PHLPPing through History: A Decade in the Life of PHLPP Phosphatases

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Abstract

In the decade since their discovery, the PH domain Leucine rich repeat Protein Phosphatases (PHLPP) have emerged as critical regulators of cellular homeostasis and their dysregulation is associated with various pathophysiologies, ranging from cancer to degenerative diseases such as diabetes and heart disease. The two PHLPP isozymes, PHLPP1 and PHLPP2, were identified in a search for phosphatases that dephosphorylate Akt, and thus suppress growth factor signaling. However, given that there are over 200,000 phosphorylated residues in a single cell, and fewer than 50 Ser/Thr protein phosphatases, it is not surprising that PHLPP has many other cellular functions yet to be discovered, including a recently identified role in regulating the epigenome. Both PHLPP1 and PHLPP2 are commonly deleted in human cancers, supporting a tumor suppressive role. Conversely, the levels of one isozyme, PHLPP1, are elevated in diabetes. Thus, mechanisms to correctly control PHLPP activity in cells are critical for normal cellular homeostasis. This review summarizes the known functions of PHLPP and its role in disease.

Keywords

Introduction

Protein phosphorylation is one of the major mechanisms by which cells transduce signals. Over 200,000 phosphorylation sites have been identified, with about 60% occurring on Serine (Ser) residues, and 25% on Threonine (Thr) residues [1]. For every phosphorylation event, there is a dephosphorylation event, yet our understanding of the structure, function, and regulation of the 40 or so Ser/Thr protein phosphatases lags behind that of the 400 or so Ser/Thr protein kinases [2]. In part, the greater understanding of protein kinases stems from their common architecture and catalytic mechanisms. In contrast, there is considerable variation in the enzymes that remove phosphate [3]. Unlike protein kinases, which phosphorylate substrates at conserved motifs, a single phosphatase can regulate a wide

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variety of substrates, not targeting a specific consensus sequence. Rather, regulatory regions on the phosphatase provide the specificity required for targeting substrates. The three serine/ threonine phosphatase families -the phosphoprotein phosphatases (PPP), metal-dependent protein phosphatases (PPM), aspartate-based phosphatases (FCP/SCP)- encode these regulatory regions in different ways. While the PPP family has regulatory domains encoded for on separate subunits, PPM and FCP/SCP family members have regulatory regions and catalytic domain encoded for on a single peptide. This review focuses on a newly discovered member of the PPM family, the PH domain Leucine rich Repeat Protein Phosphatases (PHLPP).

The PHLPP Family: domain composition and function

The PHLPP phosphatases are part of the PP2C family of Ser/Thr phosphatases which, along with pyruvate dehydrogenase phosphatase, make up the metal-dependent protein phosphatase (PPM) family. PPM members are similar to other Ser/Thr phosphatases in that they require a divalent cation, preferably Mn^{2+} or Mg^{2+}, for catalytic activity. Biochemical analysis has revealed that the activity of the purified PHLPP2 PP2C domain is dependent on the presence of Mn^{2+} [4]. Divalent cations are coordinated by four conserved aspartate residues into the active site of the phosphatase. The active site of human PHLPP1 and PHLPP2 has a unique architecture, with only three of these four conserved acidic residues; this may account for the lower catalytic activity of the PHLPP2 PP2C domain compared to PP2Cα [4].

The PHLPP family of phosphatases comprises of two genes: PHLPP1 and PHLPP2 (Figure 1A) [5]. Both family members have similar domain structures, including a predicted N-terminal Ras Association (RA) domain, a Pleckstrin Homology (PH) domain, a hydrophobic Leucine Rich Repeat (LRR) region, a catalytic Mn^{2+}-dependent PP2C phosphatase domain, and a PDZ (post synaptic density protein PSD95, Drosophila disc large tumor suppressor DLG1, and zonula occludens-1 protein zo-1) binding motif [6]. The integration of various scaffolding and regulatory domains into a single polypeptide, rather than on separate modules, likely plays a role in dictating PHLPP substrate specificity and localization.

