-Zn bond lengths consistent with those observed in experimentally determined zinc clusters (G. Chen et al., 2018).

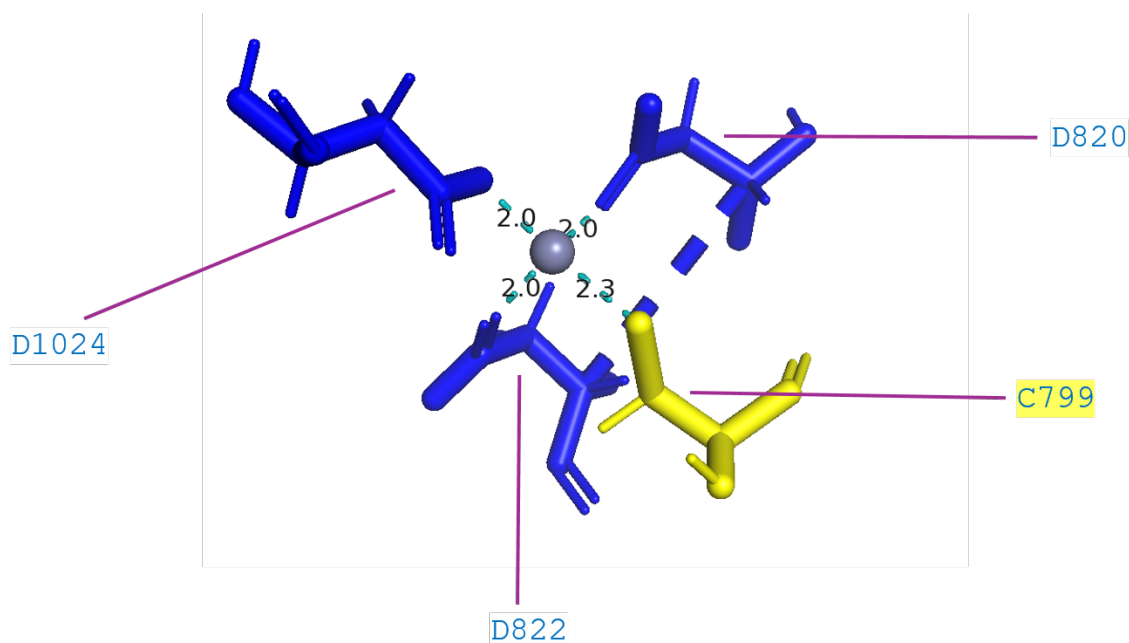


Figure 16. ONIOM analysis of the catalytic domain of PHLPP2. Residues D1024, D820, and D822 are highlighted in blue, they form bond lengths of 2.0 Ångström with Zn^{2+} . Residue C799 is depicted in yellow, it forms bond length of 2.3 Ångströms with Zn^{2+} .

5.4. Biochemistry

In light of the anomalous findings regarding PHLPP2, including the paradoxical activity of what was expected to be a phosphatase-dead mutant and the discovery of an unconventional metal ion and stoichiometry in its active site, we felt compelled to re-examine its phosphatase activity in vitro. This decision marks the beginning of a new chapter in our investigation, aimed at unravelling the intricate biochemical dynamics of PHLPP2 and its functional implications.

5.4.1. Identification of Low-Abundance PP2A Contamination in Highly Purified PHLPP2 Preparations by Peptide Fingerprinting Mass Spectrometry

To investigate whether there were any contaminating phosphatases in our purified PHLPP2 preparation, we analysed it by peptide fingerprinting mass spectrometry. Initial assessments by the mass spectrometry facility indicated an exceptionally pure preparation, with an absence of detectable phosphatase contamination. However, upon manual re-evaluation of the data, we identified the presence of peptides from all three subunits of the PP2A holoenzyme, a phosphatase distinct from PHLPP2.

A total of 13 unique peptides were detected that correspond to the three subunits of PP2A: 2 peptides matching the catalytic C subunit, 9 peptides to the A subunit, and 2 peptides to the B55 regulatory subunit. Notably, the spectral intensity log2 values indicates a substantially lower abundance of PP2A-related peptides compared to PHLPP2, with an estimated quantitative ratio indicating that PP2A peptides were outnumbered by PHLPP2 peptides by approximately 10,000-fold (Figure 17).

Despite the low abundance of PP2A peptides, the potential functional impact of this contaminant cannot be disregarded. Given that the enzymatic activity of phosphatases can be significant even at low concentrations, the presence of PP2A, regardless of its minimal representation, raises concern about all so far reported literature PHLPP2 activity.

Furthermore, the combined molecular weight of the PP2A heterotrimer, which is approximately 157 kDa, is remarkably close to the molecular weight of PHLPP2 at 147 kDa. This proximity in mass explains the inability to separate the two entities using size-exclusion chromatography.

Representative protein	Leading proteins	Gene name	Protein name	Molecular weight [kDa]	Total unique peptides	Protein Probability	Spectral count	iBAQ intensity [log2]
Q6ZVD8	Q6ZVD8	PHLPP2	PH domain leucine-rich repeat-containing protein phosphatase 2	146.66	131	1.00	1201	39.37
A0A2H1WFA2	A0A2H1W	SFRICE003	Heat shock 70 kDa protein cognate 4 (Fragment)	71.46	55	1.00	114	32.77
P04264	P04264;co	KRT1	Keratin	66.00	47	1.00	91	32.76
...	...	...	...	...	...	...	...	...
A0A2H1X028	A0A2H1X	SFRICE_01	PP2A A-subunit	65.27	9	1.00	9	25.34
A0A2H1V3Y5	A0A2H1V	SFRICE_01	PP2A B55 regulatory subunit	56.78	2	1.00	2	20.69
J9Z496	J9Z496		PP2A-C Subunit	35.44	2	1.00	2	-

Figure 17. Peptide fingerprinting mass spectrometry analysis of PHLPP2 sample reveals complete PP2A Holoenzyme in the prep

5.4.2. Assessment of PHLPP2 phosphatase activity

Based on the knowledge that there is a potential contaminating phosphatase in our PHLPP2 preparations, we designed an experiment to distinguish the source of enzymatic activity observed in malachite green assays, which are utilized to measure phosphatase activity. This approach is aimed at accurately attributing the observed activity to either PHLPP2 or the potential contaminant PP2A, known to target the activation loop of Akt as its substrate (Wlodarchak et al., 2016).

