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PHLPPing the Script: Emerging Roles of PHLPP Phosphatases in Cell Signaling

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Abstract

While protein kinases have been successfully targeted in a variety of diseases, protein phosphatases remain an underutilized therapeutic target, in part because of incomplete characterization of their effects on signaling networks. The PH domain Leucine-rich repeat Protein Phosphatase (PHLPP) is a relatively new player in the cell signaling field, and increasing roles in controlling the balance between cell survival, proliferation, and apoptosis are being increasingly identified. While originally characterized for its tumor-suppressive function in deactivating the pro-survival kinase Akt, recent evidence suggests that PHLPP may have an opposing role in promoting survival. This review summarizes the current knowledge of PHLPP, and highlights emerging functions as both a tumor suppressor and an oncogene. Understanding the context-dependent functions of PHLPP is essential for appropriate therapeutic intervention.

Keywords

PHLPP; Akt; PKC; Phosphatase; Phosphorylation; Cancer; Transcription

1. INTRODUCTION

The PH domain Leucine-rich repeat Protein Phosphatase (PHLPP) belongs to the protein phosphatase magnesium/manganese (PPM)-dependent ‘shrub’ in the human protein phosphatome [1] (Figure 1A). Originally identified in a rational search for phosphatases that would suppress growth factor signaling by dephosphorylating a key regulatory site on Akt, the hydrophobic motif [2], the repertoire of PHLPP substrates has rapidly expanded in the past few years. Indeed, PHLPP is emerging as a central regulator of diverse biologies, not only by acutely controlling the activity and stability of kinases, but also by suppressing processes such as inflammatory signaling via its dephosphorylation of transcription factors. This review provides an overview of PHLPP, highlighting recent findings on both its tumor suppressive and oncogenic roles in cancer and inflammatory signaling.

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2. DOMAINS OF PHLPP

The PHLPP family comprises two genes that share the same domain composition and 41% overall amino acid identity in humans (Figure 1B). PHLPP is evolutionarily conserved in yeast as Cyr1 [3], which curiously contains a C-terminal adenylate cyclase domain in addition to the Leucine-rich repeat (LRR) and PP2C domains of PHLPP [4] (Figure 1). The regulatory modules of PHLPP are located on the same polypeptide, distinguishing it from other Ser/Thr phosphatases, which typically consist of separate phosphatase and regulatory modules encoded by multiple genes [5].

2.1. N-Terminal Extension

The N-terminus of PHLPP1 (but not PHLPP2) has an approximately 50 kDa unstructured extension containing a bipartite Nuclear Localization Signal (NLS) that regulates the nuclear entry of PHLPP1 [6]. This NLS is opposed by a Nuclear Exclusion Signal (NES) at the C-terminal end of the LRR segment of the protein [6]. The PHLPP1 NLS is necessary for its nuclear function in controlling transcription (see Section 3.4).

2.2. PH Domain

The PH domains of PHLPP isozymes have weak homology to the canonical PH domain sequence [7] and binding studies reveal that the module has a ‘borderline’ affinity for phosphoinositides [8]. The PHLPP PH domain was acquired later in evolution compared to its other domains and is only found in mammalian *PHLPP* genes, suggesting that the spectrum of PHLPP substrates has broadened during evolution as more regulatory modules were added to the phosphatase. The PH domain is necessary for the effective interaction and dephosphorylation of protein kinase C (PKC) by PHLPP in cells (see Section. 3.2.) [9], suggesting that PHLPP regulation of PKC evolved after its role in controlling Akt activity.

2.3. LRR Domain

The LRR domains are characterized by multiple repeats of leucine residues at invariant positions. The LRR domain of PHLPP1 is composed of 18 repeats [10] and was reported to mediate the interaction of PHLPP1 with the nucleotide-free form of K-Ras in the brain, blocking K-Ras nucleotide binding and downstream activation of ERK [11]. Interestingly, the LRR domain of the yeast homolog, Cyr1, which contains 26 repeats [12], has also been reported to bind Ras in yeast [13], although with a different functional outcome: yeast Cyr1 interacts with the GTP-bound form of Ras to promote its activation [14].

2.4. PP2C Domain

The phosphatase domain of the PHLPP family members belongs to the PPM ‘shrub’ of Ser/Thr protein phosphatases (Figure 1A). This shrub houses the well-characterized PP2C α (PPM1A), whose structure has been solved [15], and the recently identified Rab-GTPase phosphatase PPM1H, which counteracts LRRK2 signaling [16]. A hallmark of PP2C phosphatases is their resistance to common Ser/Thr phosphatase inhibitors such as okadaic acid (OA) and microcystin [17, 18]. The insensitivity of PHLPP isozymes to OA provides a useful tool to distinguish between PHLPP-specific activity and that of other non-PP2C phosphatases [2, 19, 20]. The catalytic activity of the PHLPP PP2C domain is relatively

low (on the order of one reaction per second towards phospho-peptide substrates [20]), suggesting that stoichiometric association with substrates via its protein interaction modules is an important determinant for substrate dephosphorylation in cells.

2.5. PDZ Ligand

The last four carboxyl-terminal residues of PHLPP1 (DTPL) and PHLPP2 (DTAL) encode Type 1 PDZ (PSD-95/Disc-large/ZO-1) ligands. In the case of PHLPP1, its PDZ ligand is crucial for dephosphorylation of Akt in cells, as well as its pro-apoptotic and tumor-suppressive effects in a glioblastoma xenograft model [2]. The PDZ ligands of PHLPP1 and PHLPP2 also bind the scaffold, Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) (see Section 4.1.), an interaction that localizes the phosphatases near the membrane where they can dephosphorylate Akt [21]. The PHLPP PDZ ligand is conserved in lower organisms such as *C. elegans* and *Drosophila*, suggesting that antagonizing Akt signaling is an evolutionarily conserved function of PHLPP.

3. PHLPP SUBSTRATES

3.1. Hydrophobic Motif Phosphatase

The hydrophobic motif is a phosphorylation site on the C-terminal segment of a large number of AGC kinases, including Akt and PKC, that serves as a regulatory switch for kinase function (Figure 2, green square on PKC, Akt, and S6K) [22]. Originally identified in PKC and S6K [23], this phosphorylation site entered the limelight upon its acceptance as a marker for the activation state of Akt [24]. Highlighting the importance of this phosphorylation site, the hydrophobic motif is among the most frequently mutated post-translational modification sites among AGC kinases in human cancer [25, 26]. This site remains the best characterized substrate of the PHLPP [27].