PHLPP is evolutionarily conserved from yeast to humans [7]. Intriguingly, the yeast homologue of PHLPP, Cyr1, is part of the same gene as the only adenylate cyclase encoded in the *Saccharomyces cerevisiae* genome [8]. In yeast, the cyclic AMP (cAMP)/Protein Kinase A (PKA) signaling pathway is vital for nutritional sensing and growth [9], with deletion of the gene encoding Cyr1 resulting in G1 phase cell cycle arrest [8]. Increases in extracellular glucose concentration result in RAS-associated activation of adenylate cyclase and the production of cAMP, a critical cofactor for the enzyme PKA. Elevation of cAMP leads to robust changes in the transcriptome that support growth and fermentation [10]. Whether mammalian PHLPP has retained any function in regulating cAMP/PKA signaling from its distant yeast homologue remains to be investigated.

PP2C phosphatases are unique from other Ser/Thr phosphatases in their insensitivity to a number of common phosphatase inhibitors, such as okadaic acid and microcystin [11]. PHLPP inhibitors were recently discovered by a chemical and virtual screen of the National

Cancer Institute (NCI) repository [12]. Two compounds were identified to selectively inhibit activity of PHLPP1 and PHLPP2 with *in vitro* IC_{50} values in the 5μM range, compared to *in vitro* IC_{50} values in the 100μM range for PP2Cα and PP1. Treatment of cells with these inhibitors has been shown to increase Akt phosphorylation and suppress apoptosis of cells [12], promote chondrocyte proliferation [13], decrease chaperone-mediated autophagy [14], and raise levels of Akt activity in rat cortical neurons resulting in a neuroprotective phenotype [15]. The identification of such inhibitors not only provides a pharmacological breakthrough to studying PHLPP activity *in vitro* and in cells, but also provides the first steps to creating a potential therapeutic drug to inhibit PHLPP activity.

The first identified substrate of PHLPP was the pro-oncogenic AGC kinase, Akt (Figure 1B) [6]. Specifically, PHLPP dephosphorylates a key regulatory serine residue (Ser473) in the C-terminal tail of Akt, termed the hydrophobic motif, to inactivate it. Further work demonstrated that PHLPP is able to directly dephosphorylate other AGC kinases at their hydrophobic motif, most notably protein kinase C (PKC) [16], thus reducing the steady state levels of this enzyme, and ribosomal protein S6 kinase (S6K) [17]. Thus, PHLPP was dubbed the 'hydrophobic motif phosphatase'. In the decade since this initial discovery that PHLPP dephosphorylates Akt, several other non-AGC kinase PHLPP substrates have been revealed. For example, the pro-apoptotic kinase Mammalian Sterile 20-like kinase 1 (Mst1), a member of the STE kinase family, is also a substrate of PHLPP [18]. By removing an inhibitory phosphorylation on Thr387, PHLPP activates Mst1 and induces apoptosis. Thus, dephosphorylation by PHLPP inactivates pro-survival kinases such as Akt and activates pro-apoptotic kinases such as Mst1.

Most PHLPP signaling pathways discovered to date have involved the activity of cytoplasmic PHLPP. Recent evidence suggests that PHLPP may play a role in the nucleus as a novel regulator of histone proteins and thus transcription. Histones have an integral role in condensation and compaction of the eukaryotic genome by formation of DNA-protein complexes called nucleosomes [19]. Nucleosomes regulate the ability of DNA to be physically accessible to transcription factors and the transcriptional machinery. Chemical modifications to the histone N-terminal tail can dramatically alter chromatin structure, playing a key role in regulating gene expression, DNA replication, and DNA repair [20]. Loss of PHLPP1 results in an increase in global histone phosphorylation and acetylation [21], two chromatin marks associated with actively transcribed regions of the genome. Notably, loss of PHLPP1 leads to increased promoter acetylation of the receptor tyrosine kinase (RTK), EGFR, resulting in increased transcription and increased steady-state levels of the EGFR. This receptor is at the plasma membrane and signaling competent, such that PHLPP1 loss promotes amplified signaling through the MEK/ERK cascade [21]. The steady-state levels of other receptor tyrosine kinases, such as the Platelet Derived Growth Factor Receptor (PDGFR) and the Insulin Receptor (INSR), are also increased upon loss of PHLPP1 protein. Amplified signaling by RTKs is associated with diverse human cancers, as a result of somatic gain-of-function mutations of the RTKs, gene amplification, or epigenetic changes that result in increased receptor expression [22]. Underscoring the prevalence of increased RTK levels in cancer, the amplified expression of the EGFR family member HER2 is associated with up to 30% of human breast cancers [23], a disease which accounts for a striking 30% of all new cancer cases in the US each year [24]. Similarly, 30% of prostate