5.4.2.1. Malachite green assay

The Malachite Green Assay is a colorimetric technique for measuring inorganic phosphate levels, crucial in various biochemical and environmental studies. It operates on the principle of phosphate ions reacting with malachite green and molybdate under acidic conditions to produce a green complex (Figure 18). The intensity of this green colour, measurable via spectrophotometry, correlates with the phosphate concentration in the sample, offering a straightforward and sensitive detection method (McAvoy et al., 2011).

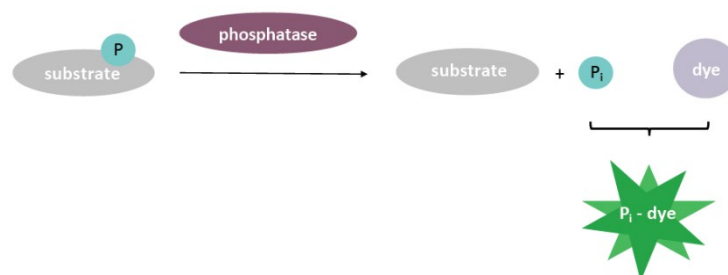


Figure 18. Schematic illustrating malachite green assay

5.4.2.2. PHLPP2 phosphatase activity

In our experiments with PHLPP2 we used the Malachite Green assay against its reported substrate Akt, specifically targeting the activation loop (GATMK**p**TFCGT) and hydrophobic motif (HFPQ**Fp**SY**SAS**) peptides. Fortunately, Okadaic acid is a known inhibitor of PP2A (Kamat et al., 2014). We proceeded with the Malachite Green assay, using varying concentrations of PHLPP2 ranging from 0 to 500 nM, with PP2A serving as a positive control at concentrations of 0 to 10,000 pM. Three biological replicates were tested under identical conditions, each showing different phosphatase activity, which was peculiar, but with higher activity towards Akt AL. After the addition of 25 nM Okadaic acid, at substoichiometric concentrations compared to PHLPP2, the activity dropped to zero. In control experiments, PP2A demonstrates reproducible phosphatase activity and it exhibits consistent inhibition profiles, mirroring the results observed with PHLPP2. This suggests that the true phosphatase activity of our PHLPP2 preparations originates from PP2A, not PHLPP2 (Figure 19).

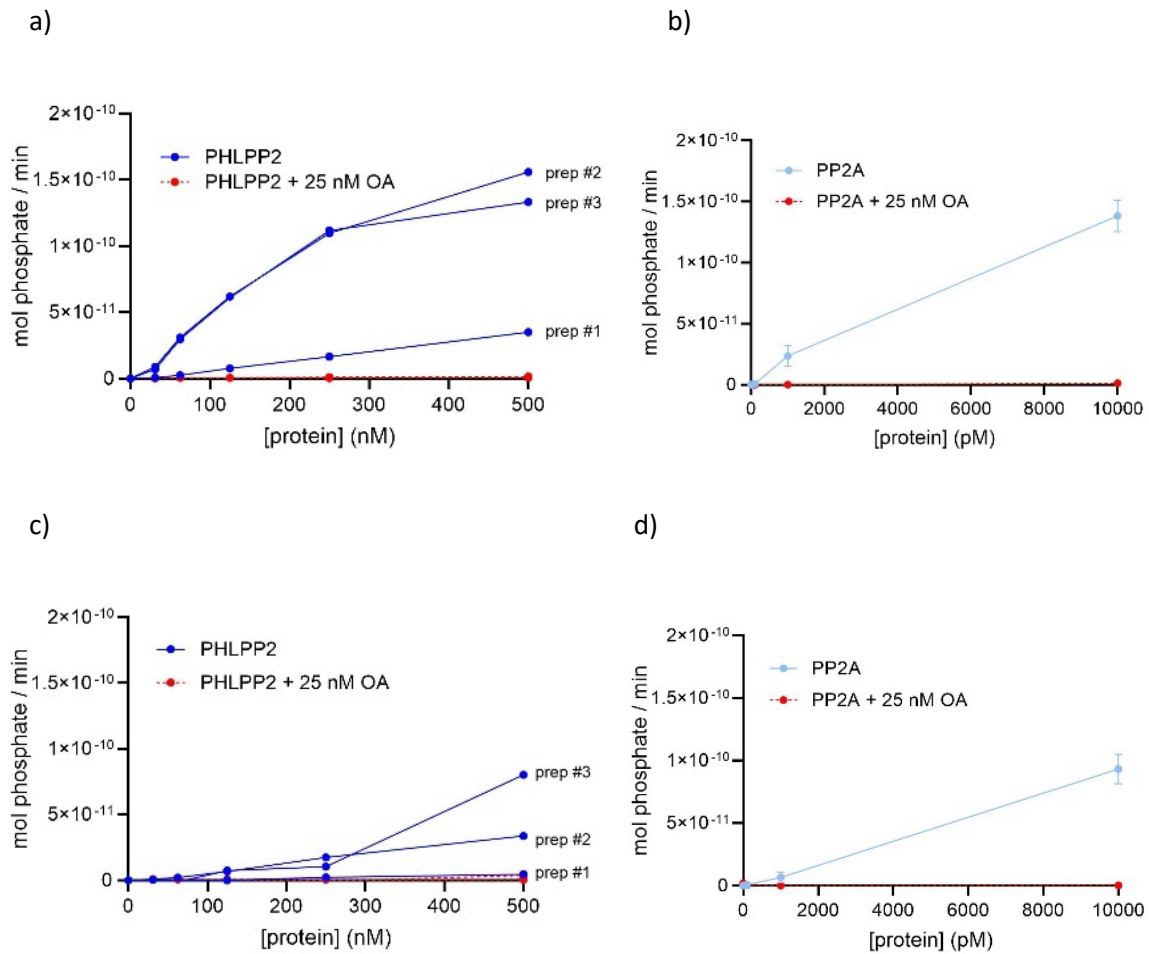


Figure 19. Malachite green assay of PHLPP2 against its reported Akt substrates with and without Okadaic Acid Inhibition