3.1.1 Akt—Akt is acutely activated by reversible phosphorylation at two sites, the activation loop (Thr308 in Akt1; pink circle on Akt in Figure 2) and the hydrophobic motif (Ser473 in Akt1), following activation of the PI3-kinase pathway [24]. Signaling is terminated by dephosphorylation of the activation loop, catalyzed by PP2A family members, and of the hydrophobic motif, catalyzed by PHLPP (Figure 2). The PHLPP-mediated dephosphorylation inhibits Akt-triggered apoptosis and suppresses tumor growth [2, 28]. PHLPP1 specifically interacts with and dephosphorylates Akt2, modulating the phosphorylation of HDM2 and glycogen synthase kinase (GSK)-3 α to prevent p53 degradation; whereas PHLPP2 specifically interacts with and dephosphorylates Akt1, inhibiting the phosphorylation of p27 to block cell cycle progression [28]. Both PHLPP isozymes interact with and dephosphorylate Akt3, inhibiting the phosphorylation of GSK-3 β and TSC2 to suppress cell survival [28]. The amplified activity of Akt following loss of PHLPP stimulates the well-characterized negative feedback loop from ribosomal protein S6 kinase 1 (S6K1), such that knock-down of both PHLPP1 and PHLPP2 does not have an additive effect on Akt activity compared to knock-down of only one PHLPP isozyme, unless S6K1 is inhibited [28]. The PHLPP-mediated dephosphorylation of Akt is also conserved in *Drosophila* [2]. Interestingly, a common polymorphism in the substrate-binding region of the

phosphatase domain of PHLPP2, L1016S, impairs the ability of PHLPP to dephosphorylate Akt in cells [29], without impairing other biological functions [30].

3.1.2 PKC—In contrast to Akt, PKC is constitutively phosphorylated at the hydrophobic motif (Ser660 in PKC β II) [31]. Furthermore, phosphorylation of this site does not control the acute activity of the kinase, rather it controls the chronic stability of the enzyme. Phosphorylation at this site occurs shortly after biosynthesis by intramolecular autophosphorylation, one of a series of phosphorylation events in the ‘maturation’ of PKC; phosphorylation of the hydrophobic motif followed PDK-1-catalyzed phosphorylation of the activation loop (Figure 2, magenta circle on PKC) and mTORC2-dependent phosphorylation of the turn motif (Figure 2; orange circle on PKC). Phosphate at the hydrophobic motif is necessary for PKC to adopt an autoinhibited conformation that is resistant to degradation, and loss of phosphate at this site results in the degradation of the enzyme. Because PHLPP controls the phosphorylation state of this site, it plays a critical role in setting the cellular levels of PKC. The steady-state levels of both conventional (Ca²⁺-dependent) and novel (Ca²⁺-independent) PKC isozymes are regulated by PHLPP [9]. While extensive biochemical and cellular studies have clearly established that PKC lacking phosphate at this site is unstable and degraded, this finding was recently validated in human patient samples: analysis of over 5,000 patient tumor samples from 19 different cancers revealed a 1:1 correlation between PKC levels and phosphorylation at the hydrophobic motif [32]. Thus, if PKC is not phosphorylated at the hydrophobic motif, it is degraded. As mentioned in Section 2.2., the cellular recognition of PKC by PHLPP depends on the PH domain of PHLPP, a module that is dispensable for PHLPP to dephosphorylate Akt in cells, and is independent of the PDZ ligand, a motif that is necessary for PHLPP to dephosphorylate Akt in cells. Furthermore, co-immunoprecipitation studies revealed that PHLPP1 recognizes PKC when it is in an “open” conformation and not when it is in a stable and autoinhibited conformation [32]. This conformational sensing of PKC was recently shown to provide a “quality control” step in the maturation of PKC, whereby newly-synthesized PKC must ultimately become phosphorylated to adopt the autoinhibited and stable conformation which ultimately is reversibly activated in response to second-messengers [32]. PHLPP1 was shown to bind newly-synthesized PKC to ensure that any aberrant enzyme that cannot be autoinhibited is dephosphorylated at the hydrophobic motif and shunted to degradation. Accordingly, cancer-associated mutations in the PKC β autoinhibitory pseudosubstrate segment prevent autoinhibition and are subject to PHLPP1-catalyzed dephosphorylation and degradation [32]. Thus, PHLPP1 binds to both newly-synthesized PKC and activated PKC to enact the dephosphorylation and degradation of improperly folded or aberrantly active enzymes [32]. PKC quality control by PHLPP1 presents a potentially ubiquitous mechanism by which PKC is lost in cancer. Compromised PKC quality control, conversely, may enable PKC gain-of-function effects that drive neurodegeneration [31].

3.1.3 S6K1—S6K1 is another AGC kinase family member that is a direct substrate of PHLPP (Figure 2), establishing a role for PHLPP in regulating protein translation initiation and cell size. Activation of S6K1 downstream of mTOR is controlled by growth factors, nutrients, and energy balance. Full activation of S6K1 requires phosphorylation on both the hydrophobic motif (Thr389), mediated by mTORC1, and the activation loop

(Thr229), by PDK-1 [33]. Consistent with its effect on other substrates from the AGC kinase family, PHLPP selectively dephosphorylates S6K1 on its hydrophobic motif *in vitro*, and overexpression studies have revealed that PHLPP reduces S6K1 phosphorylation in cells independently of Akt dephosphorylation [34]. Functionally, PHLPP depletion resulted in increased phosphorylation of ribosomal protein S6 (rpS6) and increased binding of rpS6 to the translation initiation complex, correlating with increased cell size, protein content, and rate of cap-dependent translation [34]. As noted in Section 3.1., PHLPP depletion also activates the S6K1-mediated negative feedback regulation of Akt.

3.2. Mst1

PHLPP can also induce apoptosis in cancer cells independently of its regulation of Akt and PKC. Specifically, it binds and dephosphorylates Mst1 at an inhibitory site, Thr387 (Figure 2), leading to its activation and induction of apoptosis through p38 and JNK [35]. Mst1 is crucial for apoptosis and is frequently implicated in tumor growth [36]. Therefore, PHLPP and Mst1 synergize to promote a much greater increase in apoptosis than through either protein alone. Furthermore, Akt and Mst1 are reciprocally inhibitory [37, 38]. PHLPP may thus also induce apoptosis by indirect activation of Mst1 via inhibition of Akt activity.

3.3. MAPK Pathway

Early studies reported that PHLPP1 directly interacts with K-Ras via its LRR domain to negatively regulate the Ras-Raf-MEK-ERK pathway involved in memory formation in the hypothalamus [11, 39]. Additionally, a subsequent study reported a positive feedback loop whereby oncogenic H-Ras can inhibit PHLPP1 expression in MCF-10A and HCT116 cells, leading to activation of the p38 pathway and subsequent inhibition of anoikis, a caspase-dependent cell death program [40]. PHLPP was also reported to reduce ERK phosphorylation (Figure 2), as knock-down of PHLPP1 was shown to increase ERK phosphorylation in hippocampal cultures [41]. Additionally, PHLPP1 and PHLPP2 interact directly with the proto-oncogene Raf1 and dephosphorylate Raf1 *in vitro* on Ser338 (Figure 2), thereby inhibiting its activity and contributing to the negative regulation of MAPK signaling [42].