cancers have been reported to have elevated levels of EGFR, without evidence of gene amplification [25]. This increased expression of RTKs correlates with poor disease prognosis [26, 27]. Thus, this epigenetic role of PHLPP is likely a major contributor to the tumor suppressive properties of the enzyme.

Interestingly, another study showed that PHLPP is able to directly dephosphorylate RAF1 (c-RAF) [29], which is downstream of EGFR and Ras [30]. RAF1 was identified as a PHLPP1 interacting protein via co-immunoprecipitation coupled to mass spectrometry. Further analysis showed that PHLPP1 is able to dephosphorylate RAF1 at Ser338 *in vitro*, a phosphorylated residue required for RAF1 activation. These lines of data suggest a complex role for PHLPP in regulating, both directly and indirectly, the MEK/ERK signaling cascade.

Regulation of PHLPP Expression

While much work has gone into uncovering physiological targets of PHLPP, there remains a gap in our understanding of how expression of PHLPP itself is regulated. Multiple recent studies have focused on how PHLPP transcription is regulated in the context of bone development (Figure 2A). Akt signaling plays a profound role in promoting chondrocyte proliferation, but must be suppressed upon terminal differentiation [31]. Novel evidence suggests that Histone Deacetylase 3 (HDAC3) promotes Akt activity by suppressing transcription of the PHLPP1 gene [32]. Upon stimulation with TGFβ, a potent activator of chondrogenesis, HDAC3 association with the PHLPP1 promoter was observed, resulting in decreased transcription of PHLPP1. Further work demonstrated that PHLPP1 expression was elevated in human patients with osteoarthritis due to a decrease in methylation of the PHLPP1 promoter, an event associated with transcriptional activation [33]. Furthermore, mice lacking PHLPP1 are not as susceptible to cartilage deterioration in a surgically-induced osteoarthritis model. This work suggests a novel role for PHLPP in bone development and disease.

A number of recent studies have focused on how PHLPP expression is regulated by micro RNAs (miRNA) (Figure 2B). miRNA regulates protein expression by promoting mRNA degradation or inhibiting translation of mRNA. The first reported miRNA to target PHLPP was miR-190, which targets the 3′ untranslated region (UTR) of PHLPP1 [34]. In this study, exposure to arsenic resulted in activation of Akt signaling and an increase in phosphorylation of Ser473. This was accounted for by an increase in miR-190 levels, resulting in down-regulation of PHLPP1 protein expression. A number of miRNAs that are deregulated in cancer have been shown to regulate both PHLPP1 and PHLPP2 expression levels. In hepatocellular carcinoma (HCC), miR-331-3p, which targets both PHLPP1 and PHLPP2, is significantly over-expressed [35]. miR-3127 is also over-expressed in HCC, and can function to suppress expression of not only PHLPP1 and PHLPP2, but also Inositol Polyphosphate Phosphatase 4A (INPP4A) and Inositol Polyphosphate-5-Phosphatase J (INPP5J) [36], two enzymes involved in inositol metabolism. miR-205 has been shown to target the 3′UTR of PHLPP2 and Phosphatase and Tensin Homologue (PTEN), and is in a region of the genome that is frequently amplified in non-small cell lung cancer (NSCLC) [37]. In glioma, up-regulation of miR-93 results in a marked inhibition of PHLPP2, PTEN and Forkhead Box O3 (FOXO3) expression [38]. An interesting commonality between a

number of these miRNAs is that they not only target PHLPP, but also PTEN, another tumor suppressor phosphatase that suppresses PI3K/Akt signaling, further cementing the importance of Akt signaling in tumorigenesis.