- PHLPP2 activity Against Akt Activation Loop Peptide. Three biological preps of PHLPP2 shows different phosphatase activity between each prep, which completely disappears upon the addition of 25 nM Okadaic Acid.
- Control sample PP2A Against Akt Activation Loop Peptide. PP2A display consistent phosphatase activity which is nullified by the addition of 25 nM Okadaic Acid.
- PHLPP2 activity against Akt Hydrophobic Motif Peptide. Three biological preps of PHLPP2 shows different phosphatase activity between each prep, which completely disappears upon the addition of 25 nM Okadaic Acid.
- Control sample PP2A Against Akt Hydrophobic Motif Peptides. Similar to the AKT Activation Loop peptide, PP2A shows consistent phosphatase activity against the AKT Hydrophobic Motif which is eliminated in the presence of Okadaic Acid.

To eliminate the possibility that okadaic acid interferes with PHLPP2 activity, we sought to remove the contaminating PP2A from our purified PHLPP2 in order to assess PHLPP2 activity in the absence of any phosphatase inhibitors.

5.4.3. Generation of PIBS

How can we eliminate the contaminating phosphatase PP2A from our PHLPP2 preparations? Fortunately for us again, the literature provides a solution, as there exists a published protocol for the production of phosphatase inhibitor beads that can be employed to purify PP2A.

There is another naturally occurring toxin called Microcystin that, like okadaic acid, binds to the active site of PP2A. This toxin is an irreversible inhibitor of various phosphatases, including PP1, PP2, PP3, PP4 and PP6, by virtue of forming a covalent bond to a conserved cysteine in the substrate binding cleft (Wlodarchak et al., 2016). The protocol involves attaching microcystin to beads using its reactive N-methyldehydroalanine residue, linked to the aminoethanethiol molecule, allowing for direct attachment to reactive NHS-sepharose beads to create efficient phosphatase inhibitor beads (PIBs) (Figure 20). PIBs are capable of covalently binding to a conserved cysteine residue within the active sites of PP1, PP2A and PP4-6, thereby enriching for the catalytic subunits along with their associated regulatory and interacting proteins across a broad range of organisms, from yeast to human (Brauer et al., 2021).

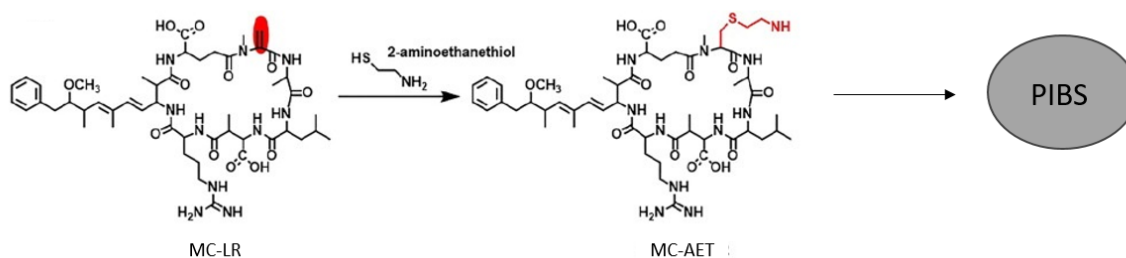


Figure 20. Generation of Phosphatase Inhibitor Beads (Pibs)

The authors (Brauer et al., 2021) used PIBs for the purification of PP2A and other similar phosphatases and they allow to maintain the integrity of holoenzyme complexes, thus preserving the interactions essential for their functionality. The PIBs-based enrichment technique overcomes several challenges associated with traditional methods, such as the need for endogenous tagging or exogenous expression of tagged subunits, which can alter protein expression or localization. Furthermore, it eliminates the dependency on specific antibodies.

By adopting the PIBs-based enrichment strategy, we can selectively target and remove PP2A from our PHLPP2 sample, thus making it PP2A-free.

To evaluate the efficiency of our newly synthesized Microcystin-beads, we aimed to see whether they could effectively isolate the PP2A subunits previously identified in our PHLPP2 preparations through peptide mass fingerprinting. For this purpose, we prepared a lysate overexpressing PHLPP2, quantified it using the Bradford assay, and split it into two equal aliquots. In one aliquot, we introduced okadaic acid as a control, given its specificity in binding to PP2A, thereby rendering the PP2A unavailable for binding by the Microcystin-beads. Subsequently, both aliquots were incubated with the phosphatase inhibitor beads and analysed via mass spectrometry for peptide fingerprinting to determine which proteins the PIBs extracted.

The results were striking: the PIBs not only pulled out the PP2A subunits, which were also identified in our initial analysis, but did so in significant quantities. However, no such enrichment was observed for PHLPP2 between the two samples, suggesting that while our beads were highly selective for PP2A, they did not bind PHLPP2.

PIBs are highly effective in enriching the PP2A subunits, with fold-enrichments of 1200 for the C subunit, 541 for the A subunit, and 65 for the B subunit of *Spodoptera frugiperda* PP2A (Figure 21). These proteins match the previously identified contaminants detected in PHLPP2 preparations. Crucially, human PHLPP2 was also detected but was not enriched in comparison to the control lysate, which aligns with expectations since PHLPP2 is part of a distinct phosphatase family that is not targeted by Microcystin-LR. This successful separation allowed us to effectively eliminate PP2A contamination from our PHLPP2 preparations.

