

Suppression of survival signalling pathways by the phosphatase PHLPP

Audrey K. O'Neill^{1,2}, Matthew J. Niederst^{1,2,*} and Alexandra C. Newton²¹ Biomedical Sciences Graduate Program, University of California, San Diego, CA, USA² Department of Pharmacology, University of California, San Diego, CA, USA

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Correspondence

A. C. Newton, Department of Pharmacology, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0721, USA
Fax: +(858) 822 5888
Tel: +(858) 534 4527
E-mail: anewton@ucsd.edu

*Present address

Massachusetts General Hospital Cancer Center, Department of Medicine, Harvard Medical School, Boston, MA, USA

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The recently discovered pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase (PHLPP) family is emerging as a central component in suppressing cell survival pathways. Originally discovered in a rational search for a phosphatase that directly dephosphorylates and inactivates Akt, PHLPP is now known to potently suppress cell survival both by inhibiting proliferative pathways and by promoting apoptotic pathways. In the first instance, PHLPP directly dephosphorylates a conserved regulatory site (termed the hydrophobic motif) on Akt, protein kinase C and S6 kinase, thereby terminating signalling by these pro-survival kinases. In the second instance, PHLPP dephosphorylates and thus activates the pro-apoptotic kinase Mst1, thereby promoting apoptosis. PHLPP is deleted in a large number of cancers and the genetic deletion of one isozyme in a PTEN (phosphatase and tensin homologue located on chromosome 1) +/- (or heterozygous) prostate cancer model results in increased tumourigenesis, underscoring the role of PHLPP as a tumour suppressor. This review summarizes the targets and cellular actions of PHLPP, with emphasis on its role as a tumour suppressor in the oncogenic phosphoinositide 3-kinase (PI3K)/Akt signalling cascade.

Introduction

The pleckstrin homology (PH) domain leucine-rich repeat (LRR) protein phosphatase (PHLPP) family of serine/threonine phosphatases contains three isozymes (Fig. 1): the alternatively spliced PHLPP1 β (also known as suprachiasmatic nucleus oscillatory protein, SCOP [1]) and PHLPP1 α [2], and PHLPP2 [3]. PHLPP1 α and PHLPP1 β differ only in the first exon

(see [4] for a diagram), resulting in an approximately 50-kDa N-terminal extension to PHLPP1 β .

PHLPP1 and PHLPP2 share a common architecture (Fig. 1), including a phosphatase domain which is 58% conserved between PHLPP1 and PHLPP2 [5]. Sequence analysis reveals that the phosphatase domain belongs to the PP2C branch of the PPM family, whose

Abbreviations

β -TrCP, β -transducin repeats-containing protein; CLL, chronic lymphocytic leukaemia; ERK, extracellular-signal-regulated kinase; FKBP51, FK506 binding protein 51; GBM, glioblastoma; GSK, glycogen synthase kinase; IRS-1, insulin receptor substrate 1; LRR, leucine-rich repeat; mTOR, mammalian target of rapamycin; NHERF, Na⁺/H⁺ exchanger regulatory factor; PDZ, post synaptic density protein (PSD95), Drosophila disc large tumour suppressor (DLG) and zonula occludens 1 protein (zo-1); PH, pleckstrin homology; PHLPP, PH domain leucine rich repeat protein phosphatase; PI3K, phosphoinositide 3-kinase; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PTEN, phosphatase and tensin homologue located on chromosome 10; RA, Ras association; SCOP, suprachiasmatic nucleus oscillatory protein; Tregs, regulatory T cells; TSC2, tuberous sclerosis complex 2.

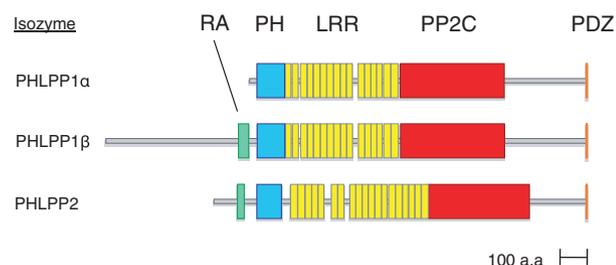


Fig. 1. Domain structure of the human PHLPP isozymes. All PHLPP family members contain a PH domain, a series of LRRs, a PP2C phosphatase domain and a C-terminal PDZ ligand. In addition, PHLPP1 β and PHLPP2 contain a putative RA domain near their N-termini.