PHLPP also suppresses ERK signaling by a mechanism unrelated to any of its previously characterized functions as a phosphatase for kinases: by suppressing the transcription of receptor tyrosine kinases (RTKs) to reduce their steady-state levels (Figure 2) [30]. Specifically, nuclear PHLPP opposes histone phosphorylation and acetylation, modifications associated with open chromatin, to reduce the expression of many RTKs including the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and insulin receptor (INSR) [30]. This function of PHLPP depends on its nuclear localization and is accompanied by direct binding of PHLPP to chromatin [30]. PHLPP1 and PHLPP2 were also shown to interact with and dephosphorylate c-Jun transcription factor at Ser63/73, inhibiting its transcriptional activity [43]. As AP-1 proteins such as c-Jun are responsible for enhancer-mediated EGFR expression in EGFR-driven cancers such as glioblastoma and head and neck squamous cell carcinoma, it is plausible that PHLPP also reduces EGFR levels through inactivation of its transcription factors [44]. Therefore, PHLPP-mediated suppression of MAPK signaling proceeds both through direct

inactivation of pathway components, such as dephosphorylation of Raf1, and also by transcriptional processes involving chromatin silencing and inactivation of transcription factors that downregulate growth factor receptor expression.

3.4. STAT1

One of the most recently identified substrates of PHLPP is the transcription factor Signal Transducer and Activator of Transcription 1 (STAT1) (Figure 3) [6]. Members of this family of transcription factors are recruited from the cytosol to cytokine-bound receptors, where they are phosphorylated on a key Tyr residue (Tyr701 on STAT1) by Janus Kinases (JAKs); this event promotes their dimerization and nuclear entry where they bind to specific promoter sequences and initiate gene transcription [45]. They also transduce signals from type I and II interferons following ligation of interferon receptors, resulting ultimately in the transcription of inflammatory genes. Their transcriptional activity is enhanced by phosphorylation of a key regulatory Ser (Ser727 for STAT1) and it is this site that is opposed by PHLPP1. Biochemical studies have revealed that nuclear PHLPP1 binds STAT1 to specifically dephosphorylate Ser727 with no effect on Tyr701 [6]. STAT1 dephosphorylation by PHLPP1 mediates resolution of the inflammatory response by profoundly altering the transcriptome. Analysis of differentially expressed genes in bone marrow-derived macrophages from *Phlpp1*^{-/-} treated with the major LPS component KLA revealed enrichment of STAT transcription factor motifs in 46% of the upregulated genes (92 of 199 genes upregulated compared to WT) [6]. This regulation of STAT1 cements a role of PHLPP1 in suppressing inflammatory signaling.

3.5. Other Substrates

The number of proteins identified to interact with PHLPP is steadily increasing, particularly with the surge in unbiased proteomics approaches. In this regard, the recent analysis of the “human interactome” by Mann and colleagues identified PHLPP1 as binding six interrogated proteins in HeLa cells: protein phosphatase 2 regulatory subunit B’ α (PPP2R3A), the mitotic checkpoint kinase BUB1, mitogen activated kinase MAPK8/JNK1, the histone deacetylase HDAC1, the TATA-binding protein associated factor (TAF15), and the transcriptional regulator protein MAX [46]. Identification of novel PHLPP interactors additionally elucidates the subcellular localizations where PHLPP functions. For example, Rac1 was shown to recruit PHLPP1 to lysosomes to promote chaperone-mediated autophagy through suppression of Akt [47]. In more targeted analyses, PHLPP2 was reported to interact with the tumor suppressor PML (promyelocytic leukemia) in the nucleus, an interaction that promotes the dephosphorylation of nuclear Akt. This association is inhibited in many cancers by deregulated protein kinase CK2, which phosphorylates PML and shunts it to degradation, thus dismantling the PHLPP phosphatase platform that allows access to Akt [48]. This demonstrates that PHLPP is capable of synergizing with other tumor suppressor proteins to inhibit Akt activity. PHLPP2 was also shown to suppress NF- κ B signaling, a pro-inflammatory signaling pathway activated in many cancers, by disrupting the Bcl10-MALT1 ubiquitin-ligase complex that enables IKK β phosphorylation and subsequent NF- κ B activation [49]. Identification of interactors that localize PHLPP1 and PHLPP2 to the nucleus will further contribute to their expanding repertoire of nuclear

functions, including core transcriptional programs in disease contexts (discussed further in Section 6.).

4. REGULATION OF PHLPP

4.1. Protein Scaffolds

Protein scaffolds are likely to play a key role in controlling specificity and efficacy in downstream signaling by PHLPP. The quantitative analysis of the human interactome in HeLa cells reported intracellular concentrations of PHLPP1 and PHLPP2 as approximately 1 nM each, with substrates such as Akt1 at 30 nM [46]. This underscores the importance of subcellular targeting to position PHLPP near its relevant targets. The key role of spatial organization in dictating the function of PHLPP is illustrated by the finding that loss of the PHLPP1 PDZ ligand abolishes its recognition of Akt2 in cells, whereas loss of the PH domain abolishes its recognition of PKC [9]. Positioning PHLPP near its substrates affords tight control, essential for the regulation of enzymes such as Akt, in which the correct balance between phosphorylated and dephosphorylated states is essential for homeostasis.

Several scaffolds that bind PHLPP have been identified. FKBP51 binds both PHLPP isozymes to promote the specific suppression of Ser473 phosphorylation and inhibition of Akt activity [50, 51], a function that is deregulated in diverse diseases from prostate cancer to mitochondrial dysfunction and neurodegeneration [51-54]. The scaffold Scribble binds the PHLPP1 C-terminal segment (but not the PDZ ligand) via its own LRR to localize the phosphatase near the plasma membrane for effective regulation of Akt [55]; knock-down in DLD1 colon cancer cells results in a reduction of PHLPP1 at the membrane fraction and higher accumulation in the cytoplasm of, leading to an increase in Akt Ser473 phosphorylation. Another scaffold protein that localizes PHLPP to the plasma membrane is NHERF1. This tandem PDZ domain scaffold coordinates two suppressors of PI3K signaling, PTEN and PHLPP, to allow effective suppression of Akt signaling. PTEN binds PDZ1 of NHERF1 [56] and PHLPP2 binds PDZ2, whereas PHLPP1 has been reported to bind both PDZ domains. Deregulated PI3K signaling is prevalent in glioblastoma, and the tumor suppressive complex of PTEN-NHERF1-PHLPP1 is frequently disabled in high-grade glioblastoma tumors, correlating with enhanced Akt activation and low patient survival [21].