Translational control of PHLPP expression appears to be regulated in part by the mammalian Target of Rapamycin Complex 1 (mTORC1) (Figure 2B). mTORC1 is a multi-subunit complex with kinase activity that is sensitive to the natural compound, rapamycin, unlike its sister complex, mTORC2 [39]. mTORC1 plays a key role in regulating translation, mostly through its ability to inactivate the translational inhibitor Eukaryotic Translation Initiation Factor 4E Binding Protein 1 (4E-BP1) and activate ribosomal protein S6K. By sensing changes in nutrient and energy levels, mTORC1 is able to ensure protein synthesis is shut off during times of stress. Treatment of cells with rapamycin results in down-regulation of both PHLPP1 and PHLPP2 [40]. Knock-down of key components of the mTORC1 complex, such as mTOR or Raptor, resulted in a similar outcome. As PHLPP mRNA levels and rate of degradation of PHLPP protein was unaffected by rapamycin treatment, the data suggests that PHLPP translational rates are sensitive to mTORC1 activity. Intriguingly, a recent study suggests a role in which free Raptor, which is normally associated with the mTORC1 complex, interacts and binds PHLPP2, resulting in PHLPP2 protein stabilization and reduced signaling through Akt [41]. In aged obese mice, levels of free Raptor decline, resulting in a reduction of PHLPP2 protein stability and prolonged signaling through Akt [41]. The authors suggest that this leads to increased *de novo* lipogenesis and formation of fatty liver. These studies propose that the mTORC1 complex has a multi-faceted role in regulating PHLPP expression.

Multiple lines of evidence suggest a role for ubiquitination in the regulation of PHLPP protein stability (Figure 2C). After phosphorylation at a phosphodegron motif by the kinase Glycogen Synthase Kinase 3β (GSK3β), PHLPP1 is recognized by β-TrCP, a subunit of the Skp-Cullin 1-F-box protein (SCF) ubiquitin E3 ligase complex, and ubiquitinated, resulting in protein degradation [42]. The interaction between PHLPP and β-TrCP is further enhanced by the binding of the co-chaperone protein, Suppressor of G2 Allele SKP1 (SGT1) [43]. As GSK3β is phosphorylated by Akt leading to its catalytic inactivation, Akt activity acts to promote PHLPP protein stability by inhibiting ubiquitination, a possible mechanism to shut down Akt activity after initial stimulation. GSK3β activity is regulated by the kinase Greatwall (GWL) [44], although the mechanism of this regulation is unclear. Over-expression of GWL results in a decrease in the levels of inhibitory phosphorylation on GSK3β at Ser9 and Ser21, resulting in increased ubiquitin-mediated degradation of PHLPP1.

A number of other studies have implicated deubiquitinating enzymes in PHLPP regulation as well. Namely, a number of deubiquitinases, including Ubiquitin Specific Peptidase 1(USP1) [45], USP12[46], and USP46 [47], have been shown to interact with and deubiquitinate PHLPP, resulting in increased protein stability. Considering that these enzymes play a role in stabilizing PHLPP, a bona fide tumor suppressor, it is not surprising that the expression and activity of these deubiquitinases are down-regulated during tumorigenesis. A somatic mutation in the protein WD Repeat Domain 48 (WDR48), which is required to recruit USP12 to PHLPP1, occurs in colon adenocarcinoma [46]. Cellular

studies have shown that this mutation results in a down-regulation of PHLPP1 protein levels, and thus an increase in Akt signaling output [46]. Additionally, reduced levels of USP46 are correlated with a down-regulation of PHLPP1 expression in colorectal cancer [47].

PHLPP and Disease

Maintaining balanced levels of PHLPP expression is key for preventing pathologies, as changes in the steady-state levels of PHLPP are correlated with a number of diseases. The most well characterized example of this is in cancer, as both PHLPP1 and PHLPP2 have been identified as tumor suppressors, largely attributed to PHLPP-dependent suppression of pro-survival signaling, such as through the PI3K/Akt signaling pathway. Both PHLPP1 and PHLPP2 expression is lost in diverse cancers [48–51]. Furthermore, PHLPP1 knock-out mice develop prostate neoplasias, which progress to carcinomas when combined with partial loss of PTEN [52]. Outside of cancer progression, loss of PHLPP expression may play a role in circadian rhythm disorders. The transcription of the PHLPP1 gene, originally identified and termed as the SCN Circadian Oscillatory Protein (SCOP), oscillates in a circadian manner in the rat suprachiasmatic nucleus, a bundle of neurons that is responsible for the circadian clock [53]. Although the mechanism is unclear, mice lacking PHLPP1 have deficiencies in light-induced resetting of the circadian rhythm [54]. While no connections have been made yet, it would be of interest to determine if loss of PHLPP1 expression in humans is linked with any circadian rhythm disorders.