members rely on Mg^{2+} or Mn^{2+} for activity and are insensitive to common phosphatase inhibitors such as okadaic acid [2,6]. Conserved regulatory domains in PHLPP include a PH domain, an LRR segment and a C-terminal PDZ [post synaptic density protein (PSD95), *Drosophila* disc large tumour suppressor (Dlg1) and zonula occludens 1 protein (zo-1)] ligand [7]. PHLPP1 β and PHLPP2 also contain a putative Ras association (RA) domain near their N-termini, although the function of these RA domains is as yet unverified. The PH domain has a relatively low affinity for phosphoinositides [8], as it contains only the middle arginine of the R-X-R-S-F motif required for phosphoinositide binding [5]. The full-length protein has been reported not to bind phosphatidylinositol 3,4-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate (PIP₃) *in vitro* [9], but whether phosphoinositide binding occurs in cells remains to be established. The PH domain, however, is important for protein interactions and is essential for the regulation of one PHLPP substrate, protein kinase C (PKC) [10]. The series of LRRs have been reported to regulate signalling through the extracellular-signal-regulated kinase (ERK) pathway [7]. Finally, a PDZ ligand is present at the C-terminus of PHLPP1 (DTPL) and PHLPP2 (DTAL); the PDZ scaffold NHERF (Na⁺/H⁺ exchanger regulatory factor) has recently been reported to bind both sequences [11]. PHLPP is conserved in eukaryotes. Interestingly, the yeast homologue CYR1 contains an adenylate cyclase domain near the C-terminus but no PDZ ligand [6].

The PHLPP family was identified in a rational, systematic search for genes predicted to encode a phosphatase domain linked to a PH domain [2], criteria hypothesized to be important for a phosphatase that would dephosphorylate the lipid second messenger kinases Akt (itself controlled by a PH domain) and PKC (also controlled by membrane-targeting mod-

ules). The mRNA of PHLPP1 β had previously been identified in a screen for transcripts whose levels oscillate in a circadian fashion in the rat suprachiasmatic nucleus and had thus been termed SCOP [1]. Biochemical and cellular studies validated PHLPP1 and PHLPP2 as functional phosphatases that dephosphorylate and inactivate Akt at its hydrophobic motif site, serine 473 [2,3]. Since their identification, the PHLPP isozymes have been shown to be widely expressed in human and mouse tissues, with particularly high expression in brain (Table 1) [1,5,12]; both PHLPP proteins appear to be localized at the membrane and in the cytosol and nucleus of multiple cell types [5].

Molecular, cellular and physiological functions of PHLPP

Targets of PHLPP

PHLPP was originally identified as the phosphatase for a conserved C-terminal phosphorylation site first identified on PKC [13,14] and S6 kinase [15] that is conserved in many members of the AGC family, including Akt [16]. This segment is flanked by hydrophobic residues and is thus referred to as the hydrophobic motif [16].

Akt

The PHLPP isozymes bind to the oncogenic serine/threonine kinase Akt and dephosphorylate it under serum-starved, agonist-stimulated and normal conditions [2,3]. Upon recruitment to the plasma membrane, Akt is activated by sequential phosphorylations on its activation loop (at threonine 308 in Akt1) by PDK-1 and hydrophobic motif (serine 473) by an mTORC2-facilitated mechanism. Akt phosphorylated at threonine 308 alone is only about 10% as active as the fully phosphorylated form but retains activity towards a group of substrates [17]. Phosphorylation of serine 473 stabilizes the active conformation of the kinase, allowing for full activation and phosphorylation of all its known substrates [18–20]. Thus, dephosphorylation of serine 473 by PHLPP is critical for regulation of Akt activity. PHLPP1 and PHLPP2 display a remarkable preference for serine 473 over threonine 308 *in vivo* [2,3]. Accordingly, PHLPP expression decreases Akt activity *in vitro* and the phosphorylation of numerous Akt substrates in cells, while depletion of either or both PHLPP isozymes results in increased Akt substrate phosphorylation [2,3,21–24]. Further supporting the high degree of substrate specificity, knockdown of

Table 1. PHLPP levels in cancer and normal tissue. IHC, immunohistochemistry; WB, western blot.