4.2. Transcriptional Regulation

A number of studies have reported on the transcriptional regulation of PHLPP. In chondrocytes, the binding of histone deacetylase 3 (HDAC3) to Smad binding elements in the PHLPP1 promoter was shown to suppress the transcription of PHLPP1, resulting in decreased PHLPP1 protein levels, increased Akt activity, and chondrocyte hypertrophy. This association of HDAC3 with the PHLPP1 promoter was released by TGF β treatment, indicating that PHLPP1 transcription is enhanced by this cytokine [57]. HDAC3 was also shown to bind the PHLPP1 promoter in breast cancer cell lines: in this study, the long noncoding RNA Xist (X inactive-specific transcript) decreased HDAC3 recruitment to the PHLPP1 promoter, increasing histone acetylation on the promoter and subsequent expression of PHLPP1 mRNA to increase PHLPP1 protein levels and suppress Akt phosphorylation [58]. Another study showed that DNA hypermethylation of the PHLPP1

gene leads to suppression of PHLPP1 transcription in melanoma and promotes Akt activation [59]. The region in the PHLPP1 promoter that is involved in transcriptional activation of PHLPP1 is between -64 and -3 and contains a consensus binding site for the transcription factor Sp1. Indeed, deletion of the Sp1-binding site or Sp1 knock-down inhibited transcription of PHLPP1 [59], and lipopolysaccharide (LPS) was shown to negatively regulate PHLPP expression in macrophages by an Sp1-dependent mechanism [60]. Consistent with promoter methylation regulating PHLPP transcription, high PHLPP1 mRNA levels in the cartilage from osteoarthritis patients correlated with reduced PHLPP1 promoter methylation [61]. In this study, the expression of PHLPP1 was shown to be induced in chondrocytes by inhibition of cytosine methylation or treatment with pro-inflammatory cytokines that can induce DNA demethylation, IL6 and TNF α [61]. A high degree of PHLPP1 methylation at the end of exon 1 has also been reported to strongly correlate with low levels of PHLPP1 protein [62]. Lastly, the transcription of both PHLPP isozymes has been reported to be inhibited by Cullin 4B (CUL4B), the scaffold protein of the CRL4B-E3 ligase complex, which was reported to bind the PHLPP promoter and monoubiquitinate H2AK119, thus repressing transcription [63].

The mRNA levels of *CYR1*, the yeast homolog of PHLPP1 which also has an adenylate cyclase module, are sensitive to glucose availability. As yeast exhaust their glucose supply, they reach a growth plateau before a diauxic shift to utilize other carbon sources. At this point, Cyr1 mRNA and protein levels decrease, resulting in decreased cAMP levels [64]. Whether mammalian PHLPP mRNA is also regulated by glucose remains to be explored.

4.3. Post-Translational Regulation

The steady-state levels of PHLPP protein are sensitive to Akt activity by both translational and post-translational mechanisms (Figure 2). First, Gao and coworkers established that a key downstream effector of Akt, mTORC1, promotes the translation of PHLPP1 and PHLPP2 mRNA in colon and breast cancer cells [65]. This regulation of protein translation can be independent of Akt function, as Akt-independent mechanisms to activate mTORC1, such as amino acid or glucose starvation, can also enhance PHLPP protein expression. This provides an additional negative feedback loop to suppress Akt activity when mTORC1 activity is elevated, complementing the well-known negative feedback loop mediated by S6K1 suppressing PIP₃ generation. In this case, Akt itself is directly kept in check by higher PHLPP activity. Secondly, cellular levels of PHLPP1 protein are enhanced via a phosphodegron that is negatively regulated by Akt activity [66]. Activated Akt phosphorylates and inactivates GSK-3 β , inhibiting GSK-3 β -dependent phosphorylation at sites in the PP2C domain of PHLPP1. This in turn prevents ubiquitination by the β -TrCP-containing Skp-Cullin 1-F-box protein (SCF) complex (SCF $^{\beta$ -TrCP) and subsequent proteasomal degradation. This second feedback mechanism ensures that high Akt activity is counterbalanced by high PHLPP. SGT1, which was reported to be over-expressed in gastric cancer, was also reported to enhance the binding between PHLPP1 and β -TrCP, therefore promoting PHLPP1 degradation and amplified Akt signaling [67]. This feedback loop is lost in aggressive glioblastoma due to altered localization of β -TrCP from the cytoplasm (in normal brain tissue) to the nucleus (in astrocytoma cell lines), spatially separating it from PHLPP1, uncoupling the levels of a negative regulator PHLPP from the level of its substrate,

phosphorylated Akt [68]. Thus, high Akt activity ramps up the amount of PHLPP protein in the cells by 1] increasing protein translation and 2] preventing protein degradation.

PHLPP protein translation is inhibited by various microRNAs (miRNA), including miR224, miR-17~92 cluster, and miR375. These bind the 3'-UTR region of PHLPP mRNA, leading to decreased PHLPP protein expression and consequently, enhanced Akt activation [69-71]. Interestingly, many miRNAs, including miR-17~92, are located in chromosomal regions that are amplified in diverse cancers [72], and it was shown that suppression of miR-17~92 increased PHLPP2 protein levels to promote terminal differentiation of myeloid leukemia cells [73].

PHLPP has also been reported to bind several deubiquitinating enzymes (DUBs) that deubiquitinate and stabilize it, such as ubiquitin-specific protease (USP)1 [74], USP46 [75], USP12, and WDR48, an activator of several DUBs [76]. Loss of such DUBs has been observed in several cancers and is correlated with decreased PHLPP expression levels and amplified Akt phosphorylation [75]. A recent study reported the formation of a protein complex between PHLPP1, PHLPP2, Akt, the Fanconi anemia complementation group proteins FANCI and FANCD2, and the DUBs USP1 and UAF1 [77]. In response to DNA damage, the interaction between FANCI, Akt and PHLPP1 is reduced, resulting in an increase of Akt phosphorylation and activity [77]. This identifies yet another complex that positions Akt near its negative regulator PHLPP to control its activity.

PHLPP is replete with predicted post-translational modification sites, and many have been identified by unbiased phosphoproteomic approaches [78], yet surprisingly few have been characterized. The coming years are likely to see a significant increase in our understanding of how post-translational modifications regulate the stability, activity, and subcellular location of PHLPP.

5. ROLE OF PHLPP IN CANCER

5.1. Tumor Suppressive Role

PHLPP is an accepted tumor suppressor based on animal models and analysis of human tumors. Loss of PHLPP1 causes neoplasia and, when co-deleted with PTEN, carcinoma in mouse prostate [79]. Samples from metastatic prostate tumors reveal frequent co-deletion of *PTEN* and *PHLPP1* genes compared to samples from primary tumors. Interestingly, PHLPP2 levels increase following co-deletion of PTEN and PHLPP1 in an mTOR-dependent manner, similar to the observed increase in p53 levels. Therefore, it is not surprising that late stage metastatic prostate samples from patients also show a high rate of co-deletion of *PHLPP1/2* and *TP53* genes with *PTEN* to further break this feedback between loss of TP53 and PTEN and enhanced PHLPP2 levels [79]. PHLPP levels are reduced or lost in many other different cancers (as reviewed in [80]). In chronic lymphocytic leukemia (CLL), with 13q14 deletion, PHLPP mRNA is lower or undetectable in more than 50% of cases [81]. In colon cancer, the expression of both PHLPP isozymes is decreased [82]. In glioblastoma cell lines, the mRNA of PHLPP1 is 40% lower compared with that of normal astrocytoma cell lines [68]. In melanoma, where the PI3K/Akt pathway is activated in up to 70% of cases, PHLPP1, but not PHLPP2, mRNA and protein levels are reduced

as a result of hypermethylation of the *PHLPP1* gene [59]. The chromosomal regions of PHLPP1 and PHLPP2 are adjacent to other tumor suppressor genes where a frequent loss of heterozygosity occurs in tumors: PHLPP1 is on 18q21.33, one of the most frequently lost regions in colon cancer [83, 84], and PHLPP2 is on 16q22.3, a region frequently lost in breast [85], ovarian [86], liver [87], kidney [88] and prostate [89] tumors. Therefore, low levels of PHLPP can serve as a biomarker in these cancer contexts.