On the other end of the spectrum, a number of other diseases are characterized by up-regulation of PHLPP levels. First, multiple reports have shown that PHLPP1, but not PHLPP2, levels are higher in both the skeletal muscle and adipose tissue of diabetic patients compared to their healthy counterparts [55, 56]. PHLPP1 selectively dephosphorylates Akt2 [5], the Akt isozyme that plays an important role in insulin-stimulated glucose uptake, in cells. Thus, one possible mechanism for why cells become insensitive to insulin in diabetic patients is because PHLPP1 levels become elevated. Akt also plays an important role in regulating cardiac cell survival. PHLPP1 knockout mice have been reported to display a cardio-protective phenotype following cardiac stress [57]. Therefore, suppressing PHLPP expression in the context of the heart would provide therapeutic benefits.

Negative regulation of Akt by PHLPP plays an important role in both suppressing and promoting a disease phenotype. This indicates the potential importance of tissue-specific expression of PHLPP, especially in the context of drug development. For example, if a systemic PHLPP1 inhibitor was designed to treat diabetes or to promote cardiac cell survival during a myocardial infarction, would it potentially promote cellular transformation in other tissues where loss of PHLPP expression or activity is associated with cancer? This provides a challenge that needs to be addressed by understanding the different players involved in tissue-specific PHLPP signaling.

Concluding Remarks

PHLPP is a relatively recent entrant into the arena of cell signaling, yet research in the past decade has secured a firm place for PHLPP as a key regulator, through diverse mechanisms,

of cellular homeostasis. Its dysregulation tips the balance from survival/proliferation to apoptosis/cell arrest. No longer considered just the 'hydrophobic motif' phosphatase, the roles of PHLPP are expanding to include much broader functions such as regulating the epigenome. The coming years will likely unveil more substrates, pathways, and diseases in which PHLPP is involved.