Sample type	PHLPP expression	Detection	Reference
<i>Human cancer</i>			
Breast	PHLPP1 mRNA is 2.0-fold lower in invasive ductal breast carcinoma	Array	[74]
Breast	PHLPP1 mRNA is 2.3-fold lower in ductal breast carcinoma	Array	[75]
CLL	PHLPP1 mRNA is 12.4-fold lower in CLL	Array	[76]
CLL	PHLPP1 mRNA is 5.5-fold lower in CLL	Array	[77]
CLL with 13q14 deletion	PHLPP1 mRNA is absent in > 50% of cases with 13q14 deletion	qPCR	[78]
Colon	PHLPP2 mRNA is 5-fold lower in colon cancer	Array	[79]
Colon	PHLPP2 mRNA is 6.1-fold lower in rectal adenoma	Array	[79]
Colon	PHLPP2 mRNA is 3.5–3.9-fold lower in several types of colon adenoma	Array	[80]
Colon	PHLPP2 mRNA is 2-fold lower in colorectal adenoma	Array	[81]
Colon	PHLPP1 and PHLPP2 protein expressions are lost or reduced in 78% and 86% of tumour tissue samples, respectively	IHC	[22]
Oesophageal	PHLPP1 mRNA is 5.0-fold lower in oesophageal adenocarcinoma	Array	[82]
Oesophageal	PHLPP2 mRNA is 3.8-fold lower in oesophageal adenocarcinoma	Array	[82]
GBM	PHLPP1 mRNA is 2.1-fold lower in GBM	Array	[83]
Liver, pancreas, stomach	PHLPP1 protein expression is significantly decreased in liver, pancreas and stomach tumour samples	IHC	[32]
Mantle cell lymphoma	PHLPP1 mRNA is 4.3-fold lower in mantle cell lymphoma	Array	[76]
Melanoma	PHLPP2 mRNA is 2.3-fold lower in benign melanocytic skin nevus	Array	[84]
Pancreatic ductal adenocarcinoma	Low PHLPP1 but not PHLPP2 protein expression is negatively correlated with survival	IHC	[25]
Prostate	PHLPP1 mRNA is significantly reduced in high Gleason score tumours	qPCR	[47]
Prostate	PHLPP1 and PHLPP2 mRNAs are absent in 30% and 50% of prostate tumours, respectively	Array	[26]
Prostate	PHLPP1 protein expression is 4-fold lower in PTEN-null, AR-null prostate cancer compared with benign lesions	IHC	[60]
<i>Normal tissue</i>			
Various rat tissues	PHLPP1 β protein is expressed in testis, hypothalamus, hippocampus, cortex, olfactory bulb and cerebellum	WB	[1]
Various human tissues	PHLPP1 mRNA is strongly expressed in brain, heart, skeletal muscle, kidney, liver, small intestine, placenta, lung	Northern blot	[23]
Mouse T cells	PHLPP1 and 2 mRNAs are increased by 7-fold and 2-fold, respectively, in Tregs compared with conventional T cells	RT-PCR	[35]
Mouse hippocampus	PHLPP1 and 2 protein levels are 30%–50% lower in CA1 compared to CA3	WB	[12]
Rat suprachiasmatic nucleus	PHLPP1 β mRNA is higher at clock time 0 than clock time 12	RT-PCR	[1]
Mouse striatum	PHLPP1 protein levels are ~ 50% lower in mouse models of Huntington's disease	WB	[34]
Human putamen	PHLPP1 protein levels are ~80% lower in Huntington's patients	WB	[34]
Rat liver	PHLPP2 mRNA levels are 23.4-fold higher in rats given lanthanum nitrate	Array	[85]
Human skeletal muscle	PHLPP1 protein is increased ~ 2-fold in obese versus non-obese subjects	WB	[21]
Human white adipose tissue	PHLPP1 protein is increased ~ 2-fold in obese versus non-obese subjects	WB	[21]

PHLPP1 versus PHLPP2 affects different subsets of Akt substrates: PHLPP1 knockdown results in increased phosphorylation of glycogen synthase kinase (GSK) 3 α and β , murine double minute 2 (MDM2) and tuberous sclerosis complex 2 (TSC2)/tuberin, whereas PHLPP2 knockdown increases the phosphorylation of GSK3 β , FoxO (Forkhead Box O) 1, p27 and TSC2 [3]. This selectivity arises because, in certain cell lines, PHLPP1 binds to and dephosphorylates Akt2 and Akt3 but not Akt1, while PHLPP2 binds and regulates Akt1 and Akt3 but not Akt2 [3,25]. However, it

should be noted that this selectivity has only been observed in lung and pancreatic cancer cell lines in culture; in the prostate, which expresses primarily Akt2 and Akt3 [20], deletion of PHLPP1 still results in an increase in Akt phosphorylation [26]. The mechanisms behind this observed specificity are unknown but could involve differential scaffolding of the isozymes: the PDZ ligand of PHLPP1 is necessary for its regulation of Akt [2], and the PDZ ligands of the two isozymes differ slightly. Indeed, the PDZ ligand of PHLPP2 appears to bind many more recombinant PDZ

domains on a PDZ domain array than that of PHLPP1 (M. T. Kunkel, E. L. Garcia, R. A. Hall and A. C. Newton, unpublished data).

Protein kinase C

Both PHLPP1 and PHLPP2 dephosphorylate the hydrophobic motif of PKC (serine 657 in PKC α) *in vitro* and in cells [10]. Dephosphorylation of this site in PKC does not acutely impair its activity; rather, it shunts the protein to the detergent-insoluble pellet where it is further dephosphorylated at okadaic acid sensitive sites (PDK-1 site and turn motif), ubiquitinated and degraded by a proteasome-dependent mechanism [27]. Thus, knockdown of PHLPP results in increased steady-state levels of PKC. Indeed, PKC levels correlate inversely with PHLPP levels in both cancer and normal cell lines [10]. PHLPP1 binds PKC via the C1 domain of the kinase and the PP2C and PH domains of the phosphatase; notably, the PH domain of PHLPP is required for PHLPP to dephosphorylate PKC in cells, suggesting that this module may play a scaffolding role [10].