Representative protein	Leading proteins	Gene name	Protein name	Molecular weight [kDa]	Total # unique peptides	Sequence coverage	Imputed LFQ intensity [log2]		Enrichment ration (log2)
							-OA	+OA	
J9Z496	J9Z496		PP2A-C Subunit	35.44	23	68.30	25.69	15.46	10.23
A0A2H1X028	A0A2H1X028	SFRICE_01	PP2A A-subunit	65.27	39	68.00	27.03	17.94	9.08
A0A2H1V3Y5	A0A2H1V3Y5	SFRICE_01	PP2A B55 regulatory subunit	56.78	24	45.10	23.73	17.53	6.20
...	...	...	...	...	...	...	...	...	...
Q6ZVD8	Q6ZVD8	PHLPP2	PH domain leucine-rich repeat-containing protein phosphatase 2	146.66	19	17.10	19.16	19.68	-0.53

Figure 21. Peptide fingerprinting mass spectrometry analysis of PIBs shows its capability of capturing PP2A Holoenzyme

5.4.4. Evaluating PHLPP2 phosphatase activity

Incorporating our newfound knowledge about the specificity of PIBs, we refined our purification protocol for PHLPP2 to include a new step specifically designed to eliminate PP2A contamination. Again, recombinant GST-PHLPP2 was purified via GST affinity chromatography and cleaved with TEV. The cleaved sample was then introduced to IEX. Prior to the SEC step we added one additional step: reverse affinity purification with the PIBs. By centrifugation, we were able to separate the beads bound with PP2A from the PHLPP2-rich supernatant. The collected supernatant, now clear of PP2A, was subjected to SEC to further purify PHLPP2 (Figure 22).

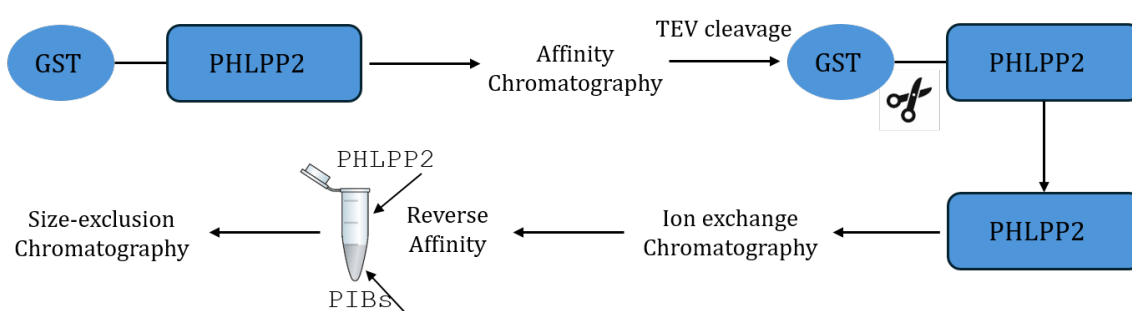
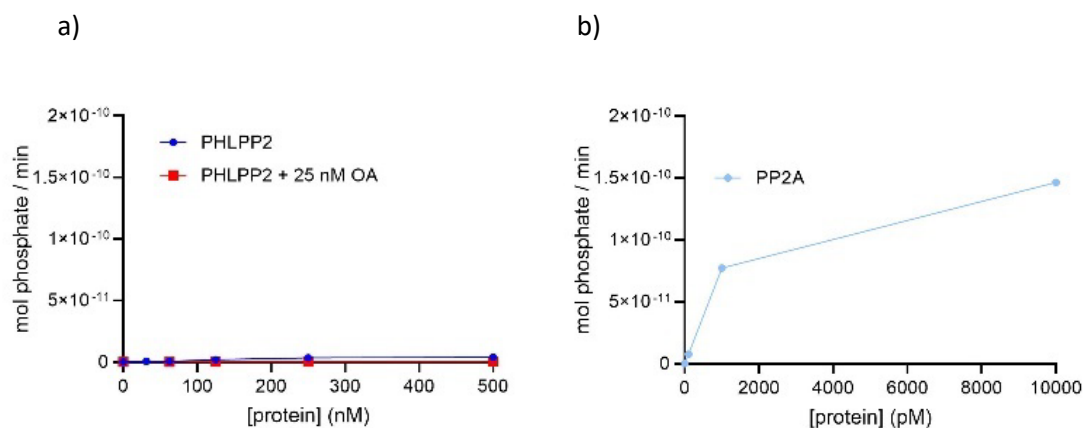


Figure 22. Schematic illustration of Four-Step PHLPP2 purification Process Involving GST, Anion Exchange, Reverse Affinity and Size Exclusion Chromatography

After implementing the revised purification strategy, we proceeded to check the phosphatase activity of the newly purified PHLPP2. The assay conditions were kept consistent with previous experiments, using PP2A as a positive control and both Akt AL and Akt HM peptides as substrates. As anticipated, PP2A demonstrated its typical activity levels. However, remarkably, PHLPP2 exhibits no detectable activity towards the Akt AL or the HM peptide (Figure 23).



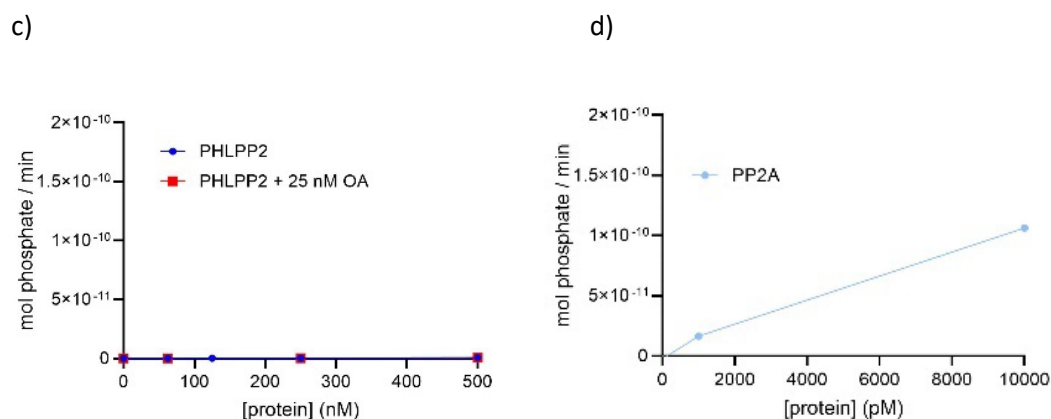


Figure 23. Malachite green assay of PHLPP2 post-cleanup with PIBs against its reported Akt substrates with and without Okadaic acid