5.1.1. Suppression of Akt signaling.—The best characterized mechanism by which PHLPP acts as a tumor suppressor is through inhibition of Akt activity (Figure 2). An abundance of studies have shown a correlation between reduced levels of PHLPP and increased Ser473 phosphorylation of Akt in different cancer cells, such as metastatic breast cancer [90], colon and glioblastoma cell lines [2, 82], CLL B-cells [91], and melanoma [59]. Over-expression of PHLPP in cancer cells leads to inhibition of tumor growth, increased apoptosis, and decreased Akt Ser473 phosphorylation [28, 82, 91]. Interestingly, 30% of the population has a heterozygous polymorphism in the PP2C domain of PHLPP2 in which a Leu residue is substituted with Ser at position 1016. As noted in Section 3.1., the Ser1016 variant of PHLPP2 has decreased activity towards Akt both *in vitro* and in cells, resulting in reduced apoptosis. In heterozygous individuals the loss of the wild-type Leu1016 allele contributes to higher grade breast cancer: in pair-matched high grade breast cancer samples, only the Ser allele is retained from heterozygous patients [29]. The same Leu1016Ser polymorphism in PHLPP2 was also reported in ovarian tumors, presenting more frequently in mucinous compared to serous tumors, with loss of heterozygosity in one case [92].

5.1.2. Suppression of RTK Signaling—PHLPP functions as a tumor suppressor through additional mechanisms that are independent of its function as the Akt hydrophobic motif phosphatase; notably, PHLPP also controls growth factor signaling by suppressing the transcription of RTKs. As discussed in Section 3.3., loss of PHLPP1 or PHLPP2 increases the transcription and steady-state protein levels of a number of RTKs, including EGFR, leading to amplified growth factor signaling [30]. This epigenetic regulation of RTK levels by PHLPP could provide one mechanism to account for the frequent upregulation of RTKs in cancer. Other studies have shown that PHLPP can act as tumor suppressor independently of Akt, PKC, and ERK pathways. Over-expression of PHLPP in Panc1 pancreatic cancer cells decreased colony numbers by more than 50% as assessed by anchorage-independent growth, but did not affect Akt or ERK phosphorylation or total levels of PKC [35]. Rather, the mechanism by which PHLPP induced apoptosis was by dephosphorylation at an inhibitory site of the pro-apoptotic kinase, Mst1. PHLPP2 was also shown to interact with IKK β , leading to inhibition of its phosphorylation and subsequent activation of a major signaling pathway in cancer, the NF- κ B pathway [49]. Additionally, PHLPP overexpression was reported to upregulate the expression and phosphorylation of p21 and p27, negative regulators of cell cycle progression [59, 82]. In turn, PHLPP1 expression is positively controlled by p27 [93], the loss of which is strongly associated with colorectal cancer progression [94]. Thus, PHLPP suppresses survival signaling by multiple mechanisms.

5.1.3. Suppression of Inflammation—PHLPP has been shown to be important for immunological processes. Regulatory T cells (Treg) are activated through either the T cell

receptor or IL-2 and have a reduced capacity to activate the PI3K/Akt pathway compared to conventional T cells, which is necessary for Treg cell development and function [95]. PHLPP1 and PHLPP2 levels are upregulated in Treg cells, and PHLPP1 knock-out mice have impaired Treg cell development and suppressive capacity [96]. Furthermore, PHLPP1 was shown to be essential for the induction of Treg cells, as inhibition of both PHLPP and PTEN phosphatases results in decreased mitochondrial membrane potential in blood-derived Treg cells [97]. These data suggest a role for PHLPP in the regulation of immunological tolerance.

Another role for PHLPP was recently reported in mediating the resolution of the innate immune response [6]. *Phlpp1*^{-/-} mice either challenged with *E. coli* or injected with LPS were protected from sepsis-induced death. This suggests that inhibition of PHLPP would be beneficial as an adjuvant therapy in antibiotic treatment of gram-negative sepsis. While the mechanism for this protection awaits elucidation, studies with bone marrow-derived macrophages identified PHLPP1 as a major regulator opposing STAT1-mediated transcription of inflammatory genes (see Section 3.4). PHLPP resolution of the inflammatory response may also play a role in chronic inflammatory disorders that predispose individuals to cancer such as inflammatory bowel disorder and nonalcoholic steatohepatitis (NASH). Intriguingly, *Phlpp1/Phlpp2* double knock-out mice were protected against dextran sulfate sodium (DSS)-induced colitis due to an Akt-dependent reduction in apoptosis of intestinal epithelial cells [98]. Protection against acute colitis was also found to be mediated by PHLPP-deficient neutrophils, which exhibited enhanced immune homeostasis and elevated phosphorylation of Akt, ERK, and STAT1 [99]. These findings suggest that PHLPP enacts coordinated effects on inflammatory processes through modulation of distinct cell types. Additionally, PHLPP itself is regulated by immune signaling, as PHLPP1 and PHLPP2 protein were shown to be downregulated after 24 hours of LPS treatment [98]. Conversely, the induction of PHLPP2 in prostate cancer upon loss of PTEN and p53 is dependent upon the pro-inflammatory cytokine IL-6 [100]. Thus, further studies are required to understand the signaling pathways underlying PHLPP regulation of the immune system in various cell types and disease contexts.

5.2. Oncogenic Role

Could PHLPP also function as an oncogene in certain contexts? Recent evidence indicates that both PHLPP1 and PHLPP2 negatively regulate two tumor suppressors, PKC and Myc (Figure 4).

5.2.1. PKC—Recent analysis of cancer-associated mutations in PKC genes has revealed that they are generally loss-of-function, suggesting that this family of kinases has a tumor-suppressive role [101]. Consistent with this, high levels of PKC protein confer improved survival in diverse cancers [102]. Furthermore, genome-editing to correct mutant PKC β II in a colon cancer cell line revealed not only that the mutant PKC was dominant-negative, but that the PKC β II was haploinsufficient with respect to cell growth in 3D: two alleles encoding wild-type PKC β II were considerably more effective at suppressing anchorage-independent growth of the cancer cell line compared with one allele [101]. As discussed in Section 3.1.2., dephosphorylation of the hydrophobic motif of PKC by PHLPP is a

key determinant in setting the steady-state levels of PKC family members. While there are many mechanisms to control the steady-state levels of PKC, PHLPP1 is the dominant regulator in pancreatic cancer [32]. Analysis of protein levels from 105 patient tumors revealed an inverse correlation between PHLPP1 levels and PKC levels [32]. Furthermore, patients with high levels of PKC hydrophobic motif phosphorylation and low levels of PHLPP1 had greatly improved survival compared to patients with low PKC and high PHLPP1 protein levels [32]. PKC's tumor-suppressive function was originally elucidated in a K-Ras-driven colorectal cancer xenograft model, and one mechanism by which PKC may act as a checkpoint in tumor progression is through attenuation of K-Ras signaling [101, 103]. Thus, in cancers that rely less heavily on Akt signaling, PHLPP1 may function as an oncogene through negative regulation of PKC, particularly in the context of oncogenic K-ras, which is frequently mutated in pancreatic cancer (Figure 4) [104, 105].