S6 kinase

PHLPP has recently been shown to directly dephosphorylate the hydrophobic motif of Akt's close relative S6K1 *in vitro*; Liu *et al.* [28] elegantly demonstrated that PHLPP knockdown or deletion results in increased phosphorylation of S6K1 and its downstream substrate S6 even when Akt is not activated, or when S6K1 phosphorylation is uncoupled from Akt upon treatment with rapamycin. Interestingly, PHLPP-mediated repression of S6K1 leads to increased insulin-stimulated signalling via loss of feedback inhibition of insulin receptor substrate 1 (IRS-1; see Fig. 2), with the apparently paradoxical result that PHLPP knockdown leads to *decreased* Akt phosphorylation at both the hydrophobic motif and activation loop in the colon cancer cells used in this study.

The ERK signalling pathway

There is some evidence that PHLPP plays a role in regulating the activation of ERK, although its direct target in this context is unknown. Overexpression of

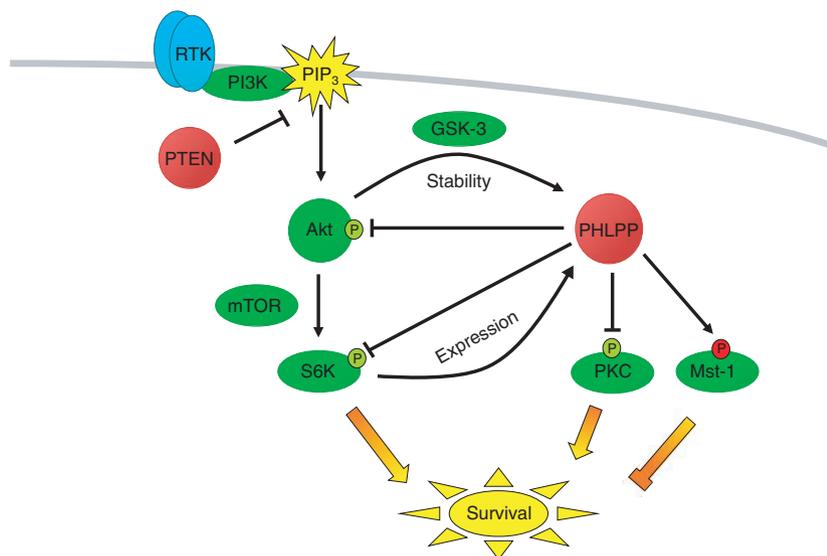


Fig. 2. PHLPP suppresses survival signalling pathways. PHLPP directly dephosphorylates and inactivates Akt, PKC, and S6K at the activating hydrophobic motif site (P in green circle) to oppose pro-survival signaling and directly dephosphorylates and activates Mst1 at an inhibitory site (P in red circle) to promote pro-apoptotic signaling. In the case of PI3K signaling, activation of receptor tyrosine kinases (RTK) results in activation of PI3K to catalyze the production of PIP₃ in the plasma membrane. This leads to activation of Akt which phosphorylates a range of downstream substrates including TSC2, which indirectly regulates mTOR and S6K. This activation is opposed by the lipid phosphatase PTEN, which dephosphorylates PIP₃, removing the upstream signal for Akt activation, and the protein phosphatases PP2A and PHLPP, which dephosphorylate Akt at threonine 308 (the activation loop) and serine 473 (the hydrophobic motif), respectively. PHLPP expression is tightly regulated downstream of Akt, resulting in negative feedback via two mechanisms. First, mTORC1 activation increases PHLPP1 and PHLPP2 protein translation via activation of S6K and inhibition of 4EBP1; second, GSK3 β -mediated phosphorylation of PHLPP1 results in increased PHLPP degradation mediated by the E3 ligase β -TrCP. Thus, repression of GSK3 β by Akt prevents PHLPP degradation and increases PHLPP protein levels. Protein kinases are shown in green and protein phosphatases in red.

PHLPP1 represses phosphorylation of ERK induced by multiple agonists, including phorbol esters, cytokines, CpG oligonucleotides and CD40 [7,24], and small interfering RNA mediated depletion of PHLPP1 increases the levels of phospho-ERK in neural and B-cell models [12,24,29]. Interestingly, the LRR region of the yeast PHLPP orthologue CYR1 binds Ras, allowing Ras-dependent activation of the associated adenylyl cyclase [30]. This observation led Shimizu *et al.* [7] to propose that the LRR of PHLPP1 β (a) competes with nucleotides for binding to Ras and (b) is necessary for PHLPP1 β to repress phorbol ester stimulated ERK phosphorylation. However, it is unclear if the regulation of ERK by PHLPP in this context results from dampening of PKC activation or from direct effects on Ras, and further investigation into possible mechanisms is warranted.