- PHLPP2 post-cleanup activity against Akt activation loop peptide. The phosphatase activity is undetectable and the addition of Okadaic Acid does not play any role, showing PHLPP2 inactivity towards the Akt AL substrate.
- Control sample PP2A against Akt activation loop peptide. PP2A displays consistent phosphatase activity.
- PHLPP2 post-cleanup activity against Akt hydrophobic motif peptide. Same as with the AL substrate, PHLPP2 shows no detectable activity.
- Control sample PP2A against Akt hydrophobic motif peptide. PP2A displays consistent phosphatase activity.

5.4.5. Para-nitrophenyl phosphate Assay

In order to further validate the observation of PHLPP2's lack of activity in the malachite green assays, we conducted another phosphatase assay, where we assessed the dephosphorylation ability of PHLPP2 on para-nitrophenyl phosphate (pNPP). This assay, which is simple and widely adopted, serves as a reliable method to quickly assess the dephosphorylation ability of phosphatases (McAvoy et al., 2011). The aim of this experiment was to eliminate any uncertainties regarding the previous observed inactivity of PHLPP2. The assay's principle is based on phosphatase activity that cleaves the phosphate group from pNPP, resulting in the formation of para-nitrophenol (pNP). Para-nitrophenol can be quantitatively measured by the increase in absorbance at 405 nm (Lountos et al., 2015). As a standard for enzymatic activity, we used Lambda phosphatase, which exhibited significant activity against pNPP. In contrast, PHLPP2, which was purified as illustrated in Figure 22, exhibited no dephosphorylation activity towards pNPP, even in the absence of okadaic acid (Figure 24).

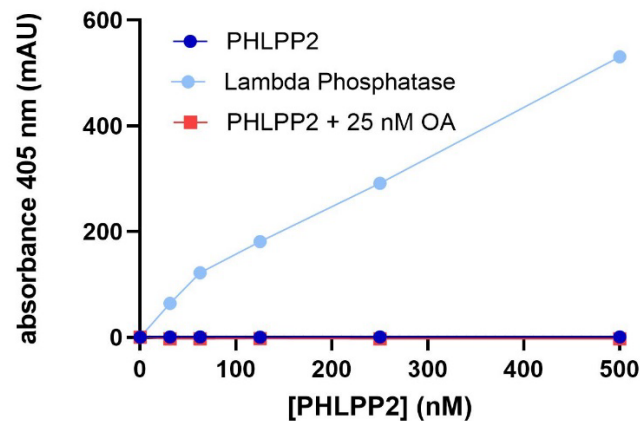


Figure 24. *p*-Nitrophenyl Phosphate assay of PHLPP2 post-cleanup with PIBs

This lack of activity confirms our previous findings that we observed using malachite green assays against phosphopeptides derived from Akt.

6. Discussion

PHLPP2 was originally reported in the literature as an Akt phosphatase, specifically targeting the Akt hydrophobic motif. Both bacterial and insect purified PHLPP2 was utilized for enzymatic characterization using Malachite green and pNPP assays, which indicated phosphatase activity. The initial researchers also employed GST-tagged PHLPP2, but relied solely on a single-step purification process via affinity chromatography. While this may suffice in certain scenarios, the presence of contaminants, even in picomolar concentrations, can pose substantial issues when dealing with enzymes. It is plausible that they too purified PP2A alongside PHLPP2 without recognizing it, attributing the observed activity to PHLPP2 mistakenly.

Furthermore, there's a noteworthy difference in the approach towards OA. In all our phosphatase activity experiments, we incorporated OA, conclusively demonstrating that the activity did not originate from PHLPP2. Conversely, the original authors merely noted that OA does not interact with PHLPP2, without providing experimental evidence. Our experiments not only established the lack of PHLPP2 activity but also proceeded to rigorously purify PHLPP2 from PP2A contaminants, conducting subsequent phosphatase assays to retest PHLPP2 activity. This completely negated the possibility that OA was inhibiting PHLPP2. Therefore, I conclude that the activity previously reported was due to sample contamination, not intrinsic to PHLPP2.

The next point of interest is that the original authors assume PHLPP2 is a manganese-bound phosphatase simply because it falls under the PPM family, yet experimental validations are lacking. Our findings, however, determine that PHLPP2 is zinc-bound with a 1:1 stoichiometry, showing distinct characteristics and a potential C1D3 zinc finger configuration model. During protein expression, the medium was not supplemented with metal ions, while, during purification, no metal ion chelators were used. This implies that PHLPP2 sequesters zinc natively during translation and retains it during purification. Contrary to PHLPP2, other phosphatases within the PPM family require two or three divalent cations at their active sites for functionality. Furthermore, a study by Chen et al., 2017 initially identified PHLPP2 as a pseudophosphatase due to mutated amino acids at its catalytic site, which impaired metal ion coordination. However, due to reported experimental phosphatase activity, the authors classified it as a phosphatase. Given the presence of only one metal ion (likely at the M2 position), the lack of phosphatase activity is anticipated. We present a new model, developed through MD and ONIOM, that proposes the catalytic site's structure.