5.2.2. Myc—Discerning the expanding roles of PHLPP2 in cancer presents an even more complex picture. Despite the fact that elevation of Akt signaling resulting from PHLPP1 and PTEN deletion is critical for the onset, progression, and outcome of prostate cancer, PHLPP2 levels become elevated in prostate cancer metastasis upon co-deletion of *Pten* and *Tp53*, correlating with decreased Akt phosphorylation on Ser473 [100]. This apparent paradox was resolved by the finding that PHLPP2 dephosphorylates Myc on Thr58 to promote the stabilization of Myc in advanced prostate cancer [106]. Therefore, although capable of suppressing Akt activation that drives the disease, PHLPP2 is required for prostate cancer progression by stabilizing Myc [106] (Figure 4). Supporting a role of PHLPP2-mediated dephosphorylation of Myc on Thr58 in oncogenesis, a recurrent Myc T58A mutation observed in Burkitt's lymphoma was sufficient to induce lymphoma and enhanced lethality in adoptive transfer of mouse hematopoietic stem cells [107]. The potential role of PHLPP2 as an oncogene provides one mechanism for the paradoxical “Akt-off” state in tumors with PI3K pathway alterations [108], which may be applicable to many apparently PI3K-driven malignancies. Whether tumor Akt status can be utilized to predict the function of PHLPP as oncogenic or tumor-suppressive is an attractive hypothesis.

6. PHARMACOLOGICAL TARGETING OF PHLPP IN CANCER

Resistance to chemotherapy is a major challenge in cancer therapy. Thus, information on the expression levels of certain biomarkers has tremendous therapeutic potential in outcome prediction and in matching available kinase inhibitors with the right patient or designing personalized medicines. PHLPP is a good candidate to be such a biomarker in cancer. For example, re-expressing PHLPP significantly increases the sensitivity of colon cancer cells to PI3K inhibition [82]. Therefore, determining the expression levels of PHLPP in cancer may help to predict which patients will respond better to PI3K and Akt inhibitors. Similarly, PHLPP expression predicts the prognosis of lung adenocarcinoma patients and PHLPP levels correlate with the latency of acquired resistance to EGFR inhibitors [109, 110]. Determining PHLPP expression could also be beneficial in determining the response to rapamycin treatment in certain cancer cell lines that develop resistance to rapamycin-mediated growth inhibition. In rapamycin-treated cells, PHLPP translation is inhibited, indicating that PHLPP may play a role in the induction of chemoresistance.

Indeed, overexpression of PHLPP proteins significantly enhanced the inhibitory effect of rapamycin on growth of these cancer cells [65]. Furthermore, hypoxia is a common feature of tumors that plays a critical role in promoting resistance to chemotherapy [111]. Hypoxia also inhibits mTOR and cap-dependent translation, which are upstream of PHLPP and positively regulate its expression. Indeed, downregulation of PHLPP contributes to hypoxia-induced chemoresistance in colon cancer cells, dependent upon hypoxia-inducible factor 1 α (HIF1 α) [112]. Conversely, in cancers where PHLPP has oncogenic functions, as described previously in pancreatic [32] and prostate [106] cancers, PHLPP levels may correlate inversely with sensitivity to treatment or with patient outcome. Thus, determining the levels of PHLPP and other biomarkers in tumors may not only aid in the identification of critical dependencies of specific tumors, but may also facilitate personalized cancer treatments and minimize off-target effects.

The choice of the right strategy to target PHLPP may have important consequences in different cancers. Drugs that stabilize PHLPP or enhance its activity would be generally beneficial in cancers driven by deregulated growth factor signaling (Figure 4, left). In this pathway, PHLPP opposes cell survival and cell growth through transcriptional repression of growth factor receptors and by suppressing the activity of the downstream kinases Akt and S6K. In contrast, cancers driven by oncogenes such as K-Ras, which are sensitive to suppression by PKC, would benefit from restoring PKC by inhibiting PHLPP function (Figure 4, right).

In the case of Akt-driven cancers, designing drugs to stimulate the activity of PHLPP is more challenging. PHLPP is commonly deleted in cancer, making drug design impossible in such cases. However, targeting many of the mechanisms that regulate PHLPP itself could stabilize the amount of protein in the cell or redirect it to relevant substrates. In this regard, a recent study identified γ -tocopherol as a molecule that binds the PH domains of Akt and PHLPP1, promoting their co-recruitment to the plasma membrane and thus enhancing the dephosphorylation of Akt on Ser473 by PHLPP1 [113]. Moreover, the authors reported that tumor growth in a xenograft mouse model was suppressed upon oral administration of the compound, suggesting suppression of tumor growth attributable to PHLPP1 inhibition of Akt [113].

PP2C phosphatases are largely refractory to commonly used small molecule inhibitors such as calyculin A and OA. However, several small molecule inhibitors selective for PHLPP over the related PP2C α were identified in a chemical and virtual screen of the NCI repository of inhibitors against the phosphatase activity of the purified phosphatase domains of PHLPP1 and PHLPP2 [114]. Biochemical and cellular assays validated two structurally diverse compounds that were identified from the screen: these selectively inhibit PHLPP *in vitro*, increase Akt signaling in cells, and suppress apoptosis. The two compounds, NSC 117079 and 45586, were predicted to be uncompetitive inhibitors of PHLPP that bind the hydrophobic cleft near the active site and interact with one of the Mn²⁺ ions. The compounds are not specific for PHLPP1 vs PHLPP2 but are selective for PHLPP compared to other phosphatases tested. Both compounds show potential therapeutic promise for diseases in which cell survival pathways are suppressed, however the IC₅₀ values towards Akt in cells are high, approximately 30 and 70 μ M for 117079 and 45586,

respectively. Optimizing PHLPP inhibitors may be beneficial to target emerging oncogenic functions of PHLPP such as PHLPP1-mediated degradation of PKC in pancreatic cancer [32] and PHLPP2-mediated stabilization of Myc in prostate cancer [106] (Figure 4, right).

PHLPP plays a central role in cellular homeostasis, with enhanced activity generally promoting apoptosis and reduced activity promoting survival. Pharmacologically targeting PHLPP is encumbered by its broadening roles in controlling many diverse, and often opposing, aspects of cell signaling. Optimally designing drugs to target PHLPP therapeutically in particular tissues and cell types for specific diseases, while challenging, will have enormous therapeutic potential.