Mst1

PHLPP has recently been shown to catalyse an activating dephosphorylation of the pro-apoptotic kinase Mst1 (the human homologue of Hippo), thus providing another mechanism to suppress cell survival. Specifically, Pardee and coworkers reported that in certain gastrointestinal cancer cells overexpression of PHLPP significantly increased apoptosis in the absence of significant decreases in the phosphorylation of its known substrates, Akt, ERK or PKC, leading to a search for cell-type-specific downstream partners of PHLPP. An MS-based screen for PHLPP1-interacting proteins identified Mst1, which was then shown to be a direct substrate for PHLPP1 and PHLPP2. In certain pancreatic cancer cells, PHLPP dephosphorylates an inhibitory residue (threonine 387) on Mst1, which, interestingly, is phosphorylated by Akt [31]. PHLPP-mediated dephosphorylation results in activation of the kinase and increased downstream signalling through p38 and c-Jun N-terminal kinase (JNK), ultimately leading to apoptosis and growth arrest [32]. Interestingly, another study showed that PHLPP1 and PHLPP2 do act to dephosphorylate Akt2 and Akt1, respectively, in a different pancreatic cancer cell line [25], indicating that even within a given cancer type PHLPP can modulate different targets depending on the specific cell line. These data highlight the cell-type-dependent actions of PHLPP and reveal the multiple ways in which PHLPP acts to oppose survival signalling.

Cellular and physiological functions of PHLPP

By both (a) terminating signalling by two survival kinases, Akt and PKC, and (b) activating signalling by a pro-

apoptotic kinase, Mst1, PHLPP strongly opposes cell growth and survival; this has been shown in numerous cellular and xenograft models [2,3,22,24,25,32]. In addition, PHLPP1 and PHLPP2 decrease protein synthesis and cell size by a mechanism dependent on downregulation of S6K [28]. PHLPP1 and PHLPP2 also appear to repress cellular migration, and both isozymes are less highly expressed in metastatic breast cancer lines than primary breast cancer or normal breast lines [32,33], suggesting that PHLPP may play a role in cancer cell motility.

In normal physiology, PHLPP1 depletion or deletion is cardioprotective: by increasing Akt activation, PHLPP1 knockdown blocks doxorubicin-induced apoptosis in neonatal rat ventricular myocytes, and ischaemic injury is reduced in PHLPP1 knockout mouse hearts [23]. Similarly, PHLPP1 depletion may be neuroprotective, as it has been shown to increase Akt signalling in hippocampal and striatal neurons [12,34]. The effects of PHLPP1 on Akt are also important for regulatory T cells (Tregs), which require suppression of Akt signalling for their development and function and which have elevated expression of PHLPP1 and PHLPP2 compared with conventional T cells. Deletion of PHLPP1 inhibits the development of conventional T cells into Tregs positive for the marker protein Foxp3, and Tregs from PHLPP1 knockout mice are less able to suppress the proliferation of conventional T cells [35].

Given the high level of PHLPP expression in the brain, it is not surprising that PHLPP has been postulated to play roles in several neural functions, namely long-term memory and the regulation of circadian rhythms. Inducible overexpression of PHLPP1 β in the murine forebrain blocks the formation of long-term novel object memory [29], while deletion of PHLPP1 interferes with light-induced resetting of the circadian clock [36]. Interestingly, injection of a PKC inhibitor into the suprachiasmatic nucleus has been shown to affect the circadian response to a light pulse [37], raising the possibility that the regulation of PKC by PHLPP is essential for the proper function of the circadian clock.

PHLPP in cancer

Several lines of data support a role for PHLPP as a tumour suppressor *in vivo*. First, as previously mentioned, PHLPP overexpression in glioblastoma (GBM), breast, colon and pancreatic cancer cell lines decreases colony formation and growth *in vitro* and in xenograft models [2,22,25,32,38]. Second, PHLPP expression is lost or decreased in a variety of human cancers (as detailed in the regulation section below).

Furthermore, the PHLPP1 knockout mouse displays an increased incidence of prostate intraepithelial neoplasia, which eventually progresses to frank carcinoma when combined with partial loss of PTEN (phosphatase and tensin homologue located on chromosome 10) [26]. Interestingly, it appears that PHLPP1 loss cooperates with PTEN loss and p53 mutation to promote prostate carcinogenesis. PHLPP1^{-/-}, PTEN^{+/-} mouse prostate cells initially undergo senescence; however, upon spontaneous inactivation of p53, this genetic combination results in increased proliferation in the prostate, carcinoma formation and increased mortality. Moreover, PHLPP1^{-/-}, PTEN^{+/-}, p53-mutant mouse embryonic fibroblasts are dependent upon Akt activity for their increased abilities to proliferate, survive and form colonies in soft agar, suggesting that in this context, at least, PHLPP blocks carcinogenesis through its actions on Akt. The idea that PHLPP1 and PTEN downregulation cooperate to increase carcinogenesis is supported by studies in humans: Kaplan–Meier analysis of relapse after radical prostatectomy revealed that a ‘PTEN/PHLPP1 low’ status predicts relapse better than Gleason scoring [26]. Finally, although few variations in the PHLPP protein sequences have been described, a single nucleotide polymorphism in PHLPP2 (L1016S, located in the phosphatase domain) has been shown to result in reduced inhibition of basal Akt phosphorylation. This polymorphism has relevance for human cancer, as grade 3 but not grade 2 breast tumours often display loss of heterozygosity at this locus, resulting in preferential loss of expression of only the more active leucine allele [39].