The idea that PHLPP2 might function as a tumor suppressor comes from its initial observation that PHLPP2 is an Akt phosphatase. This classification as a tumor suppressor is primarily based on correlations found in studies between its expression levels and Akt phosphorylation in cancer cell lines (Gao et al., 2005; Brognard et al., 2007). The inhibition of tumor development found upon reintroducing PHLPP2 into glioblastoma cells initially suggested a causative relationship, although the effect shown was relatively minor. Additionally, knockdown of PHLPP2 levels in cells increased Akt S473 phosphorylation, leading the authors to believe this was due to the enzyme's loss of activity. However, given our recent findings, these changes in Akt phosphorylation may be the consequence of more pleiotropic effects of PHLPP2 overexpression, possibly including disruptive effects on membrane signaling, considering its reported localization to the plasma membrane (Park et al., 2008).

The Cancer Gene Census is a meticulously curated list which is maintained by the Catalogue of Somatic Mutations in Cancer (COSMIC) team and includes over 700 human genes, demanding clear evidence of mutations in certain cancers reported by multiple independent studies for a gene to be classified as an oncogene or tumor suppressor (Sondka et al. 2018). Given the absence of typical mutations or patterns of copy number loss in cancers for either PHLPP1 or PHLPP2, and mutation rates akin to those of olfactory receptor genes, these proteins do not meet the criteria to be classified as tumor suppressors (Supplementary Figure 3). This, coupled with the fact that PHLPP2 does not directly dephosphorylate Akt, casts significant doubt on its purported role in cancer suppression.

6.1. Future perspectives

The following question is: What then does PHLPP2 do?

To unravel the precise role of PHLPP2, further experimentation is required, potentially revealing new biological insights and pathways where PHLPP2 could play a crucial role. Key questions include what is the function of PHLPP2 in cells, understanding potential protein interactions involving PHLPP2, and exploring the implications of these interactions. Such exploration could open up numerous research roads.

To gain insights into potential binding partners of PHLPP2, we have designed an *in vivo* photo cross-linking experiment, where PHLPP2, harbouring a non-canonical amino acid called diazirine, is overexpressed in HEK293 cells. This is done by expressing the tRNA synthetase and tRNAs specific for the TAG stop codon and diazirine, as well as supplementing the diazirine in the media. Consequently, PHLPP2 needs to be mutated with the TAG stop codon at the position where diazirine will be introduced. The diazirine is introduced into the catalytic site of PHLPP2, where we expect binding partners to interact (Supplementary Figure 1.). Upon exposure to 365nm UV light, the diazirine residue will be photo-activated, covalently crosslinking with anything in its vicinity. This experiment holds the potential to unshroud the enigma surrounding the entire PHLPP2 mystery and may finally give us an answer to its potential function.

Furthermore, given the presence of a PH domain in PHLPP2, there's a hint that it may interact with cellular membranes, a common trait among proteins harbouring this domain. Our approach to elucidating this interaction involves the use of PIP-strips, a technique that allows for the analysis of protein-lipid interactions. Through this method, we aim to screen a spectrum of phosphoinositides to pinpoint those with which PHLPP2 exhibits a high affinity. This exploration is not merely a quest to identify the phosphoinositides that PHLPP2 binds to but to discern the functional implications of such interactions. The binding affinity towards specific phosphoinositides could reveal potential regulatory roles PHLPP2 might play in signalling pathways or cellular architecture, particularly in the context of membrane-associated processes. This might give us a definitive answer regarding PHLPP2 localization.

Given the revaluation of PHLPP2's phosphatase activity and its lack of interaction with Akt, string analysis of PHLPP2 might present us further clues in unravelling the true biological function of PHLPP2 (Supplementary Figure 2). PHLPP2's involvement in cellular processes, beyond its originally hypothesized role in Akt signalling and tumor suppression, may instead pivot on its ability to act as a scaffold or its potential in performing an alternative catalytic function. High-throughput affinity capture mass spectrometry (AC-MS) and BioID screens have previously pinpointed several proteins that associate with PHLPP2's PP2C domain, such as WD-repeat-containing proteins 20 (WDR20), WDR48, ubiquitin-specific peptidase 12 (USP12), and USP46 (Huttlin et al. 2021, 2017,2015; Sowa et

al. 2009; St-Denis et al. 2016; Yadav et al. 2017; McClurg et al. 2014; Kawashima et al., 2021). While direct interactions with Akt were not highlighted in these studies, the interconnected nature of PHLPP2 with ubiquitination pathways has been indicated through its association with deubiquitinases like USP12 and USP46, which are known to influence Akt signalling indirectly. Furthermore, PHLPP2 has been identified as a binding partner to Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1), which connects to PHLPP via its PDZ domains (Molina et al. 2012). Collectively, these interactions suggest a nuanced role for PHLPP2 within the cell, potentially related to its regulation by ubiquitination rather than direct phosphatase activity.

However, the work does not conclude there. Depending on what we uncover regarding the new biological context in which PHLPP2 is found, a multitude of new questions will emerge, requiring additional experimental explorations.