7. SUMMARY

Since the discovery of PHLPP as the hydrophobic motif phosphatase of Akt in 2005 [2], PHLPP has moved quickly into the spotlight of tumor suppressor studies. The last few years have unveiled many new targets and mechanisms of function in different pathologies for this relative late-comer to the phosphatome. Numerous studies have cemented a central role for PHLPP in suppression of survival pathways and induction of apoptosis; however, it is clear that PHLPP performs multifaceted and often opposing roles, as evidenced by the recently-elucidated oncogenic functions in pancreatic and prostate cancer and complex role in the immune system. The coming years are likely to see an increase in our understanding of PHLPP regulation in normal and pathological conditions, which will be essential for the development of therapies to treat specific cancers and further establish the importance of PHLPP as a biomarker in cancer.

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Abbreviations

DUB	Deubiquitinating enzyme
GSK	Glycogen synthase kinase
HDAC3	Histone deacetylase 3
IFNγ	Interferon γ
LPS	Lipolysaccharide
LRR	Leucine-rich repeat
Mst1	Mammalian sterile 20-like kinase 1
NHERF1	Na ⁺ /H ⁺ exchanger regulatory factor 1

NTE	N-terminal extension
PDZ	PSD-95/Disc-large/ZO-1
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
RA	Ras association
RTK	Receptor tyrosine kinase
S6K1	Ribosomal protein S6 kinase 1
STAT1	Signal Transducer and Activator of Transcription 1
TLR	Toll-like receptor

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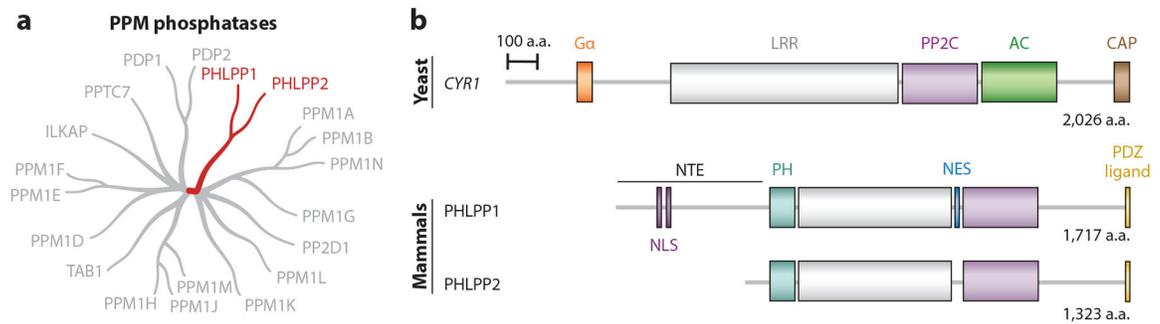


Figure 1. Phylogeny and Domain Architecture of PHLPP Phosphatases

(A) PHLPP1 and PHLPP2 are PP2C-type Ser/Thr protein phosphatases, belonging to the protein phosphatase magnesium/manganese (PPM)-dependent shrub of the protein phosphatase phylogenetic tree [115]. (B) Domain structure of ancestral and mammalian PHLPP. Yeast *CYR1* contains a Ga domain, 26 Leucine rich repeats (LRR), a PP2C phosphatase domain (PP2C) fused to adenylate cyclase (AC), and C-terminal cyclase-associated protein 1 binding domain (CAP). Mammalian PHLPP retains the LRR domain (18 in PHLPP1 and 19 in PHLPP2) and PP2C phosphatase domain in addition to a PH domain (PH), N-terminal extension (NTE) containing a bipartite Nuclear Localization Signal (NLS), and C-terminal PDZ ligand. Scale bar denotes 100 amino acids (a.a.).

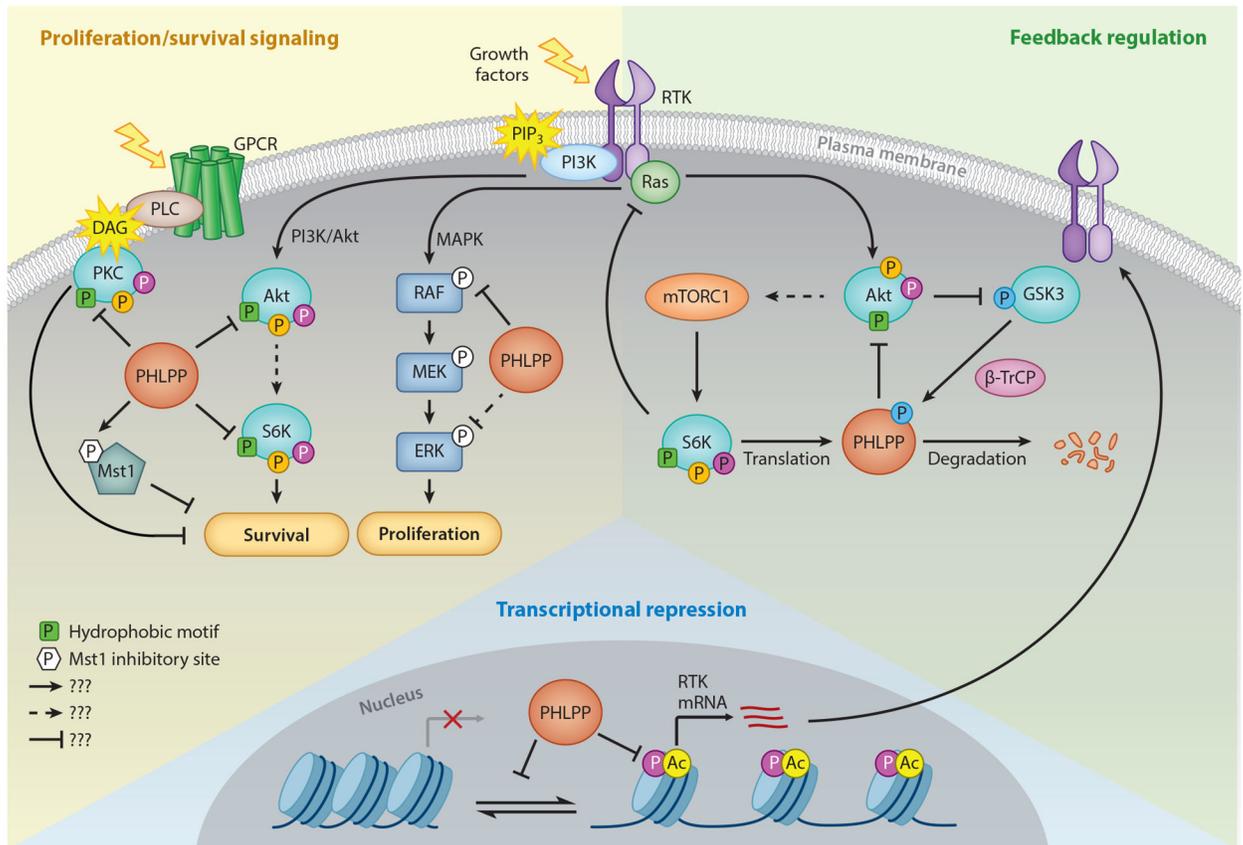


Figure 2. PHLPP Function in Growth Factor Signaling.