Acknowledgments

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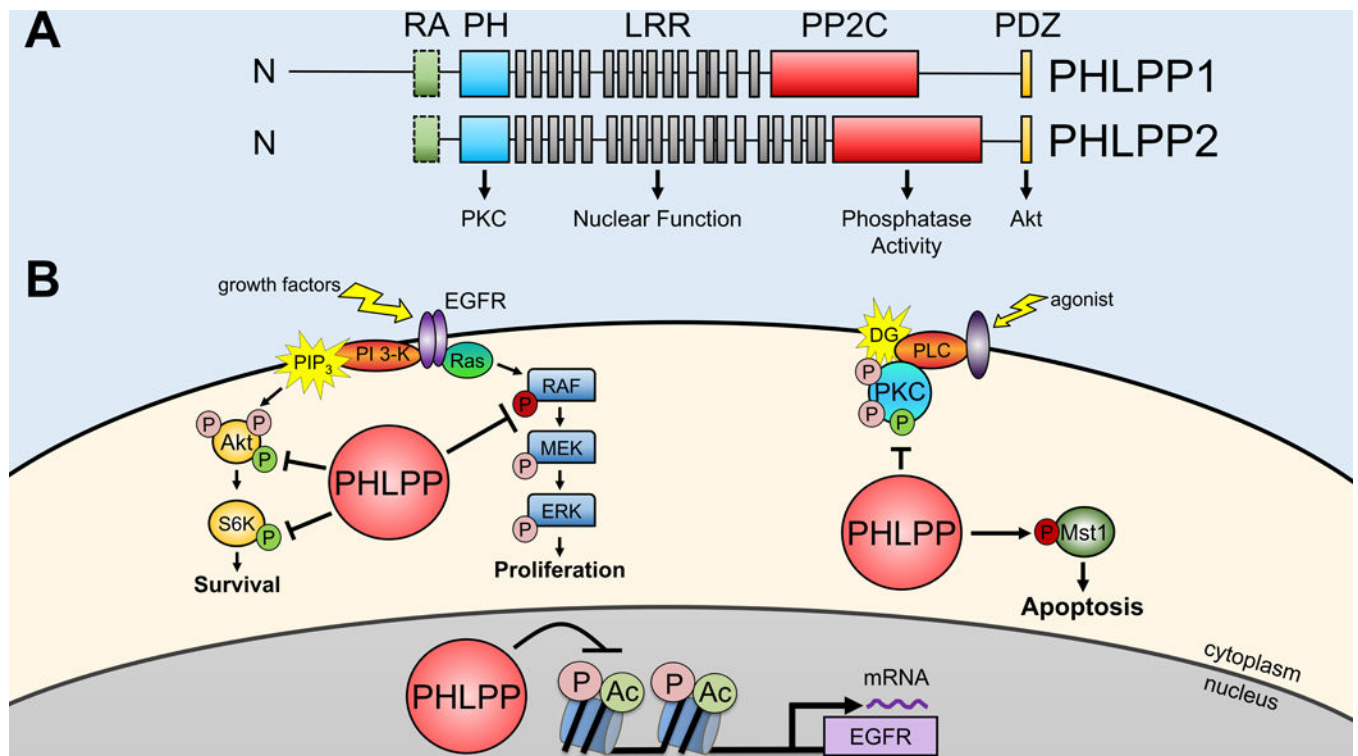
Figure 1. PHLPP Protein Structure and Function