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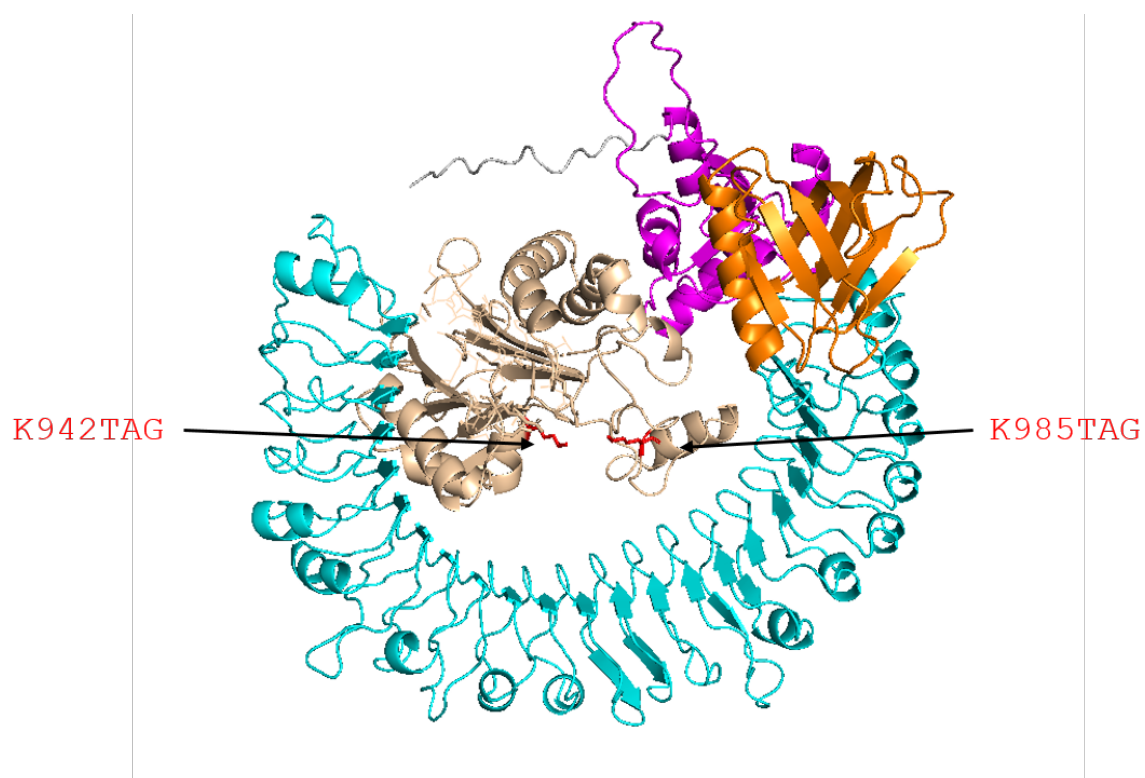
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III List of abbreviations

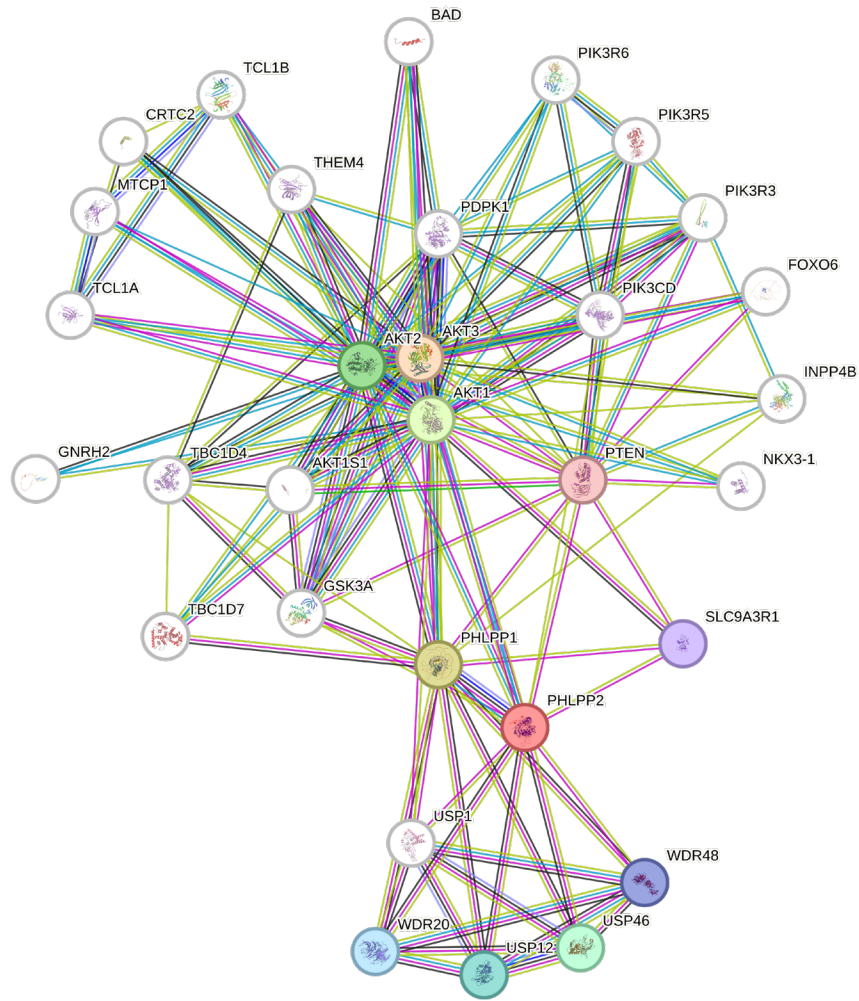
- Activation loop – (AL)
- Catalogue of Somatic Mutations in Cancer – (Cosmic)
- Cysteine-based classes - (CC)
- Differential Scanning Fluorimetry – (DSF)
- Ethylenediaminetetraacetic acid - (EDTA)
- Full-length - (FL)
- Glutathione-S-transferase – (GST)
- Hydrophobic motif - (HM)
- Ion exchange chromatography – (IEX)
- Leucine-rich repeat – (LRR)
- Metal dependent protein phosphatases – (PPM)
- Molecular Dynamics – (MD)
- Molecular Weight Cut-Off - (MWCO)
- mTOR Complex 2 - (mTORC2)
- Okadaic acid – (OA)
- Our own N-layered Integrated molecular Orbital and Molecular mechanics – (ONIOM)
- Para-nitrophenyl phosphate Assay – (pNPP)
- PH domain Leucine-rich Repeat Protein Phosphatase 2 - (PHLPP2)
- Phosphatase and tensin homolog - (PTEN)
- Phosphoinositide 3-kinase - (PI3K)
- Phosphoinositide-dependent kinase-1 - (PDK1)
- Phosphoinositide-3-Kinase–protein kinase B/Akt - (PI3K-PKB/Akt)
- Phospholipidinositol-4,5-bisphosphate - [[PI\(4,5\)P2](#)]
- Phosphatidylinositol-3,4,5-triphosphate - [[PI\(3,4,5\)P3](#)]
- Phosphoserine – (pSer)
- Phosphothreonine – (pThr)
- Pleckstrin Homology - (PH)