Regulation of PHLPP

Downregulation of PHLPP in cancer

The genetic locus for PHLPP1, which lies at 18q21.33, undergoes loss of heterozygosity in a high percentage of colon cancers [40,41]; similarly, the PHLPP2 locus at 16q22.3-16q23.1, which contains a fragile site, is susceptible to loss of heterozygosity in breast and ovarian cancers, Wilms’ tumours, and prostate and hepatocellular carcinomas [42–46].

Consistent with a role for PHLPP in the progression of prostate cancer, several studies have shown that PHLPP levels are decreased in this disease. Chen *et al.* [26] discovered that PHLPP1 and PHLPP2 protein expression is lost in 30% and 45%, respectively, of human prostate cancer samples, while Hellwinkel *et al.* [47] found that PHLPP1 mRNA levels were frequently downregulated in high-grade but not low-grade prostate cancer samples, supporting the idea that

PHLPP1 loss participates with other genetic abnormalities to promote prostate cancer progression. In fact, loss of both PHLPP1 and PHLPP2 is associated with PTEN and p53 loss in metastatic but not primary prostate cancer, which suggests that p53 loss is a late event that cooperates with PHLPP loss in metastasis [26]. The effects of PHLPP1 deletion on prostate carcinogenesis may be explained by the strong dependence of prostate cancer on phosphoinositide 3-kinase (PI3K)/Akt signalling; a survey of 218 prostate tumours found that the PI3K pathway is altered in almost half of the primary tumours and all metastatic samples. (Notably, this same study found downregulation of PHLPP1 or PHLPP2 in 11% of primary tumours and 37% of metastases [48].)

In addition to prostate cancer, PHLPP levels are decreased in a number of other malignancies (summarized in Table 1), particularly chronic lymphocytic leukaemia (CLL), GBM and colon cancer. However, the factors that regulate PHLPP expression in normal and cancer cells are incompletely characterized and deserve further attention.

Regulation of PHLPP expression

Transcriptional regulation of the PHLPP1 and PHLPP2 genes is still very much a black box, although there are several hints at possible regulators. SMAD3, a downstream effector of transforming growth factor β , binds the PHLPP1 promoter, and this binding has been shown to correlate with increased PHLPP1 expression during the development of Tregs [35]. Interestingly, the SMAD3 binding partner SMAD4 acts as a tumour suppressor in PTEN-null prostate cancer and is often co-deleted with PHLPP1, with which it shares a genetic locus [26,49]. Expression of Huntington’s disease associated alleles of *huntingtin* decreases the mRNA levels of PHLPP1, perhaps via nuclear transcription factor Y, which has putative binding sites located in the PHLPP1 promoter [34]. PHLPP1 mRNA expression is also repressed by the microRNA miR-190, which is upregulated by exposure to the carcinogen arsenic and which is overexpressed in many cancers, including CLL [50]. There may also be a connection between Akt signalling and PHLPP1 mRNA expression, though the mechanism is unclear; one study found a slight elevation in PHLPP1 but not PHLPP2 mRNA in primary myotubes from type 2 diabetics, which displayed decreased Akt activation in response to insulin. Conversely, Warfel *et al.* [51] examined microarray data from the NCI-60 panel of cancer cell lines and found decreased levels of PHLPP1 mRNA in GBM lines compared with astrocytoma

lines, correlating with increased Akt activation in GBM. However, effects on PHLPP1 protein synthesis and degradation downstream of Akt often complicate the interpretation of Akt-dependent changes in PHLPP protein levels, which may involve transcriptional, translational and/or protein degradation mechanisms.

The post-translational regulation of PHLPP is better understood than its transcriptional regulation. To date, two mechanisms controlling the steady-state levels of PHLPP protein have been described. Both act downstream of Akt, implying the existence of at least two negative feedback loops involving PHLPP and Akt (Fig. 2). First, the rate of PHLPP translation is controlled by mTOR (mammalian target of rapamycin): treating cells with the mTOR inhibitor rapamycin or genetically interfering with the mTORC1 complex decreases PHLPP1 and PHLPP2 protein levels without affecting protein degradation or mRNA expression, suggesting that mTOR activation downstream of Akt results in increased translation of PHLPP1 and PHLPP2 [38]. Consistent with this, PHLPP1 depletion in 3T3 fibroblasts and the subsequent increase in Akt signalling increase PHLPP2 protein but not mRNA levels in an mTOR-dependent fashion [26]. Second, the rate of degradation of PHLPP is controlled by Akt activity, whose inhibitory phosphorylation of GSK3 β results in stabilization of PHLPP1 protein [52]. Specifically, casein kinase- and GSK3 β -mediated phosphorylation of PHLPP1 at threonine 851 and serine 847, respectively, contributes to recognition of PHLPP1 by the E3 ligase β -TrCP (β -transducin repeats-containing protein) and subsequent ubiquitin-mediated degradation. Notably, the latter feedback loop is frequently lost in GBM (in both GBM cell lines and primary GBM neurospheres from human tumours); in these samples, β -TrCP is sequestered in the nucleus, away from its substrate PHLPP1, and the rate of PHLPP1 degradation is insensitive to Akt activation [51]. Either or both of these Akt-dependent mechanisms may explain the increase in PHLPP1 protein levels seen under conditions of Akt activation, including insulin stimulation of hepatoma cells [21], treatment of rat ventricular myocytes with leukaemia inhibitory factor, and transgenic overexpression of insulin-like growth factor 1 in the mouse heart [23]. It should be noted that β -TrCP-dependent degradation of PHLPP1 is not the only mechanism by which PHLPP1 is degraded: the calcium-dependent protease calpain has also been shown to degrade PHLPP1 β *in vitro* and *in vivo*, and inhibition of calpain in the hippocampus blocks long-term novel object memory formation, which is opposed by PHLPP1 β expression (see 'Cellular and physiological functions of PHLPP', above) [29]. Also,