(A) The PHLPP family of phosphatases is composed of two isozymes, PHLPP1 and PHLPP2. Both isozymes have similar structural domains, including a predicted Ras Association (RA) domain, a Pleckstrin Homology (PH) domain required for targeting to PKC, a Leucine Rich Repeat (LRR) required for nuclear PHLPP function, a PP2C phosphatase domain, and a PDZ binding ligand that is required for targeting to Akt. PHLPP1 has a large N-terminal extension approximately 50 kDa in size that is not present in PHLPP2. The PP2C domain between these two isozymes is 58% homologous and both require Mg^{2+} or Mn^{2+} for catalytic activity. Dotted lines denote predicted domains **(B)** PHLPP regulates a number of cellular pathways. Notably, PHLPP can directly dephosphorylate AGC kinases, such as Akt, S6K, and PKC, at their hydrophobic motifs (green phosphate). Dephosphorylation of Akt and S6K results in kinase inactivation. In the case of PKC, dephosphorylation results in increased degradation, indirectly resulting in decreased kinase activity. PHLPP dephosphorylates non-AGC kinases as well, such as an inhibitory site (red phosphate) on the pro-apoptotic kinase, Mst1, leading to its activation. A novel nuclear role for PHLPP has been uncovered in regulating the epigenome; PHLPP is able to suppress histone acetylation and phosphorylation to reduce gene expression of receptor tyrosine kinases, such as EGFR. The mechanism of this regulation has yet to be determined. Additionally, PHLPP1 dephosphorylates RAF1, providing another route for dampening MAPK (Mitogen Activated Protein Kinase) signaling.
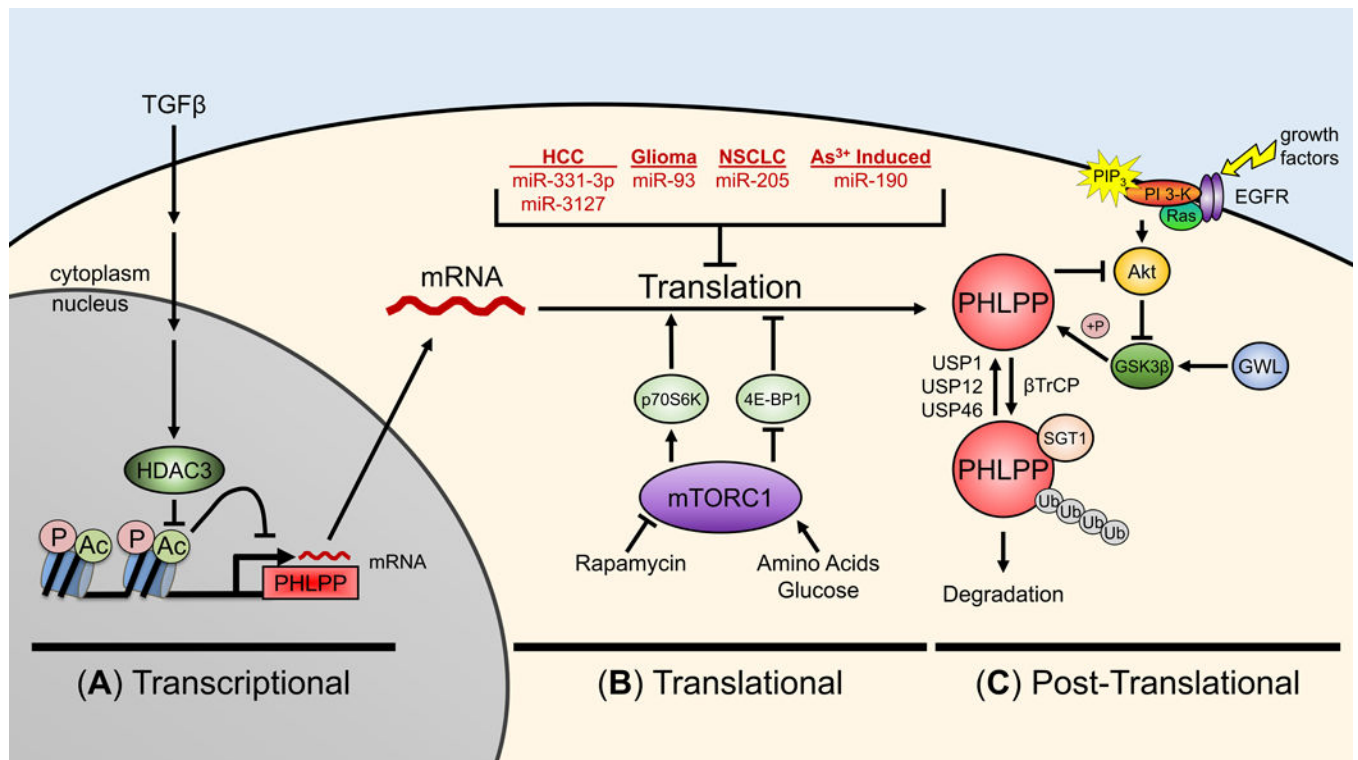
Figure 2. PHLPP Regulation at the Transcriptional, Translational, and Post-Translational Level
(**A**) PHLPP transcription is regulated by HDAC3 during chondrogenesis. Stimulation of chondrocytes with TGFβ results in activation of HDAC3. HDAC3 will then deacetylate the PHLPP1 promoter, resulting in decreased transcription of PHLPP1. (**B**) PHLPP mRNA translation is regulated by miRNA and mTORC1. A number of different miRNAs target the 3′ UTR of both PHLPP1 and PHLPP2. These various miRNAs are frequently up-regulated in a number of different cancers, including hepatocellular carcinoma (HCC), glioma, and non-small cell lung cancer (NSCLC). Arsenic exposure induces miR-190, which can target the 3′ UTR of PHLPP1 mRNA. The mTORC1 complex enhances PHLPP translation. Treatment of cells with rapamycin, which inhibits mTORC1 activity, results in decreased translation of PHLPP mRNA. (**C**) PHLPP is regulated by ubiquitination. Upon phosphorylation of a phosphodegron motif in the PP2C domain of PHLPP by GSK3β, βTrCP-dependent ubiquitination of PHLPP occurs, resulting in degradation of PHLPP protein. Activity of the kinase Greatwall (GWL), results in increased GSKβ activity. Association of the co-chaperone, SGT1, enhances the interaction between PHLPP and βTrCP, resulting in greater degradation of PHLPP. PHLPP can be deubiquitinated by a number of enzymes, including USP1, USP12, and USP46.