Schematic showing some of the substrates and signaling pathways regulated by PHLPP. In general, PHLPP suppresses proliferative and survival pathways through direct dephosphorylation of Akt, S6K1, Mst1, and Raf (red sector). PHLPP dephosphorylates the hydrophobic motif (green square with P) of Akt and S6K1 to inactivate them, while PHLPP activates the pro-apoptotic kinase, Mst1, via removal of an inhibitory phosphorylation (white hexagon with P). PHLPP also dephosphorylates PKC isozymes on the hydrophobic motif, which destabilizes them and reduces their cellular levels. Filled in circles on Akt, S6K1, and PKC denote other key phosphorylation sites, including the PDK-1 site on the activation loop (magenta circle) and mTORC2-regulated turn motif (orange circle). In addition, PHLPP suppresses the transcription and hence steady-state protein levels of RTKs such as the EGFR, thus dampening PI3K survival and ERK proliferative pathways, by an epigenetic mechanism (blue sector). PHLPP itself is under feedback regulation by components of the PI3K pathway (tan sector). High Akt activity increases PHLPP protein levels by suppressing GSK3 β -dependent degradation. Furthermore, high mTORC1 activity increases S6K1-dependent translation of PHLPP (left). Moreover, S6K1 activity also activates a negative feedback loop that suppresses Akt activity.

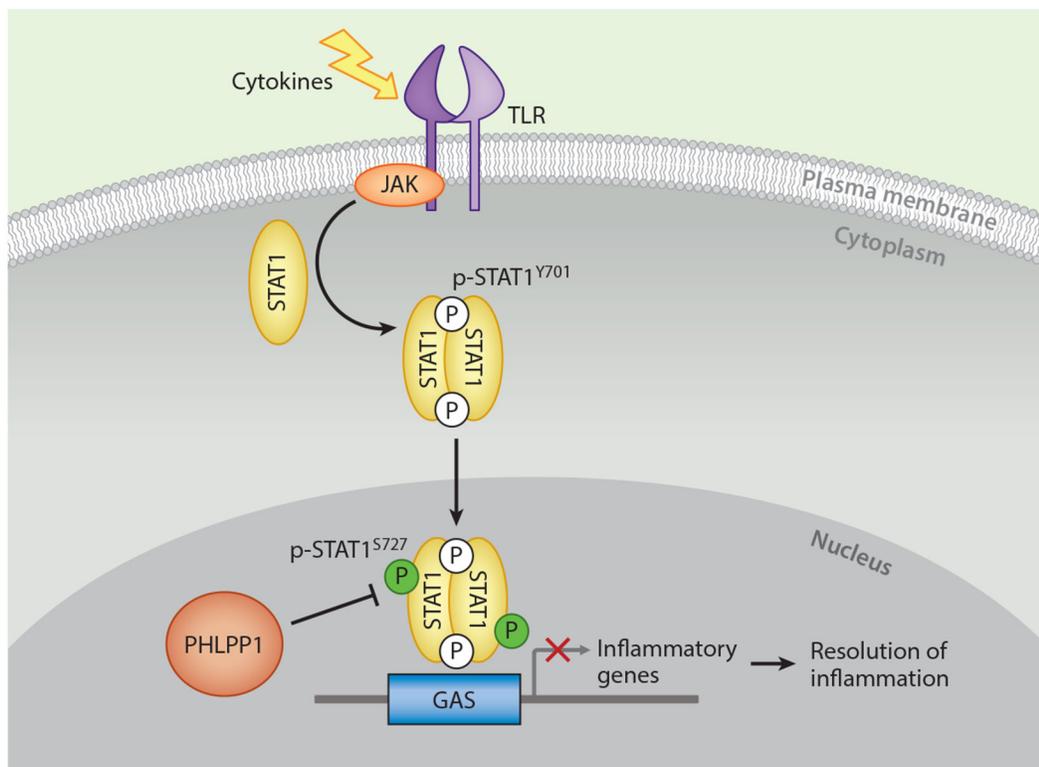


Figure 3. Emerging Roles for PHLPP1 in Inflammatory Signaling.

Binding of IFN γ to interferon receptors activates inflammatory transcriptional programs. Among the mediators of this inflammatory response are components of the JAK/STAT pathway: phosphorylation of STAT1 transcription factors on Tyr701 by Janus kinases (JAKs) induces STAT1 dimerization, nuclear translocation, and activation to promote transcription of target genes. This activity is enhanced by phosphorylation on Ser727. PHLPP1 opposes STAT1-mediated transcription to promote resolution of the inflammatory response by directly desphosphorylating Ser727 to tune STAT1 activity [6].

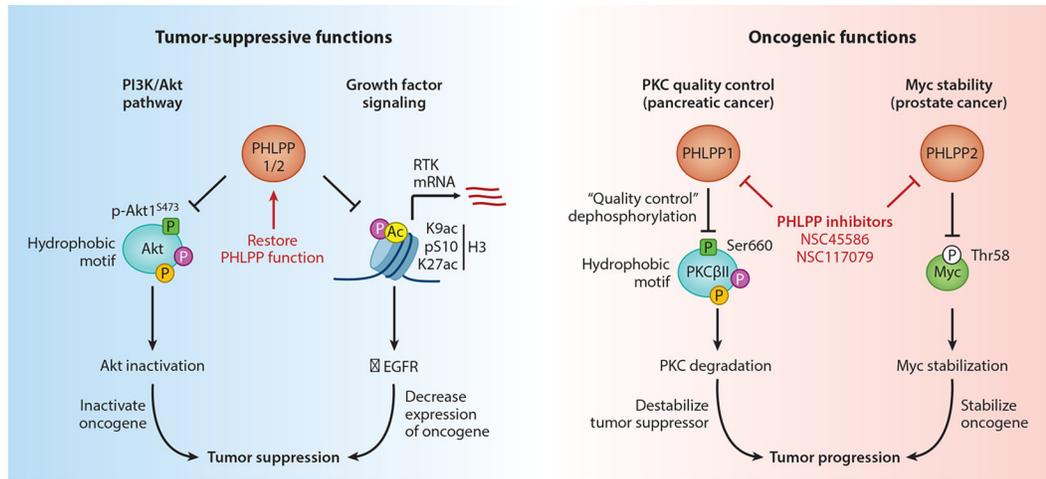


Figure 4. Pharmacological Targeting of PHLPP in Cancer.

Despite the discovery of PHLPP as a *bona fide* tumor suppressor by opposing PI3K and growth factor signaling (left), complex roles in cancer involving novel oncogenic functions present therapeutic opportunities for PHLPP inhibitors (right). In pancreatic cancer, PHLPP1 suppresses levels of the tumor suppressor PKC through quality control dephosphorylation of the hydrophobic motif, where the loss of PKC correlates inversely with patient survival [32]. In prostate cancer, PHLPP2 stabilization of oncogenic Myc is critical for tumor progression and metastatic disease [106]. Thus, future studies should explore the potential therapeutic benefit of PHLPP inhibition in these specific contexts.