the deubiquitinase UCH-L1 (ubiquitin carboxy terminal hydrolase L1), which acts as a tumour promoter in CLL, decreases PHLPP1 β protein but not mRNA expression in a manner that depends upon its deubiquitinase activity [53].

Regulation of PHLPP by protein scaffolds

The key role of protein–protein interactions in achieving spatial and temporal specificity of signalling by enzymes has come to the forefront of the signal transduction field in recent years [54]. PHLPP is no exception: a number of protein scaffolds for PHLPP have been proposed to be essential for the regulation of PHLPP targets. First, NHERF1, which binds PHLPP1, PHLPP2 and PTEN via its two PDZ domains, has been reported to localize PHLPP1 to the membrane in GBM cells, an interaction that allows the phosphatase to exert anti-proliferative effects [11]. Interestingly, samples from patients with high-grade GBM often display concomitant reductions in PHLPP1, PTEN and NHERF protein levels, and upon PTEN depletion in LN229 cells PHLPP1 becomes better able to reduce Akt activation, probably because of increased scaffold availability. These data again highlight the cooperation between PHLPP1 loss and PTEN loss and suggest that, in the absence of PTEN, PHLPP1 provides an additional layer of negative regulation of the PI3K pathway [11]. The scaffold Scribble has been proposed to play a similar role in colon cancer cells, where it coordinates PHLPP1 (via Scribble's LRR region) and Akt at the membrane, allowing PHLPP1 to dephosphorylate serine 473 [55]. Finally, the putative tumour suppressor FKBP51 (FK506 binding protein 51) has been reported to scaffold PHLPP to Akt in pancreatic cancer cells [56]. FKBP51 is essential for PHLPP to dephosphorylate Akt and exert its apoptotic effects in pancreatic cancer cells; accordingly, overexpression of FKBP51 results in decreases in serine 473 phosphorylation of Akt and cell survival that are dependent on the presence of PHLPP [57]. Other studies suggest that this interaction may play a role in suppressing Akt signalling in lung and prostate cancers. In non-small-cell lung cancer cells, repression of insulin-stimulated Akt phosphorylation downstream of the P2X7 purinergic receptor requires both PHLPP and the activity of FKBP51, which scaffolds Akt in these cells and may recruit PHLPP, PTEN and calcineurin to oppose Akt phosphorylation [58]. In models of prostate cancer, castration or androgen receptor inhibition decreases PHLPP1 protein levels in a manner that depends upon FKBP51 depletion, suggesting that FKBP51 not only scaffolds PHLPP but also plays

a role in maintaining its stability [59,60]. In human prostate cancer tissue samples, PHLPP1 protein levels are correlated with the levels of PTEN, FKBP51 and the androgen receptor, raising the possibility that the androgen and PI3K pathways may interact via inhibition of PHLPP [60].

PHLPP has also been shown to interact with several other signalling factors, although the effects of these interactions are mainly unknown. PHLPP2 binds to adenylate cyclase in rat cardiomyocytes, and this interaction appears to contribute to forskolin-stimulated downregulation of Akt [61]. Interestingly, this interaction recapitulates the proximity between a PP2C phosphatase domain and adenylate cyclase seen in yeast, where these two domains are part of the same protein, CYR1. In addition, a recent proteomic screen of the human deubiquitinases revealed ubiquitin-specific peptidases 12 and 46 as binding partners for both PHLPP1 and PHLPP2 [62]. These and other interactions remain to be further characterized and may offer hints to mechanisms of PHLPP regulation or the discovery of new PHLPP targets.

Phosphatase activity of PHLPP

Little is yet known about how the intrinsic catalytic activity of PHLPP is controlled in cells. However, a recent screen for specific inhibitors of PHLPP revealed insights into the structure of the phosphatase domain. Homology modelling of the structure of the PP2C domain of PHLPP2 onto the known structure of PP2C α [6], combined with knowledge about the structure of several validated PHLPP2 inhibitors, allowed Sierrecki *et al.* to construct a set of models for the active site, all of which included one, two or three manganese ions, and to determine key residues for the catalytic activity, which include aspartate 806, glutamate 989 and aspartate 1024 [63]. Virtual screening using the best of these theoretical catalytic domain structures allowed the identification of numerous new predicted inhibitors, a few of which were experimentally tested and shown to inhibit PHLPP activity *in vitro* and *in vivo*. As expected, these inhibitors increase phosphorylation of Akt and its downstream substrates and attenuate etoposide-induced apoptosis in cell culture [63].