- Protein Kinase B - (PKB, also known as Akt)
- Protein Phosphatase 2 – (PP2A)
- Protein Phosphatase 2C – (PP2C)
- Protein Phosphatase, Mg²⁺/Mn²⁺ Dependent 1A – (PPM1A)
- Quantum Mechanics – (QM)
- Ras associated - (RA)
- Serine 473 - (S473)
- Size exclusion chromatography – (SEC)
- Size Exclusion Chromatography coupled with Inductively Coupled Plasma Mass Spectrometry - (SEC-ICP-MS)
- Threonine 308 - (T308)
- VSEPR - (Valence Shell Electron Pair Repulsion)
- Wild-type - (WT)

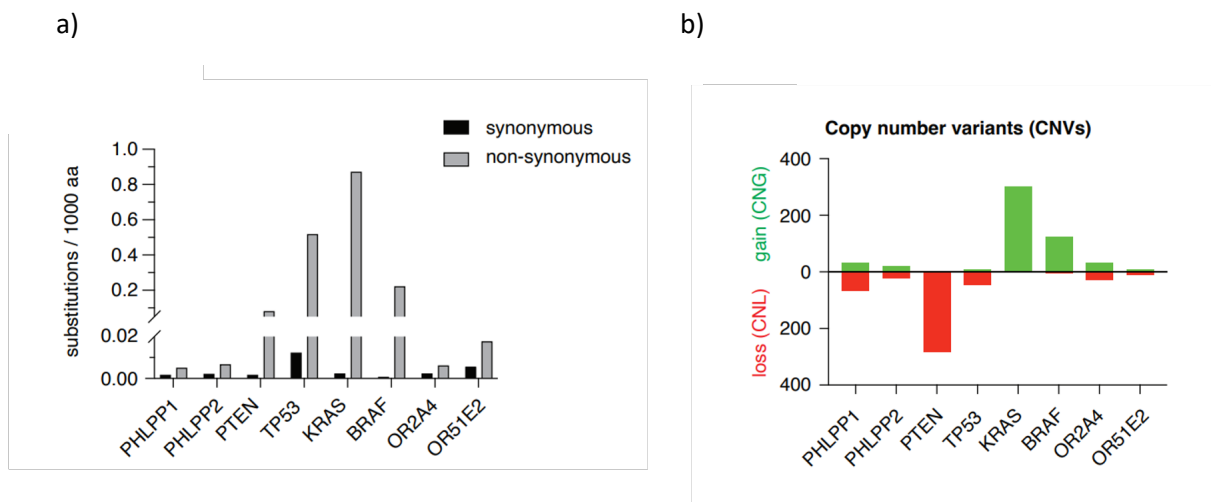
IV Supplementary Figures



Supplementary Figure 1. Residues mutated to implement diazirine mutant into PHLPP2 for photo-crosslinking experiment



Supplementary Figure 2. String analysis PHLPP2 network



Supplementary Figure 3. Comparative Analysis of Genetic Variations in Cancer-Related genes

a) Number of synonymous and non-synonymous substitutions per 1000 amino acids reported in COSMIC for: PHLPP1 and PHLPP2; the tumor suppressor genes PTEN and TP53; the oncogenes KRAS and BRAF; the olfactory receptor genes OR2A4 and OR51E2.

*b) Distribution of non-synonymous mutations within the coding sequence of the genes represented in A.
Red: known hotspot mutations in Cancer Gene Census (CGC) genes.*

V Zusammenfassung

In der Erforschung der PH-Domäne Leucin-reiche-Wiederholungs-Proteinphosphatase 2 (PHLPP2) haben anfängliche Studien von Gao et al. diese Phosphatase als Regulator des Protein-Kinase B (PKB, auch bekannt als Akt) Signalwegs identifiziert. Es wurde gezeigt, dass PHLPP2 eine Domänenstruktur aufweist, die eine Ras-assoziierte (RA) Domäne, eine Pleckstrin-Homologie (PH) Domäne, eine leucinreiche Wiederholungsdomäne (LRR), eine Protein-Phosphatase 2C (PP2C) Domäne und ein PDZ-Bindungsmotiv umfasst. Es wurde berichtet, dass sie spezifisch das regulatorische hydrophobe Motiv von Akt dephosphoryliert, was auf eine Rolle in den Zellüberlebens- und Wachstumspfaden hinweist. Jedoch legen umfassende Reinigungs- und biochemische Profilierungsstudien nahe, dass die beobachtete Phosphataseaktivität in früheren Studien von einer mitgereinigten Protein-Phosphatase 2A (PP2A), einer bekannten Akt-Phosphatase, und nicht von PHLPP2 selbst stammte. Der Einsatz von Okadasäure (OA), einem Inhibitor von PP2A, sowie die Entfernung von PP2A mit Phosphatase-Inhibitor-Perlen (PIBs), offenbarte keine intrinsische Phosphataseaktivität von PHLPP2 nach der Reinigung. Darüber hinaus haben wir experimentell bestimmt, dass das katalytische Domäne von PHLPP2 ein einzelnes Zn^{2+} Metallion verankert und wir präsentieren ein Modell für die Pseudo-Katalysetelle von PHLPP2 unter Verwendung von molekulardynamischen (MD) und quantenmechanischen (QM) Methoden. In Verbindung mit dem Fehlen von PHLPP2-Aktivität in Phosphatase-Assays stellen wir die Hypothese auf, dass PHLPP2 möglicherweise eine Pseudophosphatase ist, die unter den getesteten Bedingungen keine intrinsische enzymatische Aktivität aufweist. Diese Erkenntnis erfordert eine Neubewertung der Rolle von PHLPP2 und lädt zu rigoroseren experimentellen Ansätzen ein, um seine biologische Funktion zu klären.

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This thesis is as much yours as it is mine. Thank you all.

VII Publications arising from this thesis

This work has been published at PNAS journal.

Link to publication: <https://www.pnas.org/doi/10.1073/pnas.2417218122>