Endogenous regulators of PHLPP activity *per se* remain to be identified. Both isozymes have an abundance of predicted phosphorylation sites, and phosphorylation is a likely candidate to control the catalytic activity of the enzyme. Indeed, it has been suggested that factors downstream of the insulin receptor may modulate PHLPP activity [64], and serum stimulation or overexpression of Akt1, 2 or 3 was

reported to decrease the activity of tagged, exogenously expressed PHLPP [9]. Further study of the regulation of PHLPP activity *in vivo* in the coming years is likely to unveil mechanisms that control the catalytic activity of PHLPP.

Phosphatases in the PI3K pathway

The PI3K/Akt signalling cascade (Fig. 2) is a well-studied complex pathway that promotes cell growth and survival. Attesting to the importance of precise control of Akt signalling for cellular homeostasis, signalling through this pathway is often increased in primary and metastatic tumours [48,65], and its activation, combined with that of other factors such as Ras [66], is crucial for driving the majority of human cancers [67]. In fact, several inhibitors of the pathway targeted at PI3K, Akt and/or mTOR are currently in clinical trials as cancer therapeutics [65]. Although much attention has been focused on the mutation of factors that positively affect this pathway (e.g. growth factor receptors and PI3K), negative regulation is equally important and often involves dephosphorylation, which opposes signalling through this pathway via several mechanisms. These include (a) downregulation of PI3K signalling via removal of its product PIP₃ by the lipid phosphatases PTEN [68] and SHIP (which dephosphorylates the 5' position to yield PI(3,4)P₂) [69], (b) dephosphorylation of Akt at its activation loop and hydrophobic motif by PP2A and PHLPP, respectively [2,3,70], and (c) dephosphorylation/activation of the Akt substrate and Hippo homologue Mst1, which acts to increase apoptosis and limit organ size [31,71]. The importance of proper phosphatase regulation of this pathway is highlighted by several recent studies showing that depletion of both PHLPP and PTEN is necessary for full activation of Akt in GBM [11] and predicts metastasis and relapse in prostate cancer [26].

This exquisitely tuned pathway is subject to several layers of negative feedback regulation (Fig. 2). One well-characterized loop involves S6K-mediated inhibitory phosphorylation of IRS-1, which dampens activation of PI3K in the face of excess mTOR activation [72]. Also, Yu *et al.* [73] recently showed that mTORC1 phosphorylates and stabilizes the adaptor protein Grb10, which negatively regulates signalling through growth factor receptors. Recent studies (described in more detail above) have demonstrated the existence of three more feedback loops, these ones involving PHLPP. First, mTOR activation positively regulates the translation of PHLPP1 and PHLPP2, thereby resulting in inactivation of Akt by PHLPP-mediated dephosphorylation [38]. Second, increased Akt activation

results in phosphorylation and inhibition of GSK3 β , resulting in decreased phosphorylation of PHLPP1 at serine 847. PHLPP1 that is not phosphorylated at serine 847 cannot be recognized by its E3 ligase β -TrCP and is therefore stabilized, leading to increased levels of PHLPP and decreased activation of Akt [51,52]. Notably, this feedback loop is lost in GBM because of sequestration of β -TrCP in the nucleus, away from its substrate PHLPP1 [51]. Finally, PHLPP also dephosphorylates the activation loop of S6K1, thus activating PI3K/Akt signalling through upregulation of IRS-1 [28].

Perspective

Repression of signalling by protein phosphatases, originally conceived as a promiscuous general 'off' signal, has increasingly been recognized as an important layer of specific and dynamic regulation in mammalian cells. The PHLPP phosphatases, which dephosphorylate the oncogenic kinase Akt (among other targets), provide good examples of specificity of signalling. First, they are highly selective for the hydrophobic motif of Akt over the activation loop; second, they display a remarkable isozyme specificity, with PHLPP1 binding and dephosphorylating Akt2 and 3 and PHLPP2 binding and dephosphorylating Akt1 and 3. Their localization and binding to Akt also appear to be tightly regulated by the scaffolding proteins NHERF1, Scribble and FKBP51 in a cell-type-dependent manner, and the requirement for various protein-protein interaction domains for binding to these factors and to PHLPP substrates highlights the additional layer of regulation provided by such non-catalytic domains. Finally, the existence of several feedback loops involving upregulation of PHLPP protein levels downstream of Akt activation suggest that precise regulation of this phosphatase is essential for the modulation of signalling through the PI3K/Akt pathway.

The rise in studies concerning PHLPP since its characterization in 2005 is beginning to unveil the targets and upstream regulators of this phosphatase. With recent evidence demonstrating a tumour suppressor role of PHLPP, future studies of the regulation of this enzyme in normal and cancer cells are likely to provide major insights into this enzyme as a therapeutic target and/or biomarker in human cancer.

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